Title: NAD(P)H dehydrogenase, quinone 1 (NQO1) protects melanin-producing cells from cytotoxicity of rhododendrol

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**Summary**

Rhododendrol (RD) is a potent tyrosinase inhibitor that is metabolized to RD-quinone by tyrosinase, which may underlie the cytotoxicity of RD and leukoderma of the skin that may result. We have examined how forced expression of the NAD(P)H quinone dehydrogenase, quinone 1 (NQO1), a major quinone-reducing enzyme in cytosol, affects the survival of RD-treated cells. We found that treatment of the mouse melanoma cell line B16BL6 or normal human melanocytes with carnosic acid, a transcriptional inducer of the *NQO1* gene, notably suppressed the cell killing effect of RD. This effect was mostly abolished by ES936, a highly specific NQO1 inhibitor. Moreover, conditional overexpression of the human *NQO1* transgene in B16BL6 led to an expression-dependent increase of cell survival after RD treatment. Our results suggest that NQO1 attenuates the cytotoxicity of RD and/or its metabolites.
Significance

The cytotoxicity of rhododendrol (RD) against melanin-producing cells is reported to result from its metabolite quinone compounds. The NAD(P)H dehydrogenase, quinone 1 (NQO1), a major quinone reducing enzyme, is retained at low levels in a basal state in many tissues. Treatment of a mouse melanoma cell line or human melanocytes with carnosic acid, an established transcriptional inducer of the NQO1 gene, renders cells resistant to RD, and inhibition of NQO1 activity eliminates this resistance. Results obtained by conditional expression of the human NQO1 transgene in mouse melanoma cells also support the pivotal role of NQO1 in detoxification of RD.

Keywords

Rhododendrol, quinone, NQO1, melanin, carnosic acid

Running title

NQO1 mitigates cytotoxicity of rhododendrol
Introduction

Rhododendrol (4-(4-hydroxyphenyl)-2-butanol, RD), a phenolic compound originally isolated from the azalea *Rhododendron fauriaei*, is a strong inhibitor of tyrosinase (Archangelsky 1901; Kawaguchi et al. 1942; Akazawa et al. 2006), a key enzyme for melanin biosynthesis that catalyzes the oxidation of tyrosine (del Marmol and Beermann 1996). The suppressive effect of RD on melanin production has been studied in view of its possible cosmetic applications, and subsequently it was added as an ingredient to a commercially available skin lightener produced by a cosmetics company in Japan. However, a considerable number of consumers developed leukoderma after using the RD-containing skin lightener, and the product was eventually recalled. Chemically induced leukoderma is characterized as cutaneous depigmentation caused by recurrent exposure to a specific substance that damages epidermal melanin-producing cells (Ghosh 2010). As a competitive inhibitor of tyrosinase, RD itself is oxidized by tyrosinase to RD-quinone or related compounds, which recent studies suggest are responsible for the cytotoxicity of RD and the resulting leukoderma (Ito et al. 2014; Sasaki et al. 2014).

The NAD(P)H dehydrogenase, quinone 1 (NQO1, also known as DT-diaphorase), catalyzes the obligatory two-electron reduction of a range of quinone compounds to their hydroquinone forms, thus preventing the one-electron reduction that results in production of potentially harmful semiquinone radicals, a source of reactive oxygen species (ROS) (Siegel et al. 2012). Depending on the relative toxicity of the parental quinones and their reduced metabolites, NQO1 functions as either a detoxifier or a bioactivator for any given quinone compound (Siegel et al. 2012). *NQO1* gene expression is under the control of the KEAP1/NRF2 transcription regulation system, together with genes encoding other phase II detoxification enzymes (Itoh et
The transcriptional coactivator NRF2 is bound by the E3 ubiquitin ligase KEAP1 in a basal state, sequestered in the cytosol, and constitutively ubiquitinated for proteasome-mediated degradation. In the presence of oxidative and/or electrophilic stress, sulphydryl groups of key cysteine residues of KEAP1 are subjected to covalent modification, leading to inhibition of its E3 ligase activity. This results in accumulation and subsequent nuclear translocation of NRF2. A heterodimer of NRF2 and a small Maf factor binds to the electrophile/antioxidant response element (EpRE/ARE), up-regulating the transcription of a set of genes including \textit{NQO1} whose products exert a cytoprotective role against noxious stress in a coordinated manner.

