Aliphatic and Alicyclic Diols Induce Melanogenesis in Cultured Cells and Guinea Pig Skin

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We have found that several aliphatic and alicyclic diols induce melanogenesis in cultured S91 mouse melanoma cells and normal human epidermal melanocytes (NHEM). In addition, these compounds induce melanogenesis when applied to guinea pig skin, with transfer of melanin to keratinocytes and formation of "supranuclear caps," as occurs in naturally pigmented skin. The relative order of potency of some of these diols in NHEM is 5-norbornene-2,2-dimethanol > 3,3-dimethyl-1,2-butanediol > *cis*-1,2-cyclopentanediol > 2,3-dimethyl-2,3-butanediol > 1,2-propanediol. Following treatment with these diols or 3-isobutyl-1-methylxanthine, melanin and tyrosinase activity are increased within S91 cells and NHEM; however, for cultured NHEM, the largest increases of melanin and tyrosinase occur in an extracellular particulate fraction, shown by electron microscopy to consist almost entirely of stage III and IV melanosomes. These results indicate that cultured NHEM treated with diols export melanosomes in a fashion that is commensurate with natural melanogenic processes. In contrast, S91 mouse melanoma cells exhibit aberrant melanosomal trafficking, in accordance with the known defect in myosin-V mediated melanosomal transport. Both S91 cells and NHEM exhibit morphologic changes and growth arrest indicative of differentiation following treatment with diols. The diols described in this report are candidates for use as cosmeceutical tanning agents. ***Key words: norbornane/tanning/tyrosinase. J Invest Dermatol 110:428–437, 1998***

When exposed to certain stimuli, including sunlight, melanocytes undergo a process of terminal differentiation that culminates in melanosome maturation and melanin synthesis. During this process, melanosomes are transported from melanocytes in the basal layer of the epidermis to closely associated keratinocytes (reviewed in Jimbow *et al*, 1991, 1993; Hearing and King, 1993). Keratinocytes in the basal layer proliferate, resulting in continual migration of keratinocytes towards the outer layers of the skin. During this migration, keratinocytes undergo a process of terminal differentiation that results in enhanced keratin production, loss of organelles including nuclei, and transformation into flattened ceramide-rich remnants of cells that comprise the stratum corneum (reviewed in Schaefer and Fedelmeier, 1996). Migration of keratinocytes from the basal layer of the epidermis to the bottom layer of the stratum corneum takes 2–4 wk in humans. Bottom layers of the stratum corneum are continually displaced by differentiating keratinocytes resulting in further migration of melanin towards the surface of the skin. This latter process takes an additional 2 wk and culminates in shedding of the stratum corneum.

Sunlight is thought to induce tanning by stimulating the release of substances from keratinocytes and other cells in the skin that cause differentiation of melanocytes. These substances include adrenocorticotropic hormone, melanocyte stimulating hormone (MSH), lipotropic hormone, endothelin-I, basic fibroblast growth factor (reviewed in Jimbow *et al*, 1991, 1993; Gilchrest *et al*, 1996), and nitric oxide (Romero-Graillet *et al*, 1997). The effect of sunlight on keratinocytes may be wavelength dependent, with ultraviolet A inducing protein kinase C mediated signal transduction pathways (Matsui *et al*, 1994), and ultraviolet B (UVB) inducing NF-kB mediated signal transduction pathways (Tobin *et al*, 1996). Both pathways can be activated as a result of free radical and active oxygen generation (von Ruecker *et al*, 1989; Mohan and Meltz, 1994).

Drugs to induce melanogenesis in human melanocytes would be useful to provide natural protection against damage from sunlight exposure. Although sunscreens provide instant protection, they require reapplication and can be washed off, so that continual diligence is required to prevent photodamage (Pathak and Fitzpatrick, 1993). In addition, several common components of sunscreens have been shown to generate mutagenic free radicals when exposed to sunlight (Knowland *et al*, 1993; Allen *et al*, 1996). Furthermore, sunscreens did not protect against the development of UV induced melanomas in laboratory studies (Wolf *et al*, 1994). Recent epidemiologic studies have shown that sunscreen use is associated with an increased risk of developing skin cancer (Westerdahl *et al*, 1995). In contrast, natural melanin provides broad range protection against UVR, and may have the additional benefit of quenching UV induced free radicals (Kochevar *et al*, 1993; Pathak and Fitzpatrick, 1993). Furthermore, the presence

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Abbreviations: α-NBane-ol, α-norborneol; Bl-Neo, black-neonatal; CT, cholera toxin; Dopac, 3,4-dihydroxyphenylacetic acid; ETOH, ethanol; 5-NBene-2,2-DM, 5-norbornene-2,2-dimethanol; L-Dopa, L-3,4-dihydroxyphenylalanine; MSH, melanocyte stimulating hormone; NBane, norbornane; NBane-2,2-DM, norbornane-2,2-dimethanol; NHEM, normal human epidermal melanocytes; 1,2-cs-CPD, 1,2-*cis*-cyclopentanediol; 1,2-PD, 1,2-propanediol; 2P, 2-pyrrolidone; 2,3-BD, 2,3-butanediol; 2,3-DM-2,3-BD, 2,3-dimethyl-2,3-butanediol; 2-M-1,3-PD, 2-methyl-1,3-propanediol; 2-NBaneM, 2-norbornanemethanol; 2,3-c/e-NBaneD, 2,3-*cis*/*exo*-norbornanediol; 3,3-DM-1,2-BD, 3,3-dimethyl-1,2-butanediol; Wh-Ad, white-adult; Wh-Neo, white-neonatal.

of constitutive melanin in skin is associated with protection against sun induced erythema, degenerative aging processes, and neoplastic disease (Pathak and Fitzpatrick, 1993).

In past studies, several agents have been shown to induce melanogenesis in cell culture, including L-3,4-dihydroxyphenylalanine acid (L-Dopa), L-Dopa phosphates, 3,4-dihydroxyphenylalanine (Dopac), NH_4Cl, MSH, diacylglycerol, and 3-isobutyl-1-methylxanthine (IBMX) (Pawelek and Murray, 1986; McLane *et al*, 1987; Slominski *et al*, 1988; Karg *et al*, 1989; Gordon and Gilchrest, 1989; Karg *et al*, 1993; Fuller *et al*, 1993). It is unclear at this time if any of these agents will have a practical application as tanning agents. Induction of melanogenesis by L-Dopa and Dopac is accompanied by onset of toxic effects in S91 cells (Pawelek and Murray, 1986; Karg *et al*, 1989). L-Dopa phosphates are far less toxic than L-Dopa, but do not directly induce melanogenesis; rather they rely on concomitant treatment with MSH (McLane *et al*, 1987). In clinical studies with MSH and MSH analogs, injection was required for administration, and tanning occurred primarily in areas previously exposed to sunlight, so that tanning was uneven (Levine *et al*, 1991; Dorr *et al*, 1996). The use of diacylglycerol may be limited because it is a potential inducer of inflammation and has the properties of cancer promoters (Allan *et al*, 1995). IBMX is a strong inducer of melanogenesis in cell culture, but has not yet been demonstrated to be an effective tanning agent when applied to human skin.

In this study, we report that many aliphatic and alicyclic diols can induce melanogenesis in cell culture and when applied to guinea pig skin. The melanogenic potency of these diols ranges from very low for propanediols to relatively high for the norbornane (NBane) related diols. Some of these compounds induce differentiation of melanoma cells, suggesting their potential as cancer therapeutic agents.

