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In vivo depigmentation by hydroxybenzene derivatives

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Certain mono- and dihydroxybenzene derivatives are selectively cytotoxic for melanocytes *in vivo*, and can cause depigmentation of skin and hair. We produced selective melanocytotoxicity/hair depigmentation in C57Bl mice by injection of 0.032–1.0% *p-t*-butylcatechol (tBC) or *p*-hydroxyanisole (MMEH) in physiological saline. No depigmentation occurred on injection of 3,4-dihydroxyphenylalanine (DOPA) or 3,4-dihydroxyphenylacetic acid (DOPAC). Light- and electron-microscopic examination of biopsy specimens taken from depigmented areas indicates selective melanocyte damage as early as 2 h post-injection. Melanocytes from anagen hair are most susceptible to depigmentation. All four compounds are substrates for tyrosinase, but only tBC and MMEH generate their respective *isolable* 1,2-benzoquinones, tBCQ and MMEHQ. These caused depigmentation in C57Bl mice to a comparable degree to the parent compounds. DOPA- and DOPAC-quinones (DOPAQ and DOPACQ) are not spectroscopically detectable in solution, suggesting extremely low steady-state levels of these compounds. The net observed rate of reaction of the respective 1,2-quinone with 300 μM bovine serum albumin (BSA) *in vitro* varies widely, with tBCQ ≫ MMEHQ = DOPACQ ≫ DOPAQ. The results are consistent with a mechanism involving attack of -SH on melanosomal proteins and/or enzymes by tyrosinase-generated 1,2-quinones. This mechanism evidently differs from that involved in *in vitro* hydroxybenzene melanocytotoxicity of melanoma cells, in which active oxygen intermediates generated by hydroxybenzene autoxidation play a significant role. The most reliable prognosticator of *in vivo* depigmentation appears to be the ability of the depigmenter to form a spectroscopically stable 1,2-quinone which is capable of reacting with protein -SH.

Key words: Depigmentation, hydroxybenzene, quinones, reactivity, tyrosinase.

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Introduction

Certain mono- and dihydroxybenzene derivatives are selectively cytotoxic for melanocytes *in vivo*, and can cause depigmentation of skin and hair. *In vivo* depigmentation of follicular C57Bl mouse melanocytes by candidate hydroxybenzene derivatives is sometimes used as a preliminary screening assay for potential chemotherapeutic agents for malignant melanoma.[1,2] Compounds which show promise are then tested on melanoma cells in tissue culture *in vitro* and/or in implanted melanoma cells *in vivo*.

The cytotoxicity of hydroxybenzenes is thought to depend on the formation of toxic oxidation products. These are either 1,2-benzoquinones arising from tyrosinase-catalysed oxygenation/dehydrogenation[3] or autoxidation[4] of hydroxybenzene substrate, 'active oxygen' species[4] or free radicals, which might[3] or might not[5] lead to significant lipid peroxidation. These mechanisms are not mutually exclusive, and need not be the same *in vivo* and *in vitro*. Moreover, the marked sensitivity of experimental *in vivo* depigmentation to the conditions used for its elicitation[6] may make direct comparison of work carried out in various laboratories unreliable.

It is important to assess a mechanistic comparison between *in vivo* and *in vitro* melanocytotoxicity. A first step in this direction is to study a set of *in vivo* depigmenters under a standard set of experimental conditions that afford reproducible results which may be reliably compared with those from analogous tissue culture or melanoma implantation studies. In this study, we have injected C57Bl mouse hair follicles intradermally and subcutaneously with four familiar hydroxybenzene derivatives of widely differing depigmenting potencies. These are: *p-t*-butylcatechol (tBC), a known occupational depigmenter[6] which kills melanoma cells in culture,[1,7] *p*-hydroxyanisole (monomethyl ether of hydroquinone; MMEH), a potential anti-melanoma agent studied extensively by Riley and coworkers *in vitro*,[8] L-3,4-dihydroxyphenylalanine (DOPA), and 3,4-dihydroxyphenylacetic acid (DOPAC), a dopamine metabolite

implicated in the aetiology of vitiligo.[9] The latter two compounds do not cause *in vivo* depigmentation, but do kill melanoma cells in tissue culture.[1,10] We have assessed the ability of these compounds to act as substrates for mammalian tyrosinase, and the reaction of their 1,2-quinone derivatives with bovine serum albumin (BSA) sulphydryl groups *in vitro*. The findings suggest the importance of the addition of tyrosinase-generated quinone to critical -SH in *in vivo* melanocytotoxicity, and the lack of direct correspondence between *in vitro* and *in vivo* mechanisms.