In the present study, we investigated how NQO1 activity modulates the cytotoxicity of RD against melanin-producing cells. To achieve ectopic overexpression of NQO1, we adopted two different approaches. The phytochemical carnosic acid (CA) is oxidized to an electrophilic quinone form within cells, which is a potent activator of the KEAP1/NRF2 system (Satoh et al. 2008). We first explored the effect of CA on proliferation of RD-treated cells, and also established and characterized a cell line in which NQO1 expression is conditionally regulated. The results obtained from either of these approaches indicated a strong correlation between NQO1 expression and resistance to RD. Our observations suggest that the cytotoxicity of RD, probably mediated by quinone compounds, can be ameliorated by NQO1, and also that CA, or other KEAP1/NRF2 activator chemicals, could have potential cosmetic applications in combination with RD.
**Results**

**CA induces expression of NQO1 within the range of concentration for minimal cell killing**

To confirm the effect of CA as a KEAP1/NRF2 activator (Satoh et al. 2008) in our experimental setting, we treated B16BL6 mouse melanoma cells with increasing concentrations of CA for 24 h and examined the cells for expression of NRF2 and one of its targets, NQO1. Although both NRF2 and NQO1 were hardly detectable in cells in an uninduced state, addition of CA as well as another established NRF2 activator, sulforaphane (Zhang et al. 1992; Zhang et al. 1994), clearly increased the cellular amounts of NRF2 and NQO1 in a dose-dependent manner (Figure 1A). Treatment with 10 µM CA for 24 h, which resulted in quite robust induction of NQO1, only marginally compromised cell growth (Figure 1B). Even with 100 µM CA, cell growth was at least 70% of non-treated cells. The same concentrations of CA caused similar or slightly higher growth inhibition of TERT-immortalized human melanocytes (HEMn-LP/hTERT1, Figure 1B). We concluded that treatment with CA in the range of concentration for least toxicity elicits firm activation of the KEAP1/NRF2 system and consequent expression of NQO1.

Recent work has shown that tyrosinase-dependent metabolites of RD have a high propensity for linkage with the cysteine residues of cellular proteins, thus inactivating them, which may account for at least some of the cytotoxicity of RD (Ito et al. 2015). This prompted us to investigate whether RD metabolites activate the KEAP1/NRF2 system by attacking the sensor cysteine residues of KEAP1. Contrary to our expectation, however, RD proved to be a much poorer activator of NRF2 than CA at the concentration that results in substantial growth inhibition (Supplementary Figure S1).
CA mitigates cytotoxicity of RD

We next studied the cytotoxicity of RD in the absence or presence of CA, expecting that NQO1 expression might modulate the toxicity of the RD-derived quinone compound. First, B16BL6, HEMn-LP/hTERT1 or normal human melanocytes (NHEM) were treated with RD alone at concentrations of 0 to 2 mM for 24 h, and cell growth was measured. The respective IC50 values were 230 μM for B16BL6, 119 μM for NHEM, and 178 μM for HEMn-LP/hTERT1 (Figure 2A), being comparable to those reported previously for human melanocytes (Sasaki et al. 2014; Kasamatsu et al. 2014). When the cells were similarly treated with RD but in the presence of 0.5 μM CA, the IC50 values increased by 5.5 to >11.2-fold (1260 μM, 840 μM and >2000 μM for B16BL6, NHEM, and HEMn-LP/hTERT1, respectively, Figure 2A). Treatment with 10 μM CA further increased the IC50 for B16BL6 to >2000 μM, indicating a dose-dependent attenuation effect of CA on RD cytotoxicity, which is consistent with the CA dose-dependent expression of NQO1 in this range. To demonstrate the pivotal role of NQO1 among dozens of NRF2 targets, we added ES936, a highly specific inhibitor for NQO1 (Winski et al. 2001), together with CA (Figure 2B). This mostly eliminated the effect of CA, suggesting that NQO1 was solely responsible for the CA-mediated dose decrement of RD. Note that ES936 suppressed the effect of CA but did not increase the sensitivity of the cells to RD. This probably indicates that the small amount of NQO1 in B16BL6 cells in a basal state (Figure 1A) hardly contributes to RD resistance.