MATERIALS AND METHODS

Materials 1,2-propanediol (1,2-PD), 2,3-butanediol (2,3-BD), IBMX, α-melanocyte stimulating hormone (α-MSH), L-Dopa, Dopac, ethanol (ETOH), 1-propanol, 2-propanol, ascorbic acid, oleic acid, glycerol, ethylene glycol, cholera toxin (CT), melanin, and catalase were from Sigma (St. Louis, MO). 2,3-dimethyl-2,3-butanediol (2,3-DM-2,3-BD), 2-methyl-1,3-propanediol (2-M-1,3-PD), 1,2-*cis*-cyclopentanediol (1,2-cs-CPD), 3,3-dimethyl-1,2-butanediol (3,3-DM-1,2-BD), 5-norbornene-2,2-dimethanol (5-NBene-2,2-DM), α-norborneol (α-NBane-ol), 2-norbornanemethanol (2-NBaneM), tartaric acid, D-ribose, D-Deoxyribose, and 2-pyrrolidone were from Aldrich (Milwaukee, WI). Azone was from Durham Pharmaceuticals (Durham, NC). L-[3,5–^{3}H]-tyrosine was from NEN (Boston, MA). Norbornane-2,2-dimethanol (NBane-2,2-DM) was prepared by catalytic (Pd/charcoal) hydrogenation of 5-norbornene-2,2-dimethanol. 2,3-*Cis/exo*-norbornanediol (2,3-c/e-NBaneD) was synthesized from norbornene by selective oxidation (OsO_4) of double bonds. Thymidine glycol (*cis*-5,6-dihydroxy-5,6-dihydrothymidine) and thymine glycol (*cis*-5,6-dihydroxy-5,6-dihydrothymine) were prepared by potassium permanganate ($KMnO_4$) oxidation of thymidine or thymine, respectively, according to Frenkel *et al* (1981). 5-Hydroxymethyluracil was synthesized by hydroxymethylation of uracil with formaldehyde/potassium hydroxide as reported by Cline *et al* (1959).

Cell cultures and treatments Cloudman S91 mouse melanoma cells were obtained from the American Type Culture Collection (ATCC) and cultured in modified Eagle's medium (BioWhittaker, Waltersville, MD) with 10% calf serum (BioWhittaker or Hyclone, Logan, UT). Cells were plated at 10^5 cells per well in 6 well plates the day before treatment, in media containing 10% calf serum. Media was changed to modified Eagle's medium with 2% calf serum concomitant with addition of treatments (Eller *et al*, 1996). Treatments were added to cell culture media either neat or dissolved in ethanol or water.

Normal human epidermal melanocytes (NHEM) from black-neonatal (Bl-Neo) male (Lot. 14466), white-neonatal (Wh-Neo) male (Lot. 13043), and white-adult (Wh-Ad) female (Lot. 14604) donors were obtained from Clonetics (San Diego, CA), and cultured exactly as prescribed by the supplier, except for experiments in which alternative culture conditions were employed as noted below. Media was Clonetics Melanocyte Growth Medium-3 BulletKit^R with constituents at a final concentration of 0.5% fetal bovine serum, 7.5 μg bovine pituitary extract per ml, 2 ng human recombinant fibroblast growth factor per ml, 16 nM phorbol myristate acetate (PMA), 10 μg insulin per ml, 10 μg hydrocortisone per ml, 100 μg gentamicin per ml, and 100 ng amphotericin-B per ml in Clonetics Melanocyte Growth Medium (MGM^R). Cells were cultured for at least 2 wk in alternative culture conditions – either without PMA but with 10 nM cholera toxin (No PMA/+CT) or without PMA or CT (No PMA/No CT) – before addition of treatments. Cells were plated at 10^5 cells/P60 2–4 d before addition of treatments. Treatments were as described above for S91 cells.

Melanin analysis Media was removed from cells. S91 cells were washed twice with 2 ml 1XPBS (BioWhittaker), detached with 0.05% trypsin/ethylenediamine tetraacetic acid (BioWhittaker), and counted on a Coulter (Hialeah, FL) counter. NHEM were washed once with 3 ml HEPES buffered saline solution (Clonetics), detached with 2 ml of 0.025% trypsin/ethylenediamine tetraacetic acid (Clonetics), and similarly counted. S91 or NHEM were centrifuged at 200 × *g* for 5 min, and then washed with 1XPBS. Cells were solubilized in 1N NaOH by vigorous vortexing. In some cases, media was retained and centrifuged at 1600 × *g* for 5 min for analysis of melanin in the extracellular particulate fraction, using the same procedures as for cells. Melanin was measured as absorbance at 475 nm, by comparison with melanin standards. Melanin was calculated per cell.

Tyrosinase analysis Cells were trypsinized, counted, and pelleted as described above for melanin assays. In some cases, media was retained and centrifuged at 1600 × *g* for 5 min for analysis of tyrosinase activity in the extracellular particulate fraction and in the supernatant media fraction, using modifications of procedures described by Pomerantz (1966), Jara *et al* (1988), and Gilchrest *et al* (1993). Each sample was analyzed with and without L-Dopa, a necessary cofactor for tyrosinase (Pomerantz, 1966; McLane *et al*, 1987). All reported tyrosinase values are exclusive of counts that occurred in buffer blanks and L-Dopa negative aliquots. Protein was determined by the Bradford method (Bradford, 1976).

Electron microscopy of extracellular particulate fraction The extracellular particulate fraction was collected from untreated or 5 mM 5-NBene-2,2-DM treated Bl-Neo NHEM by centrifuging media at 1600 × *g* for 5 min and washing twice in 0.12 M phosphate buffer pH 7.4. Melanosome pellets were initially fixed with 2% glutaraldehyde in 0.12 M phosphate buffer, followed by a postfixation in 1% osmium tetroxide in the same buffer, and then en bloc staining with 2% aqueous uranyl acetate. Samples were dehydrated with a graded ethanol series (35%–100%), followed by treatment with propylene oxide. Samples were then embedded in Spurr's epoxy resin and thin sections were mounted on copper grids. These were further stained with uranyl acetate and lead citrate before viewing in a Zeiss (Oberkochen, Germany) EM10 CA electron microscope operated at 80 KV.

Treatment of guinea pigs American short-haired guinea pigs were obtained from Kuiper Rabbit Ranch (Chicago, IL). Animals were maintained and treated at Biocon (Rockville, MD). Three days prior to initiation of treatments, the backs of guinea pigs were shaved. On the day treatments began, fur-free treatment spots were created by application of the depilatory agent Nair (Carter-Wallace, New York, NY) for 15 min. Test agents were applied twice a day for 5 d at 20 μl per application. In the first set of experiments (see **Fig 7*A*, *B***; **Table III**), treatment agents were full strength (13.6 M) 1,2-PD, full strength (11.26 M) 2-M-1,3-PD, and full strength (10.95 M) 2,3-BD. In the second set of experiments, treatments agents were 10.95 M 1,2-PD in 20% ETOH, 4 M 3,3-DM-1,2-BD in 20% ETOH, or 8.7 M 1,2-cs-CPD in 20% ETOH (see **Figs 7*C*, 8*A***; **Table III**). In the third set of experiments treatment agents were 2% 2-pyrrolidone in 20% ETOH (2P), 10.6 M 1,2-PD in 20% ETOH and 2% 2-pyrrolidone (1,2-PD/2P), 1 M 5-norbornene-2,2-dimethanol in 20% ETOH and 2% 2-pyrrolidone (5-NBene-2,2-DM/2P), and 1 M 5-NBene-2,2-DM in 8.5 M 1,2-PD, 20% ETOH, and 2% 2-pyrrolidone (5-NBene-2,2-DM/1,2-PD/2P) (see **Fig 8*C*, *D***; **Table III**). Two weeks after the cessation of treatments, backs of guinea pigs were shaved and treated with Nair to remove all fur. Treatment spots were then subjectively evaluated for degree of melanogenesis according to the following scale: 0, no change from background; +0.25, slight darkening, indistinct; +0.5, slight darkening; +1, slight to moderate darkening; +2, moderate, even darkening; +3, substantial, even darkening; +4, profound, even darkening. Animals were recorded on videotape for generation of the images shown in **Fig 8**. Punch biopsies were collected from the center of treatment spots and fixed in 10% formalin for standard histologic preparation, followed by staining by Fontana-Masson or hematoxylin and eosin.

RESULTS

Diols increase melanin levels in S91 melanoma cells During the course of screening for melanogenic compounds we found several aliphatic and alicyclic diols that induced melanogenesis in S91 mouse melanoma cells (**Figs 1, 2*A***). For each compound an initial dose range was determined, followed by a dose response analysis over a much narrower concentration range. In at least a dozen different experiments, 100–300 mM 1,2-PD induced a dose dependent increase

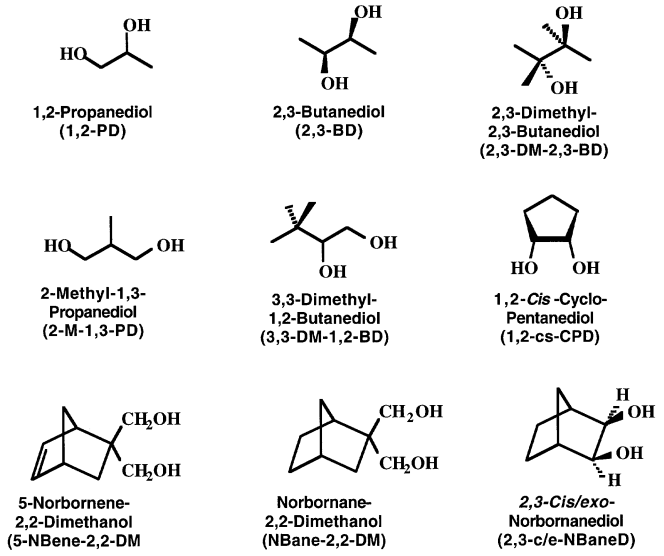

Figure 1. Structure of diols examined in this study. Abbreviations are given in parentheses below the name of each compound.

of melanin in S91 cells, with maximal induction in the 3–4-fold range (e.g., **Fig 2*A***). 2-M-1,3-PD and 2,3-DM-2,3-BD were more potent, exhibiting a similar magnitude of induction but with treatments in the 50–100 mM and 12.5–50 mM range, respectively (**Fig 2*A***). 1,2-cs-CPD, 3,3-DM-1,2-BD, and 5-NBene-2,2-DM were even more potent, with melanin induction in the 5–8-fold range at 50 mM, 25 mM, and 5 mM, respectively (**Fig 2*A***). IBMX or α-MSH were utilized as positive controls in all experiments, and consistently induced 6–8-fold induction of melanin at 0.1 mM and 0.1 μM, respectively (**Fig 2*A***). Melanogenesis was not induced in S91 cells by ethanol, 1-propanol, 2-propanol, ascorbic acid, tartaric acid, 2-pyrrolidone, oleic acid, Azone, D-ribose, D-Deoxyribose, thymidine glycol, thymine glycol, or hydroxymethylurea when tested up to toxic concentrations (data not shown). Other compounds that did not induce melanogenesis when tested at subtoxic levels included glycerol and ethylene glycol (data not shown).

Diols increase tyrosinase activity in S91 melanoma cells To further confirm the melanogenic activity of diols, we examined tyrosinase activity in extracts of S91 cells (**Fig 2*B***) treated with concentrations of diols that increased melanin levels 3–8-fold in S91 cells (**Fig 2*A***). In accordance with results for induction of melanin, 3,3-DM-1,2-BD and 5-NBene-2,2-DM were not only melanogenic at lower concentrations than 1,2-PD, but also induced higher levels of tyrosinase activity.

5-NBene-2,2-DM and NBane-2,2-DM exhibit equivalent melanogenic potency in S91 cells Because 5-NBene-2,2-DM was the most potent melanogenic diol of those tested in S91 cells, further studies were carried out to determine how melanogenic activity varied with the structure of norbornene and NBane analogs. Results indicate that 5-NBene-2,2-DM and NBane-2,2-DM have virtually equivalent potency for induction of tyrosinase in S91 cells (**Fig 3**). When tested at 5 mM, less tyrosinase activity was induced in S91 cells by other NBane analogs, in the following order of potency: 2-NBaneM > 2,3-c/e-NBaneD > α-NBane-ol > NBane. 2,3-c/e-NBaneD and α-NBane-ol exhibited similar potency at 1 mM and 5 mM.

Diols induce morphologic differentiation and reduce proliferation of S91 melanoma cells The diols induced morphologic differentiation of S91 cells as evidenced by conversion from a predominantly rounded bipolar morphology in untreated cells, to a flattened multipolar morphology in treated cells (**Fig 4**). Acquisition of the flattened multipolar phenotype was associated with dramatic increases in the time required to detach S91 cells from dishes by trypsin, suggesting a change in the interaction of the cells with the extracellular matrix. Furthermore, morphologic differentiation of S91 cells was

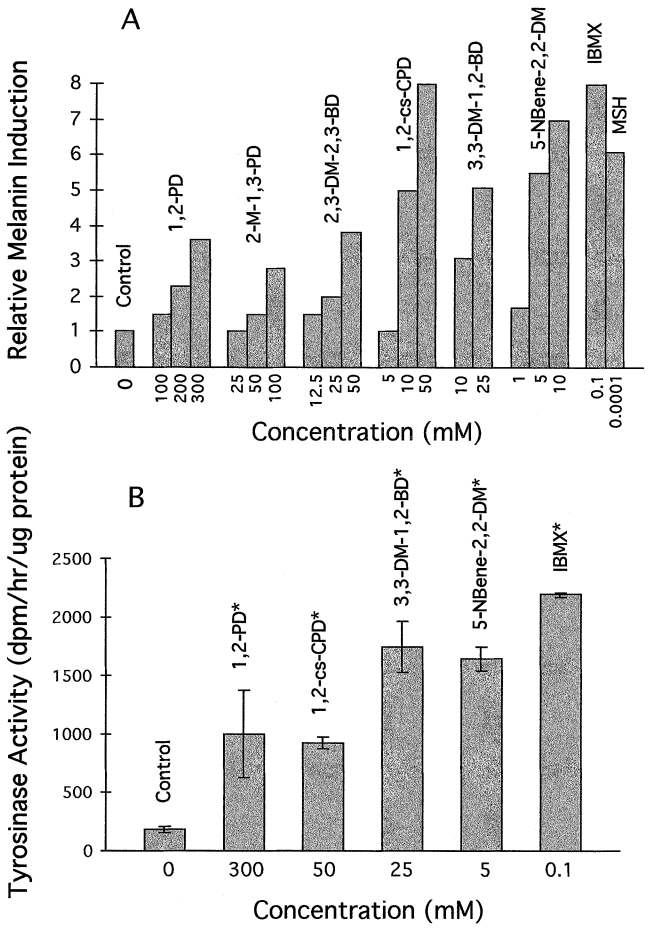

Figure 2. Diols increase melanin content and tyrosinase activity of S91 mouse melanoma cells. Cells (10^5) were treated with the indicated concentrations of diols, IBMX, or α-MSH and harvested 6 d later for analysis of melanin content or tyrosinase activity. (*A*) Induction of melanin by diols, IBMX, and α-MSH in S91 mouse melanoma cells. Melanin was extracted with NaOH and measured by optical density at 475 nm. Values are relative to untreated controls. 1,2-PD, 1,2-propanediol; 2-M-1,3-PD, 2-methyl-1,3-propanediol; 2,3-DM-2,3-BD, 2,3-dimethyl-2,3-butanediol; 1,2-cs-CPD, 1,2-*cis*-cyclopentanediol; 3,3-DM-1,2-BD, 3,3-dimethyl-1,2-butanediol; 5-Nbene-2,2-DM, 5-norbornene-2,2-dimethanol; IBMX, 3-isobutyl-1-methylxanthine; α-MSH, α-melanocyte stimulating hormone. (*B*) Induction of tyrosinase by diols and IBMX in S91 mouse melanoma cells. Tyrosinase activity was measured in cell extracts by tritium release from L-[3,5-^{3}H]tyrosine and normalized to cellular protein. Values are mean ± SEM (N = 4 for Control, 1,2-PD, and 5-NBene-2,2-DM; N = 2 for 1,2-cs-CPD, 3,3-DM-1,2-BD, and IBMX; *$p < 0.05$; Students t test).

accompanied by up to 3-fold increases of protein/cell for 5 mM 5-NBene-2,2-DM and 25 mM 3,3-DM-1,2-BD (data not shown). At the end of the incubation period untreated cells typically showed a 5-fold increase in cell number, whereas the cells treated with the compounds increased by about a third of the control value. It is important to emphasize that the reductions of cell growth induced by diols were never accompanied by rounding or detachment of cells or a decline in cell numbers relative to the starting plating density.