CA prevents RD-induced apoptosis of melanocytes

We studied whether RD cytotoxicity involves induction of apoptotic cell death, and if so, how
CA affects this process. B16BL6 or HEMn-LP/hTERT1 cells were treated with 10 or 50 µM RD for 72 h in the absence or presence of 25 µM CA, and the appearance of apoptosis marker proteins was examined. RD treatment induced cleavage of caspase 3 and poly ADP ribose polymerase (PARP) in HEMn-LP/hTERT1 at both concentrations of RD, and the induction was strongly suppressed by addition of CA (Figure 3). In contrast, only weak induction of cleavage of caspase 3 and PARP was observed in B16BL6 even in the presence of 50 µM RD, which was completely abrogated by CA. The difference in the rate of apoptosis induction between B16BL6 and HEMn-LP/hTERT1 cells may partly explain the slight difference in their RD sensitivity (IC₅₀: 230 µM versus 178 µM), or may simply reflect a difference in the mode of death between the two cell lines.

Establishment of a cell line showing conditional NQO1 expression in an auxin-dependent manner

To demonstrate the involvement of NQO1 in detoxification of RD more specifically, we established a cell line conditionally expressing NQO1 from B16BL6, making use of an auxin-inducible degron (AID) system (Nishimura et al. 2009). In the AID system, the plant-derived F-box protein TIR1 and the protein of interest fused with AID are co-expressed. Addition of the plant hormone auxin induces TIR1-dependent rapid ubiquitination of AID followed by proteasome-mediated degradation of the fusion protein usually within 1 h. This facilitates far more prompt shut-off of the target protein in comparison with a transcription-repression-based system. We inserted human NQO1 cDNA into the pAID1.1N plasmid and isolated stable clones from B16BL6. A clone showing high TIR1 and AID-NQO1
expression was selected and confirmed to show auxin-mediated degradation of AID-NQO1 (B16BL6/pAO1). B16BL6/pAO1 constitutively expresses AID-NQO1 from the cytomegalovirus promoter at a level comparable to endogenous NQO1 induced by treatment with 10 µM CA (Figure 4A). AID-NQO1 quickly disappears after treatment with a synthetic auxin analogue, 1-naphthaleneacetic acid (1-NAA), for 1 h. We measured the growth of B16BL6/pAO1 after RD treatment. As expected, B16BL6/pAO1 was much more resistant to RD (IC₅₀ > 2000 µM, Figure 4B) than its parental line B16BL16 (IC₅₀ = 230 µM). Remarkably, addition of 1-NAA reduced the IC₅₀ of RD from >2000 µM to 272 µM (Figure 4B), to a degree resembling that of the parental line. Another independent clone gave essentially the same results (data not shown). These observations strongly indicate that expression of NQO1 is almost solely responsible for CA-mediated attenuation of RD cytotoxicity.

CA does not interfere with the inhibitory effect of RD on melanin synthesis

We next examined whether CA hinders the inhibitory effect of RD on melanin synthesis. B16BL6 cells were cultured for 72 h in the presence of 2 or 10 µM RD with or without 0.5 µM CA. Cytotoxicity of both RD and CA is minimal under these conditions (Figure 1B, 2A). The pellets of cells treated with 10 µM RD were markedly lighter in color than those of untreated control cells or cells treated with 2 µM RD (Figure 5A). Addition of CA did not notably affect the lightening induced by RD. We quantified the melanin concentration by measuring the absorbance of a cell lysate at 500 nm (Figure 5B). The melanin level was reduced to approximately 50% with 2 µM RD treatment, and to 30% with 10 µM RD treatment. Again, addition of CA did not significantly change the inhibitory effect of RD.
Effect of RD and CA on melanin production and melanocyte survival in a 3D skin model

To investigate the effect of CA on RD cytotoxicity against melanocytes in a setting closer to in vivo conditions, we utilized a commercial 3D culture model of human skin. The culture model was incubated for 10 or 14 days in medium containing 2 or 10 µM RD with or without 25 µM CA. In the control culture, obvious darkening was observed, reflecting proliferation of melanocytes and ongoing melanin synthesis during this period (Figure 5C). Addition of RD suppressed this darkening in a dose-dependent manner, and CA did not have any apparent effect on this suppression (Figure 5C). Microscopy of the immunostained sections revealed that most of the melan-A-positive cells in the basal layer, representing melanocytes, disappeared after treatment with 10 µM RD in the absence of CA (Figure 5D). This implies that suppression of darkening by RD alone may involve permanent elimination of melanin-producing cells. In contrast, about 60% of the melan-A-positive cells persisted in the sample treated simultaneously with RD and CA, in comparison with the control. Our observations suggest that CA also attenuates the cytotoxicity of RD without notably retarding the suppression of melanogenesis by RD under in vivo-mimicking conditions.
Discussion

Our present results clearly demonstrated that forced expression of NQO1 mitigates the cytotoxicity of RD without notably affecting the inhibition of melanin synthesis. An obvious explanation for this is that NQO1 reduces quinone compounds originating from RD to less toxic catechol forms. One immediate metabolite of RD produced by tyrosinase oxidation is reported to be RD-quinone (Ito et al. 2014), which would be reduced to RD-catechol (hydroxy-RD) by NQO1. While the relative cytotoxicity of RD-quinone and RD-catechol has not been directly measured due to instability of the former (Sasaki et al. 2014; Kasamatsu et al. 2014; Okura et al. 2015), it seems reasonable to assume that highly reactive RD-quinone may have higher cytotoxicity (Ito et al. 2015), and therefore that its reduction may imply detoxification. Other secondary quinones might also partly contribute to the cytotoxicity of RD (Ito et al. 2015). This issue remains to be clarified in a future study.

The KEAP1/NRF2 system up-regulates a set of genes encoding phase II detoxification enzymes other than \textit{NQO1}. These include \textit{GCLM} and \textit{GCLC}, whose products constitute glutamate-cysteine ligase, a rate-limiting enzyme for GSH production, as well as several genes encoding glutathione S-transferases (Malhotra et al. 2010; Suzuki et al. 2013). Since conjugation of GSH to quinone is regarded as a first line of cellular defense against quinone toxicity, it would be reasonable to assume that NRF2/KEAP1 activation may contribute to detoxification of RD by increasing not only NQO1, but also GSH within cells and activating conjugation. In the present study, however, forced expression of NQO1 alone was sufficient to decrease RD toxicity to almost the same level as that by CA-mediated detoxification. This observation suggests that NRF2 targets other than \textit{NQO1} play minor or redundant roles in
resistance to RD.

NQO1 is also reported to modulate cell growth by stabilizing the tumor suppressor p53 (Asher et al. 2001; Asher et al. 2005). Although we have not examined the involvement of p53 in growth recovery after RD treatment, if NQO1 overexpression positively regulates p53 function by stabilizing it, that would result in either cell cycle arrest or activation of apoptosis, and be observed as growth inhibition of the cell population. However, this appears to contradict what we observed, and accordingly we do not consider p53 to play a major role in mitigation of RD toxicity by NQO1.