Diols induce the greatest increases of melanin in the extracellular particulate fraction of NHEM The preceding experiments were performed with tumor derived S91 mouse melanoma cells. We were interested in measuring the activity of the diols on primary human melanocytes in culture. As described in *Materials and Methods*, we used preparations of NHEM derived from Wh-Neo or Wh-Ad donors as well as cells from a Bl-Neo donor. Published work indicated that treatment of primary human melanocytes with IBMX, UVR, or other agents resulted in only modest increases in cellular melanin content (Abdel-Malek *et al*, 1992, 1994; Fuller *et al*, 1993). In our initial

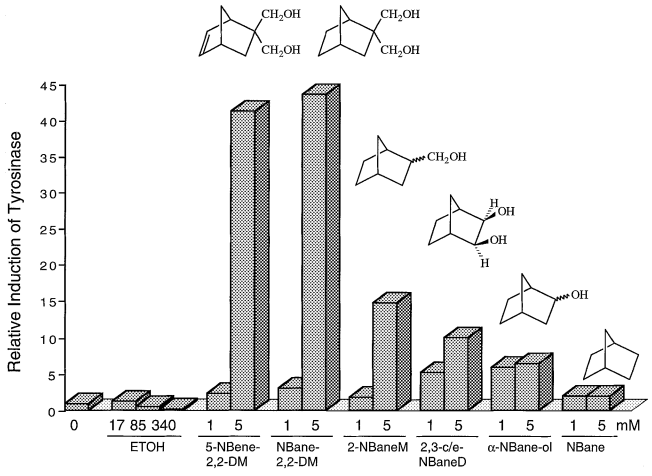

Figure 3. 5-NBene-2,2-DM and NBane-2,2-DM induce equivalent tyrosinase activity in S91 cells. When tested at 5 mM, NBane analogs induced tyrosinase activity in the following relative order of potency: 5-norbornene-2,2-dimethanol (5-NBene-2,2-DM) > norbornane-2,2-dimethanol (NBane-2,2-DM) > 2-norbornanemethanol (2-NBaneM) > 2,3-*cis*/*exo*-norbornanediol (2,3-c/e-NBaneD) > α-norbornaneol (α-NBaneM) > norbornane (NBane). Tyrosinase activity was measured in cell extracts by release of tritium from L-[3,5-^{3}H]tyrosine. Values are relative to untreated controls.

Table I. Induction of melanin (ng per 10^3 cells) in cells and in the extracellular particulate fraction of NHEM from a Wh-Neo male donor treated with diols

	Cellular	1600 × *g* Partic
Control	20 (1.0×)	1.0 (1.0×)
25 mM 2,3-DM-2,3-BD	20 (1.0×)	0.7 (0.7×)
50 mM 2,3-DM-2,3-BD	31 (1.6×)	3.9 (3.9×)
25 mM 1,2-cs-CPD	21 (1.1×)	2.9 (2.9×)
50 mM 1,2-cs-CPD	29 (1.5×)	13 (13×)
12.5 mM 3,3-DM-1,2-BD	31 (1.6×)	5.9 (5.9×)
25 mM 3,3-DM-1,2-BD	41 (2.1×)	30 (30×)

experiments we also observed that the diols as well as IBMX induced only small increases (1.5–2-fold) in melanin levels in NHEM, results that were consistent with the literature; however, we noted a marked increase in the presence of dark brown colored particulate material floating in the media of diol and IBMX treated Wh-Neo NHEM cells. Because we suspected this particulate material might contain exported membrane bound melanosomes of the type previously seen in recipient keratinocytes (Szabo *et al*, 1969), we sedimented this particulate material from media by centrifugation at 1600 × *g* and measured melanin levels. Although cellular melanin levels were increased no more than 2-fold in the Wh-Neo NHEM treated with diols, melanin in the extracellular particulate fraction was increased up to 30-fold relative to untreated controls (**Table I**). Consequently, in subsequent studies in which tyrosinase was measured, measurements were done on both cellular and extracellular particulate fractions (see below).

Diols induce the greatest increases of tyrosinase in the extracellular particulate fraction of NHEM Tyrosinase was measured in NHEM using procedures identical to those for S91 cells (Fuller *et al*, 1993). In addition, media from 5 d treatment periods was retained and centrifuged at 1600 × *g* for analysis of tyrosinase activity in the extracellular particulate fraction, and in the resultant supernatant media fraction. In some cases (**Table II**), tyrosinase was also measured by an *in situ* assay wherein radiolabeled tyrosine was added directly to freshly replaced media of NHEM for a period of 24 h following a 6 d treatment period (Abdel-Malek *et al*, 1992).

Results using Bl-Neo NHEM showed that of the diols tested, 5 mM 5-NBene-2,2-DM induced the highest levels of tyrosinase in the *in situ* assay, in cellular extracts, in the extracellular particulate fractions, and in the media. Both 5 mM 5-NBene-2,2-DM and 25 mM 3,3-DM-1,2-BD induced more tyrosinase in each of these assays/fractions than did 300 mM 1,2-PD. IBMX, used as a positive control, induced as much tyrosinase activity as 5 mM 5-NBene-2,2-DM when measured by the *in situ* assay, but less in cellular, extracellular particulate, and media fractions (**Table II**). The largest increases of tyrosinase relative to controls occurred in the particulate fraction, followed by the media fraction, and the cellular fraction. Although *in situ* and cellular assays showed different absolute amounts of tyrosinase activity, for the most part these assays showed similar relative induction of tyrosinase with treatments.

The appearance of enhanced tyrosinase activity in the particulate fraction also occurred in melanocytes from white donors. Treatment of Wh-Ad NHEM with diols or IBMX resulted in increases of tyrosinase in the 1600 × *g* extracellular particulate fraction comparable with those induced in Bl-Neo NHEM (**Fig 5**). For both Bl and Wh NHEM, induction of extracellular particulate tyrosinase was greater in +PMA/No CT media than in No PMA/+CT media (data not shown). Similar to the results of Abdel-Malek *et al* (1992), cells cultured in No PMA/No CT media grew poorly and showed little or no response to treatments (data not shown). Tyrosinase activity was undetectable in similarly collected extracellular fractions from S91 cells treated with concentrations of diols or IBMX that induced maximal increases of S91 cellular tyrosinase (data not shown).

Diols induce morphologic differentiation of normal human epidermal melanocytes Similar to the results for S91 cells, diols induced conversion of NHEM from a bipolar phenotype to a multidendritic phenotype (**Fig 6*A*, *B***), accompanied by reductions in cell growth of up to 2-fold for 25 mM 3,3-DM-1,2-BD and 5 mM 5-NBene-2,2-DM relative to untreated controls (data not shown). Again, as with the S91 cells, there were no reductions of cell numbers below the original number plated. Dark particulate material could be observed within dendrites and in vesicles at the end of dendrites (**Fig 6*A*, *B***). Some of these vesicles appeared to be pinching off from the ends of dendrites concomitant with increases in the levels of melanoma and tyrosinase in the extracellular particulate fraction (**Figs 5, 6*C*, *D***, **Tables I** and **II**). Unlike S91 cells, morphologic differentiation of NHEM was accompanied by only very small increases of protein/cell (up to 1.5-fold), and no increases of the time required to detach cells from dishes by trypsin (data not shown).

Electron microscopy shows that the extracellular particulate fraction of NHEM is composed primarily of melanosomes Increases of both melanin and tyrosinase in the extracellular particulate fraction of NHEM strongly suggested that this fraction was enriched in melanosomes. Subsequent studies showed that the lysosomal/melanosomal enzyme β-glucuronidase (Fishman *et al*, 1967) was enriched 3–4-fold (normalized to protein) in the extracellular particulate fraction of NHEM treated with diols or IBMX relative to untreated controls (data not shown). To verify the presence of melanosomes, the extracellular particulate fraction was sedimented by centrifugation at 1600 × *g* and examined by electron microscopy. Results showed that this fraction was composed almost exclusively of melanosomes, most of which exhibited a striated or solid matrix indicative of stage III or IV melanosomes, respectively (**Fig 6*C*, *D***). Although there were many fold more melanosomes in the extracellular particulates fraction of 5-NBene-2,2-DM treated Bl-Neo-NHEM relative to untreated controls, there were no readily apparent qualitative differences in the ultrastructure of melanosomes as a result of treatment.