An allelic variant of NQO1 with essentially no enzymatic activity, NQO1*2 (NQO1P187S, rs1800566 in dbSNP, Traver et al. 1992), is reported to exist at a high frequency, especially in Asian populations (allele frequency = 0.4187 in Asians versus 0.2107 in Europeans) according to the phase 3 May 2013 call set of the 1000 Genome Project (1000 Genomes Project Consortium et al. 2012). Whereas the amount of NQO1 in the basal state hardly contributed to growth recovery after RD treatment in our experiment using the B16BL6 mouse melanoma cell line, it might exert some influence in human skin in vivo, considering the varying degree of basal expression of NQO1 in different tissues (Siegel et al. 2012). While it is clear that the NQO1 genotype is not the sole determinant, since only 2% of RD consumers developed leukoderma (Nishigori et al. 2015), it would be of interest to investigate the genetic background of RD-induced leukoderma. In fact, for some antitumor quinones on which NQO1 may act as a bioactivator, polymorphism of NQO1 is reportedly associated with response to chemotherapy (Fagerholm et al. 2008; Jamieson et al. 2011). It should also be noted that application of CA may not be effective for individuals with homozygous NQO1-null alleles.
Methods

Materials

RS-Rhododendrol (RD) was kindly provided by Kanebo Cosmetics Inc. (Tokyo, Japan). Carnosic acid (CA), N-phenylthiourea (PTU), sulforaphan, and the NQO1 inhibitor ES936 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared by dissolving the chemicals in DMSO at 4 M (RD), 100 mM (CA), 100 mM (sulforaphan) and 20 μM (ES936). All other reagents and chemicals were of high grade and commercially available.

Cell culture and media

All cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C.

A murine melanoma cell line B16BL6 was obtained from RIKEN Cell Bank (Tsukuba, Japan) and cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS.

Primary cultures of normal human epidermal melanocytes (NHEM) were purchased from Kurabo Industries Ltd. (Osaka, Japan) and cultured in Medium 254 containing Human Melanocyte Growth Supplement (Kurabo Industries Ltd.), penicillin (100 U/ml), streptomycin (100 μg/ml), and 4-amphotericin (250 ng/ml).

An immortalized human melanocyte line (HEMn-LP/hTERT1) established in our laboratory from the commercially available human melanocyte line HEMn-LP (Thermo Fisher Scientific) was cultured in DMEM (Thermo Fisher Scientific) supplemented with 1% Human Melanocyte Growth Supplement (Thermo Fisher Scientific).

A three-dimensional human skin model MEL-300-A was purchased from Kurabo Industries Ltd.
(Osaka, Japan) and maintained in EPI-100LLMM medium as instructed by the manufacturer.

Cell viability assay

A Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), which utilizes water-soluble tetrazolium salts, was used to evaluate the proliferation of melanocytes or melanoma cells after drug treatment. Cells were seeded into 96-well plates (2000 cells/well) and cultured for 24 h prior to treatment with RD and/or CA. After the treatment, medium in each well was replaced with 100 µl of drug-free fresh medium and 10 µl of Cell Counting Kit-8 solution, incubated for an additional 1-2 h, and the absorbance of each well at 450 nm was measured with the use of a Multiskan Spectrum spectrophotometer (Thermo Fisher Scientific).

Protein preparation and immunoblotting

Cells at 80–90% confluence were washed twice with ice-chilled PBS, treated with 10% TCA for 30 min on ice, and then scraped off into a tube. The cell pellet was washed once with deionized water and lysed in 9 M urea, 2% Triton X-100 and 1% DTT. Protein concentration was measured with a BCA protein assay kit (Merck Millipore, Billerica, MA, USA) before addition of DTT. Protein samples were electrophoresed on 4–12% NuPAGE Bis-Tris gels (Thermo Fisher Scientific) for 30 min at 200 V and then transferred onto polyvinylidene fluoride transfer membranes (Pall Corporation, Port Washington, NY, USA). The membranes were blocked with 5% non-fat dried milk (#9999, Cell Signaling Technology) in 1 × TBS-T for 1 h at room temperature and then immunoreacted with an appropriate primary antibody overnight at 4°C and with a HRP-conjugated secondary antibody (GE Healthcare Life Sciences,
Buckinghamshire, UK) for 1 h at room temperature. Signals were detected with ECL prime detection reagents (GE Healthcare Life Sciences) and ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA, USA). The intensity of the detected signals was quantified using ImageJ/Fiji software (Schindelin et al. 2012) and normalized against the β-actin signal. Antibodies against NRF2 (#8882, 1:500 for immunoblotting), NQO1 (#3187, 1:1000), cleaved caspase 3 (#9661S, 1:1000) and PARP1 (#9544S, 1:1000) were from Cell Signaling Technology (Danvers, MA, USA). The antibody against β-actin was from Sigma-Aldrich (#A2228, 1:1000). Electrophoresis and immunoblotting were repeated at least three times with independent sample preparations and confirmed to be reproducible.

**Stable overexpression of NQO1 in B16BL6 cells**

Total RNA was prepared from a human melanoma cell line A7, in which KEAP1/NRF2 is constitutively activated (Miura et al. 2013). cDNA was synthesized with oligo(dT) and SuperScript III (Thermo Fisher Scientific), and NQO1 cDNA was amplified by PCR using KOD Plus NEO DNA polymerase (Toyobo, Osaka, Japan) and a specific primer pair, 5′-GGGGGGATATCACGTCGGCAGAAGAGCACTGAT-3′ and 5′-GGGGGACGCGTTCATTTTCTAGCTTTGATCTGGT-3′. The ends of the amplified cDNA fragment were digested with EcoRV and MluI, subcloned into the pAID-N1.1 vector (BioROIS, Mishima, Japan), and termed pAO1. The sequence of the entire length of the inserted NQO1 cDNA was verified using a 3500 Genetic Analyzer (Thermo Fisher Scientific). pAO1 was linearized with AflIII to increase the integration efficiency, and transfected into B16BL6 cells using Lipofectamine 3000 (Thermo Fisher Scientific) in accordance with the manufacturer’s
protocol. Stably transfected cells were selected in medium containing 1000 µg/ml G418 for 7 days, and several clones were isolated by limiting dilution. The clones were examined for expression and 1-NAA-dependent degradation of the AID-NQO1 fusion protein by immunoblotting.

**Measurement of melanin content**

Melanin was quantified spectroscopically as reported previously (Yokota et al. 1998). Three hundred thousand cells were plated on a 60-mm plastic dish. After attachment, the cells were treated with 2 or 10 µM RD and/or 0.5 µM CA for 72 h. The cells were then harvested by trypsinization, fixed with 5% TCA for 90 min, washed with ethanol-ether (3:1) and ether, and dissolved in 0.5 ml of Soluene-350 (PerkinElmer, Inc., Waltham, MA, USA) at 80°C for 1 h. The absorbance at 500 nm was measured with a Multiskan Spectrum instrument (Thermo Fisher Scientific).

**Evaluation of melanocyte viability and melanogenesis in 3D human skin models**

The human skin model was cultured in medium containing 2 or 10 µM RD with or without 0.5 µM CA for 10 or 14 days with a change of medium every other day. After the treatment, photographs were taken for visual inspection of melanogenesis. For microscopic observation of melanocytes in the 3D model, samples were fixed with 4% formaldehyde after the 10 days of treatment, and embedded in paraffin. Four-micrometer-thick sections were cut and stained with hematoxylin and eosin. The sections were then immunostained using anti-melan A, following a
procedure described previously (Watanabe et al. 2013). The extent of immunohistochemical reactivity was estimated by light microscopy.

**Statistical analysis**

Data are presented as the mean ± SEM. Statistical significance of differences in the mean was assessed by Welch’s $t$-test using Microsoft Excel (Microsoft, Seattle, WA, USA). $P < 0.05$ was considered to indicate statistical significance. Logistic function was fitted to the cell proliferation data points with the least squares estimates of the parameters. IC$_{50}$ values were calculated from the fitted function.
Acknowledgements

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**Figure legends**

**Figure 1.** CA activates the KEAP1/NRF2 transcriptional program in a dose-dependent manner within the range of least toxicity. (A) BL16BL6 mouse melanoma cells were treated with CA at several different concentrations for 24 h, and the levels of NRF2 and NQO1 protein were examined with immunoblotting. Sulforaphane was used as a positive control (Zhang et al. 1992). (B) B16BL6 cells and human immortalized melanocytes (HEMn-LP/hTERT1) were treated with increasing concentrations of CA for 24 h, and the cell growth was assessed with a water-soluble tetrazolium salt-based assay (see Materials and Methods). The results are expressed as mean ± SEM of relative cell numbers relative to those of the untreated control in triplicate experiments.