Diols induce melanogenesis when applied to guinea pig skin Following treatment with UVR or UVR and MSH, melanocytes are distributed in guinea pig skin similarly to their distribution in human skin (Bolognia *et al*, 1989). We were interested in determining if diols would also induce melanogenesis in this animal model. In our initial experiments we found that treatment of shaved and Naired patches indicated a clear positional effect, whereby the effect of agents was greatest towards the heads of animals and significantly less towards the tail regions. For example, treatment with undiluted 1,2-PD (13.91 M) resulted in an unambiguous melanogenic response in anterior treatment

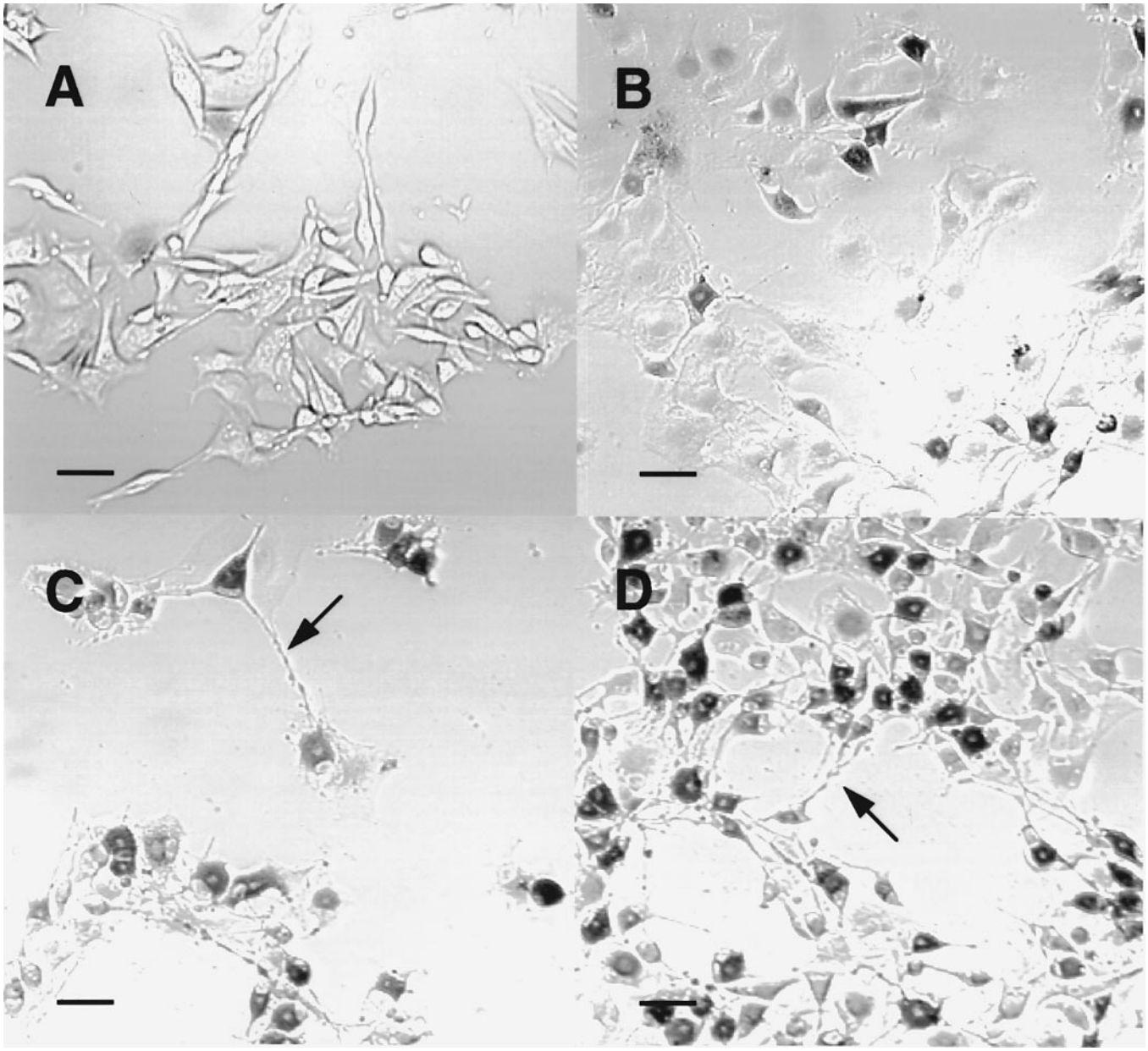

Figure 4. Diols increase melanin content and induce morphologic differentiation of S91 mouse melanoma cells. Untreated S91 cells exhibit a rounded and bipolar phenotype, whereas cells treated with diols exhibit a flattened multipolar phenotype with extended dendritic processes. Cells were treated with the indicated concentrations of diols and photographed (unstained) 6 d later. (*A*) Untreated; (*B*) 300 mM 1,2-propanediol; (*C*) 25 mM 3,3-dimethyl-1,2-butanediol; (*D*) 5 mM 5-norbornene-2,2-dimethanol. *Arrows* indicate extended dendritic processes. *Scale bars*, 50 μm.

Table II. Comparison of tyrosinase (dpm per h per 10^3 cells) activity measured in an *in situ* assay with tyrosinase measured in cellular extracts, extracts of extracellular particulate fractions, and media of NHEM from a Bl-Neo donor treated with diols or IBMX

	In situ	Cellular	1600 × *g* Partic	Media[a]
Control	16.8	10259	341	1457
85 mM ETOH	15.0 (1.00×)	10201 (1.00×)	574 (1.0×)	1654 (1.00×)
300 mM 1,2-PD	16.8	10247	535	1864
300 mM 1,2-PD	17.2 (1.07×)	10875 (1.03×)	1164 (1.9×)	2123 (1.3×)
25 mM 3,3-DM-1,2-BD	20.5	11728	2182	5495
25 mM 3,3-DM-1,2-BD	21.0 (1.31×)	11730 (1.15×)	2651 (5.3×)	3056 (2.8×)
5 mM 5-NBene-2,2-DM	24.5	13838	6940	4164
5 mM 5-NBene-2,2-DM	25.4 (1.57×)	14716 (1.40×)	6764 (15.0×)	4639 (2.83×)
0.1 mM IBMX	25.3	10910	2409	2698
0.1 mM IBMX	26.1 (1.62×)	11737 (1.11×)	2094 (4.9×)	2935 (1.81×)

[a]Post 1600 × *g*.

Table III. Comparison of pigmentation response in the posterior half of guinea pigs treated with diols, with and without the penetration enhancer 2P. Results are normalized to 1,2-PD for comparison of relative potency

Pigmentation treatment	Rating	Normalized to 1,2-PD[a]
No penetration enhancer		
Nair	0.08 ± 0.05 (n = 6)	
13.61 M 1,2-PD	0.29 ± 0.09 (n = 12)	1.0×
11.0 M 2,3-DM-2,3-BD	0.25 ± 0.14 (n = 3)	1.0×
11.3 M 2-M-1,3-PD	0.58 ± 0.08[b] (n = 3)	2.9×
8.7 M 1,2-cs-CPD	1.89 ± 0.27[b] (n = 9)	13.5×
4.0 M 3,3-DM-1,2-BD	1.17 ± 0.44[b] (n = 3)	18.1×
Penetration enhancer 2% 2P		
2P	0.17 ± 0.08 (n = 6)	
10.6 M 1,2-PD/2P	0.33 ± 0.05 (n = 6)	1.0×
1.0 M 5-NBene-2,2-DM/2P	0.66 ± 0.05[b] (n = 6)	32.7×
1.0 M 5-NBene-2,2-DM/ 8.5 M 1,2-PD/2P	1.00 ± 0.13[b] (n = 6)	44.7×

[a]Background corrected for Nair or vehicle treated control spots.
[b]$p < 0.05$; Students t test.

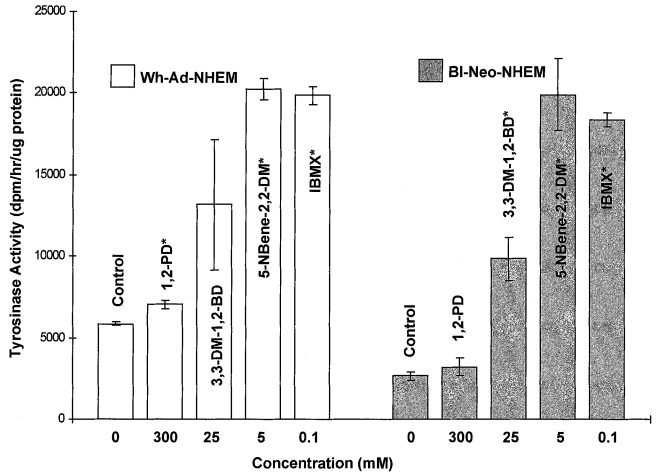

Figure 5. The media of NHEM treated with diols or IBMX contains an extracellular particulate fraction that is enriched in tyrosinase. NHEM (10^5) from a neonatal black donor were treated with the indicated concentrations of diols or 3-isobutyl-1-methylxanthine (IBMX), and the extracellular particulate fraction was harvested 6 d later by centrifugation at $1600 \times g$. Tyrosinase activity was measured in extracts of the extracellular particulate fraction by tritium release from L-[3,5-^{3}H]tyrosine. Results were normalized to protein content of extracellular particulate fractions. Induction of tyrosinase by diols and IBMX in the extracellular particulate fraction was similar for Wh-Ad female and Bl-Neo male NHEM. 1,2-PD, 1,2-propanediol; 3,3-DM-1,2-BD, 3,3-dimethyl-1,2-butanediol; 5-NBene-2,2-DM, 5-norbornene-2,2-dimethanol. Mean ± SEM (N = 2; *$p < 0.05$; Students t test).

spots, whereas posterior treatment spots showed either marginal or negligible effects (**Fig 7*A***). Both 2-M-1,3-PD and 2,3-BD resulted in a slightly greater melanogenic response than 1,2-PD when applied to guinea pigs and compared within equivalent anterior-posterior treatment locations (**Fig 7*B***). When 3,3-DM-1,2-BD and 1,2-cs-CPD were applied to posterior regions of animals, they resulted in a much greater melanogenic response than 1,2-PD when applied at the same treatment locations (**Fig 7*C***).

In order to minimize the effects of diminution of response from the head to the tails of animals, all subsequent data analyses (**Table III**) were carried out using only treatment spots located on the posterior half of animals (spots c or d, **Figs 7**, **8**). In this area of the animals, differences of responsiveness to strong and weak inducers of pigmentation, as deduced from cell culture, were greatest (e.g., **Fig 8*B–D***). The compounds were either applied neat or dissolved in as high a concentration as possible in ETOH, or ETOH with the penetration enhancer 2-pyrrolidone (2P). Comparison of treatment spots in the posterior half of animals showed the following descending order of pigmentation response: 8.7 M 1,2-cs-CPD > 4 M 3,3-M-1,2-BD > 8.5 M 1,2-PD/1 M 5-NBene-2,2-DM/2% 2P > 1 M 5-NBene-2,2-DM/2% 2P > 11.3 M 2-M-1,3-PD (**Table III**). In the posterior half of animals, responses to 13.61 M 1,2-PD, 10.6 M 1,2-PD/2% 2P, and 11 M 2,3-DM-2,3-BD were not significantly different from control (Nair or 2% 2P treated) spots. Pigmentation ratings were corrected for background (control treatment spots), normalized to account for the different amounts of each agent applied, and then normalized to results for 1,2-PD (**Table III**). This comparison showed that the descending order of relative potency was 5-NBene-2,2-DM > 3,3-DM-1,2-BD > 1,2-cs-CPD >> 2-M-1,3-PD, and that using 1,2-PD as carrier for 5-NBene-2,2-DM increased responsiveness to this compound. Although we did employ 2P as a penetration enhancer with some of the compounds, delivery of the compounds was not optimized. In the case of 5-NBene-2,2-DM, undissolved compound was visible on the surface of the skin after application, suggesting that the potency assessment is likely to be underestimated.

Biopsies taken from the posterior half of guinea pigs showed that induction of melanogenesis was marked by deposition of melanin in keratinocytes, in some cases with formation of "supranuclear caps" (**Fig 9*B***, ***D***), indicative of induction of true natural UV protective melanogenesis (Gates and Zimmermann, 1953). Skin biopsies taken from untreated guinea pigs exhibited melanocytes and melanin in the basal layer of the epidermis only (**Fig 9*A***). Biopsies taken from the posterior half of guinea pigs showed very little response to treatment with 1,2-PD (**Fig 9*C***). Biopsies from animals treated with 3,3-DM-1,2-BD, 1,2-cs-CPD, or 5-NBene-2,2-DM or 5-NBene-2,2-DM/1,2-PD exhibited increased melanin in the basal layer of the epidermis, increased melanin in epidermal keratinocytes, and increased thickness of the epidermis (**Fig 9*B***, ***D***, and data not shown). Examination of biopsies stained by hematoxylin and eosin exhibited no signs of inflammation or scarring from treatment with diols (data not shown).

DISCUSSION

This study is the first to demonstrate that several aliphatic and alicyclic diols can induce melanogenesis. This induction has been demonstrated in several different model systems, including cultured S91 mouse melanoma cells, cultured NHEM, and guinea pig skin. In skin, melanogenesis *in vivo* is thought to occur primarily as a result of cofactors released from keratinocytes, fibroblasts, and other cells in the skin as a result of UV irradiation (reviewed in Jimbow *et al*, 1991, 1993; Gilchrest *et al*, 1996); however, results presented here using cultured S91 cells and NHEM show that diols act directly on melanocytes, without the necessity of an exogenous cellular source of cofactors. Thus, in guinea pig skin, it is likely that diols stimulate melanogenesis by direct action on melanocytes, although the possibility of additional indirect effects cannot be excluded.

Although treatment with diols or IBMX resulted in large increases of melanin and tyrosinase within S91 cells, such increases were relatively small within NHEM. Rather, treatment of NHEM with diols or IBMX resulted in dramatic increases of melanin and tyrosinase in an extracellular particulate fraction, shown by electron microscopy to consist almost entirely of phase III and IV melanosomes. Additionally, microscopic observation of NHEM indicated that treatment with diols resulted in induction of a profoundly differentiated phenotype characterized by arrest of cell division and acquisition of a multidendritic morphology. Moreover, induction of this phenotype by diols or IBMX was characterized by an apparent budding process wherein granular appearing vesicles were observed in various stages of development and release from dendrites. Release of melanosomes from melanocytes occurs as a component of the natural melanogenic process whereby melanosomes are transferred to keratinocytes that proliferate and carry melanin towards the surface of the skin (reviewed in Jimbow *et al*, 1991, 1993). Our results indicate that in cell culture, in the absence of keratinocytes, the melanosomal fraction accumulates in the media.

Recent studies indicate that S91 mouse melanoma cells are unable to export melanosomes because of a defective myosin-V transport