**Figure 2.** CA mitigates the cytotoxicity of RD by NQO1 activation. (A) B16BL6, HEMn-LP/hTERT1 or normal human melanocytes (NHEM) were treated with various concentrations of RD with or without 0.5 µM CA for 24 h, and cell growth was measured. In B16BL6, 10 µM CA induced further resistance to RD. (B) B16BL6 cells were treated with RD at concentrations of 0 to 2 mM and 0.5 µM CA with or without 100 nM ES936 for 24 h. ES936 abolished most of the cytoprotective effect of CA on RD-treated cells. Results are expressed as mean ± SEM of triplicate experiments.

**Figure 3.** CA prevents RD-induced apoptosis of melanocytes. B16BL6 or HEMn-LP/hTERT1 were treated with 10 or 50 µM RD with or without 25 µM of CA for 72 h. Extracts of the treated cells were examined for cleavage of caspase-3 and PARP1.
Figure 4. Ectopic expression of NQO1 mitigates the cytotoxicity of RD. (A) Stable expression of AID-NQO1 in B16BL6/pAO1. B16BL6 or B16BL6/pAO1 cells were treated with increasing concentrations of CA and/or 0.5 µM auxin for 24 h, and examined for NQO1 expression using immunoblotting. B16BL6/pAO1 constitutively expressed the AID-NQO1 fusion protein to an extent comparable with that of endogenous NQO1 in the parental B16BL6 induced by treatment with 10 µM CA. AID-NQO1 promptly disappeared after addition of 0.5 mM 1-NAA. (B) B16BL6/pAO1 cells were treated with RD at concentrations of 0 to 2 mM with or without 0.5 mM 1-NAA for 24 h, and cell proliferation was assessed. Results are expressed as mean ± SEM of triplicate experiments.

Figure 5. CA treatment does not notably hinder the suppressive effect of RD on melanogenesis while increasing the survival of melanocytes against RD in a 3D culture model. (A) B16BL6 cell pellets after RD and/or CA treatment. B16BL6 cells were cultured for 72 h in the presence of 2 or 10 µM RD with or without 0.5 µM CA. PTU, an established tyrosinase inhibitor with a non-competitive mechanism (Chang 2009), was used as a positive control. (B) Relative amounts of melanin in BL16BL6 cells after RD and/or CA treatment. Absorbance of extracts of the treated cells at 500 nm was measured with a spectrophotometer. Results are expressed as mean ± SEM of triplicate experiments. (**P <0.01, ***P <0.001, Welch’s t-test). (C) Photographs of the 3D human skin model after the treatment. The skin model was placed in 6-well plates and cultured in medium containing 10 µM RD or 2.0 µM RD with or without 0.5 µM CA for 14 days. Medium containing the drug was replaced every other day. (D) Representative
microscopic images of melan-A-immunostained sections of the 3D skin model after treatment with RD and CA for 10 days.
References


the active brightening material, rhododendrol, is related to tyrosinase activity at a certain threshold. J. Dermatol. Sci. 76, 16–24.


Supplementary Figure legend

**Figure S1** Expression of NRF2 and NQO1 in B16BL6 cells after RD, CA or ES936 treatment.

B16BL6 mouse melanoma cells were treated with a combination of RD (0.2 mM), CA (0.5 µM) or ES936 (100 µM) for 24 h, and the levels of NRF2 and NQO1 protein were examined by immunoblotting. Signal intensities were normalized against the β-actin signal. Note that 0.2 mM RD resulting in 50% growth inhibition induced a much weaker level of NRF2 or NQO than 0.5 µM CA resulting in no significant growth inhibition.