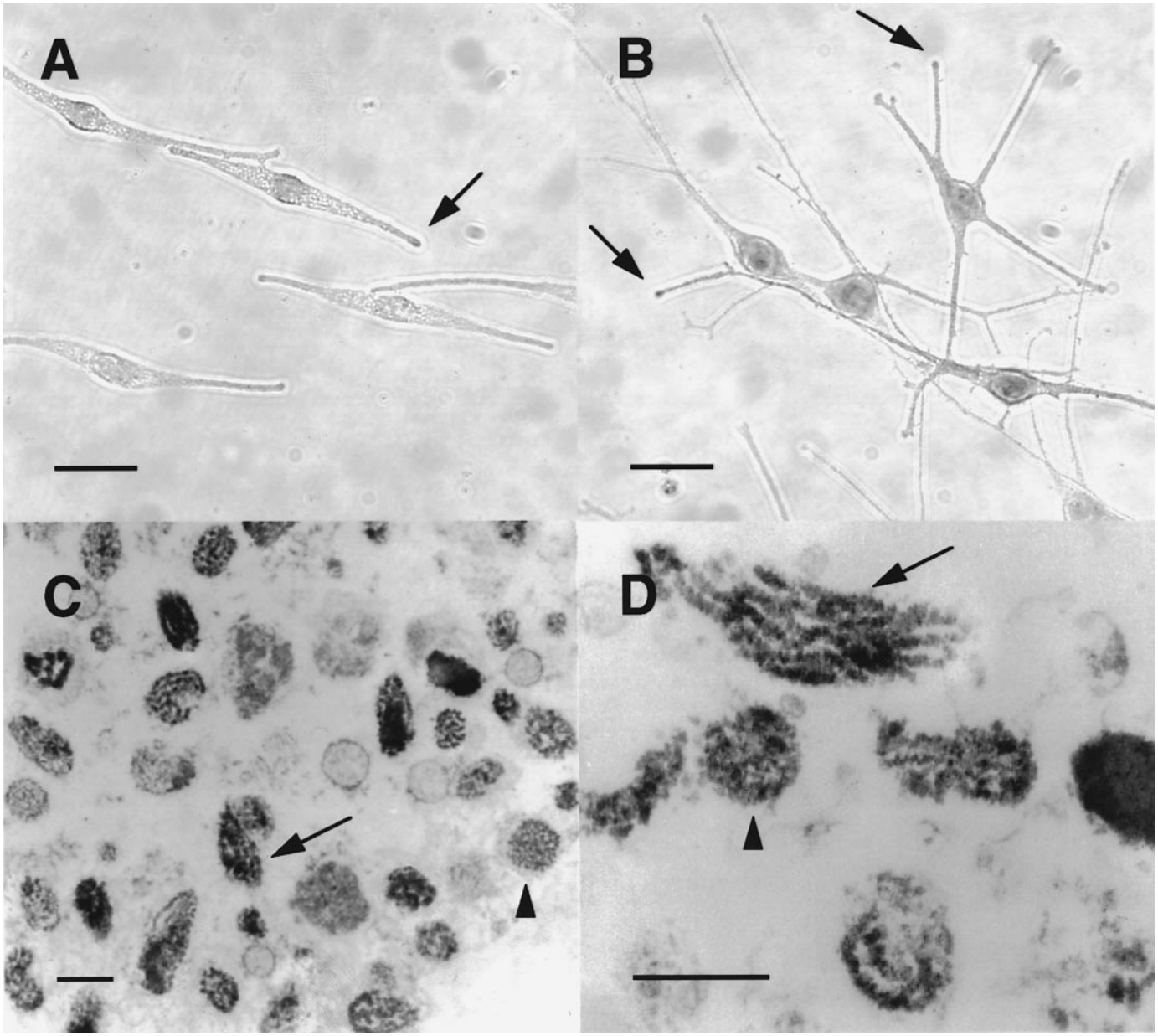

Figure 6. Cultured NHEM treated with 5-NBene-2,2-DM secrete melanosomes into the media. Photographs of unstained cultures of Bl-Neo male NHEM showing formation of secretory vesicles (*arrows*): (*A*) untreated; (*B*) 5-NBene-2,2-DM. *Scale bars*, 25 μm. (*C*) and (*D*) Electron micrographs of the extracellular particulate fraction from 5-NBene-2,2-DM treated Bl-Neo male NHEM showing striated melanosomal matrix in longitudinal (*arrows*) and cross-sectional (*arrowheads*) views. *Scale bars*, 0.25 μm.

system (Wu *et al*, 1997). In accordance with this finding, our results show that upon treatment with diols, S91 melanoma cells do not export melanin or tyrosinase, but rather accumulate these intracellularly. Although it has been suggested that L-Dopa or X-irradiation can result in the release of tyrosinase into the media as a result of membrane damage or cell disintegration (Schachtschabel *et al*, 1988), the absence of extracellular tyrosinase in the media of myosin-V defective S91 cells treated with diols or IBMX indicates that these agents are not acting via such a mechanism. In addition, unlike L-Dopa and X-irradiation that cause leakage and reductions of cellular protein levels (Schachtschabel *et al*, 1988), diols and IBMX result in increases of protein levels in both S91 cells and NHEM.

Treatment with the diols described in this study transformed S91 cells from a rounded bipolar phenotype to a flattened multipolar cuboidal phenotype. Concomitant with this transformation, there was a dramatic reduction of proliferation of the cells, increased pigmentation, and increased adherence to substrate (monitored by increased trypsin detachment time). Similar morphologic changes and effects on proliferation, consistent with terminal differentiation, have been reported for S91 cells treated with phenylacetate and phenylbutyrate (Liu *et al*, 1994, 1995), and retinoids (Lauharanta *et al*, 1985). These compounds are now in clinical trials as anti-cancer differentiation agents (Thibault *et al*, 1994; Moon *et al*, 1997). Several of the compounds described in this study, most notably the norbornanediol and related compounds, are active in the same 1–5 mM concentration range as phenylacetate and phenylbutyrate (Liu *et al*, 1994, 1995). Further studies are needed to ascertain if the diols described in this study have *in vivo* anti-cancer activity in animal models.

The involvement of protein kinase C and protein kinase A pathways in the induction of melanogenesis by the diols is an important question. In preliminary experiments (D. Brown *et al*, in preparation) our results suggest that, like the situation with UVR (Friedmann and Gilchrest, 1987; Carsberg *et al*, 1994; Romero-Graillet *et al*, 1996), induction of melanogenesis by diols does not appear to be dependent on stimulation of protein kinase A and protein kinase C pathways. Furthermore, addition of catalase to the culture media did not alter the melanogenic

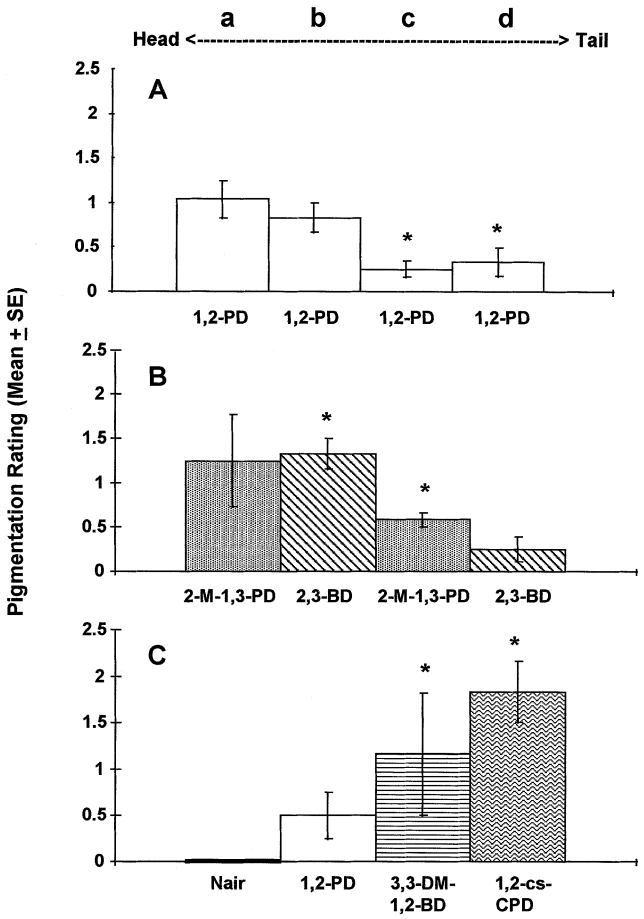

Figure 7. Guinea pigs treated with diols show a diminished pigmentation response along the anterior-posterior axis. Pigmentation ratings of guinea pigs treated with the following. (*A*) 13.61 M 1,2-PD showing diminution of response along the anterior-posterior axis (see lower row a–d in **Fig 8*B***, and upper row a–d in **Fig 8*C*, *D***). *Significantly different ($p < 0.05$ Students t test; mean ± SEM, N = 6) from 1,2-PD treated spot nearest head. (*B*) 11.26 M 2-M-1,3-PD and 10.95 M 2,3-BD showing diminution of response along the anterior-posterior axis. *Significantly different ($p < 0.05$ Students t test; mean ± SEM, N = 3) than 1,2-PD treated spot at same posterior-anterior location in (*A*). (*C*) Nair (control), 10.95 M 1,2-PD, 4 M 3,3-DM-1,2-BD, and 8.7 M 1,2-cs-CPD in 20% ETOH (see row a–d in **Fig 8*A***) showing markedly increased responsiveness to 3,3-DM-1,2-BD and 1,2-cs-CPD in the posterior regions of animals relative to compounds tested in (*A*) and (*B*). *Significantly different ($p < 0.05$ Students t test; mean ± SEM, N = 3) than 1,2-PD treated spot at same posterior-anterior location in (*A*).

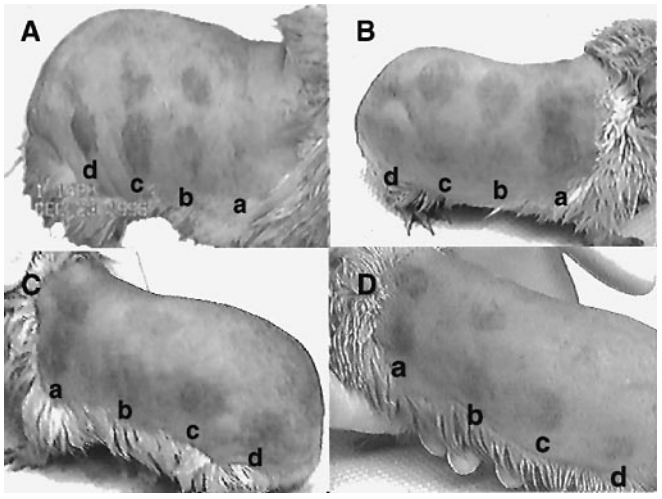

Figure 8. Guinea pigs treated with diols showing pigmentation responses. (*A*) Nair (a), 10.95 M 1,2-PD (b), 4 M 3,3-DM-1,2-BD (c), and 8.7 M 1,2-cs-CPD (d) shown in the lower row of treatment spots (the upper row is from an unrelated experiment). (*B*) 8.7 M 1,2-cs-CPD (a–d in upper row) or 10.6 M 1,2-PD (a–d in lower row) in 20% ETOH, showing that diminution of response along the anterior-posterior axis is greater for 1,2-PD than for 1,2-cs-CPD. (*C*, *D*) 10.6 M 1,2-PD (a–d in upper row) and 1 M 5-NBene-2,2-DM/8.5 M 1,2-PD (a–d in lower row) in 20% ETOH with 2% 2P showing diminution of response to 1,2-PD (upper row), but little diminution of response to 5-NBene-2,2-DM/1,2-PD.

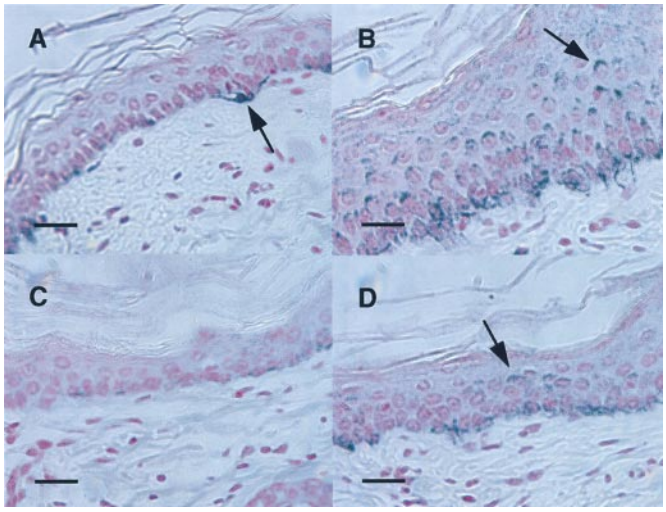

Figure 9. Biopsies from guinea pigs showing histologic features of pigmentation responses. (*A*) Biopsy taken from untreated control spot located between c and d spots of guinea pig in **Fig 8*C***, showing dendritic melanocyte in basal layer of epidermis (*arrow*); (*B*) biopsy taken from 8.7 M 1,2-cs-CPD treated spot of guinea pig in **Fig 8*B*** (c in upper row), showing increased melanin content in epidermis and formation of "supranuclear caps" (e.g., *arrow*); (*C*) biopsy taken from 10.6 M 1,2-PD/2% 2P treated spot of guinea pig shown in **Fig 8*C***; (*D*) biopsy taken from 1 M 5-NBene-2,2-DM/8.5 M 1,2-PD/2% 2P treated spot of guinea pig shown in **Fig 8*C*** (c in lower row) showing increased melanin content and supranuclear caps (*arrow*). *Scale bars*, 100 μm.

response to diols (D. Brown *et al*, unpublished), suggesting that unlike Dopac and other catechols, diols do not act by melanogenic mechanisms involving quinone formation (Karg *et al*, 1989, 1991, 1993). Additional studies will be required to delineate the signal transduction pathways stimulated by the diols.

Treatment of guinea pigs with diols resulted in melanogenic responses that were virtually identical to those that resulted from treatment of guinea pigs with UVB or UVB + MSH (Bolognia *et al*, 1989), in that melanin was distributed to keratinocytes and the thickness of the epidermis was approximately doubled. In addition, treatment of guinea pig skin with 1,2-cs-CPD or 5-NBene-2,2-DM resulted in deposition of melanin as distinct "supranuclear caps" (Gates and Zimmermann, 1953), on the side of nuclei closest to the skin surface (**Fig 9*B*, *D***), thereby providing the greatest protection of DNA from sunlight. Supranuclear caps can also be observed in guinea pig skin following treatment with UVB or UVB + MSH (Bolognia *et al*, 1989). Thus, diols appear to result in a physiologically functional melanogenic response that parallels that likely to be caused by direct exposure to sunlight, or indirectly as a result of sunlight stimulated MSH release.

Our observation of a diminution of melanogenic response from the anterior to the posterior regions of guinea pigs is reminiscent of the well known anterior-posterior diminution of response to agents that induce allergic contact dermatitis in guinea pigs (Magnusson and Kligman, 1970). Melanogenesis associated with contact dermatitis in guinea pigs has been reported (Imokawa and Kawai, 1987); however, induction of melanogenesis as a result of allergic contact dermatitis is associated with inflammation and resultant deposition of melanin in the dermis (Imokawa and Kawai, 1987), neither of which were observed in our experiments. Thus, the anterior-posterior axis of melanogenic response observed in this study is probably a reflection

of regional differences in skin biology, perhaps a function of compound transport.

Although the mechanism of action of the diols presented in this study has not yet been established, there is evidence showing that 1,2-PD exerts considerable biologic activity. For example, previous studies have shown that 1,2-PD (i) enhances the effect of follicle stimulating hormone on ovulation in sheep (Lopez-Sebastian *et al*, 1993), (ii) reduces the androgenic effect of testosterone in quail (Deviche and Balthazart, 1987), (iii) causes parthenogenetic activation of mouse and human oocytes (Gook *et al*, 1995 and references therein), (iv) reduces mouse bladder epithelial cell DNA synthesis and the number of tetraploid and octoploid cells (Farsund, 1978), (iv) reduces mouse lymph node proliferation in response to 2,4-dinitrochlorobenzene (Heylings *et al*, 1996), and (iv) enhances rat liver microsomal enzyme activities (Dean and Stock, 1974). These studies indicate that 1,2-PD may either interact with steroid receptors, or act by stimulation of steroid metabolism or deactivation. In this regard, we have found that β-estradiol stimulates melanin production in S91 cells 3-fold over control levels when added to the media (data not shown), and Dewhurst *et al* (1996) have found that β-estradiol inhibits melanoma invasiveness.

Although there is little information regarding the biologic activity of the other diols examined in this study, there is considerable literature on the use of NBane derivatives in pharmaceuticals, cosmetics, and perfumes (reviewed in Buchbauer *et al*, 1991a, b, 1992). NBane derivatives are used as anti-viral, anti-cancer, and anti-convulsive drugs, analgesics, diuretics, anti-inflammatory agents, and antibiotics, to name a few applications (Buchbauer *et al*, 1991a, b; Zhang *et al*, 1994; Posner *et al*, 1994; Jager *et al*, 1995). Incorporation of NBane related structures into drugs can increase potency by providing a conformationally rigid structure that enhances stereo-specific biologic activity, and a bulkiness that provides protection from biodegradative processes (Iwasaki *et al*, 1992). Further work is needed to clarify how incorporation of substituent groups in NBane derivatives increases their activity, particularly in the case of substituent groups that are otherwise inactive.

This study demonstrates that certain diols have the potential to be used as tanning agents. Further study is needed to evaluate their usefulness as chemotherapeutic differentiation agents. Analysis of the activity of additional compounds of this class and the elucidation of the mechanism of action will be of considerable interest.

Guinea pig experiments were done at Biocon, Inc., Rockville, Maryland with assistance from resident staff. Histology slides were prepared and stained by American HistoLabs, Inc., Gaithersburg, Maryland. Electron microscopy was done by Tim Maugel, Director of the Laboratory for Biological Ultrastructure, Department of Zoology, University of Maryland, College Park, Maryland.

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