Materials and methods

Depigmenting chemicals

tBC, MMEH, and DOPAC (Aldrich) and DOPA (Sigma) were treated as previously described.[11] Quinones 4-methyoxy-1,2-benzoquinone (MMEHQ) and 4-*t*-butyl-1,2-benzoquinone (tBCQ) were prepared by respective reaction of MMEH and *p*-*t*-butylphenol with Fremy's Salt, $(KSO_3)_2NO$.[12] All other chemicals were reagent grade.

Animal studies

Three to four-week-old C57Bl mice were originally obtained from Charles River Laboratories and inbred in our laboratory. Shaved mice were injected subcutaneously 5 days/week for 4 weeks on the right dorsal flank or hair was plucked, and mice were subsequently injected intradermally (i.d.) once on the right dorsal flank with 0.1 ml tBC, DOPA, DOPAC, or MMEH (0.032–1.0% in physiological saline). Control animals were injected with saline alone. Results from both regimens were essentially identical. Freshly prepared MMEHQ and tBCQ were dissolved in 80% aqueous ethanol prior to intradermal injection into C57Bl mice. Hair was allowed to grow back, and mice were examined for signs of visual depigmentation, irritation, necrosis, and signs of systemic toxicity. Biopsy specimens were taken from test and control sites at 0–48 h and at 2–4 weeks after injection. Histological damage was assessed by light and electron microscopy.

Reactivity studies

Highly purified tyrosinase [EC 1.14.18.1] from B-16 mouse melanoma, obtained from Dr V. J. Hearing, Jr, NIH was used to determine the relative ability r_q, of the hydroxybenzenes (300 μM) to act as substrates for mammalian tyrosinase.[13]

To determine the reactivity of the respective quinones with bovine serum albumin (BSA), MMEH, tBC, DOPA, and DOPAC (300 μM) were oxidized to the 1,2-benzoquinone (Q) with 3 μg (17.7 units) or 30 μg (177 units) mushroom tyrosinase (Sigma) in the presence or absence of 300 μM BSA in 3.0 ml phosphate buffer, pH 7.4. One unit of enzyme activity causes an increase in 475 nm absorption of 0.001 OD unit/min in a 300 μM buffered DOPA solution.[13] Initial rates of quinone formation were determined spectrophotometrically[11] using the following wavelengths (nm) and values for molecular extinction coefficients (litre/mol/cm): 4-*t*-butyl-1,2-benzoquinone (tBCQ), 400 nm (1600); 4-methoxy-1,2-benzoquinone (MMEHQ), 413 nm (1600); DOPAC oxidation product, 349 nm (1670); dopachrome, 475 nm (3750). The formation of the latter two compounds is rate-limited by formation of their respective (unstable) 1,2-quinones.

In the case of MMEH, 45 μM L-DOPA was added as a cofactor to eliminate the induction time for quinone formation which is observed for MMEH alone.[13] Control experiments determined that the contribution of dopachrome (resulting from DOPA oxidation) to the total absorbance at 413 nm was small (18% in the absence of BSA, and essentially zero in its presence). The values presented in Table 2 are corrected for this effect. Under these conditions, the rate of dopaquinone/dopachrome formation was roughly 10% that of MMEHQ formation. This rules out the possibility that dopaquinone is the major oxidizing agent for MMEH.

The difference between the experimentally observed rates of quinone formation in the presence and absence of BSA, $\Delta dQ/dt$, represents the net (observed) rate of reaction of the generated quinone with the sulphhydryl group of BSA, that is, the reaction of quinone with BSA minus other quinone reactions as self-cyclization (e.g. dopachrome formation), addition of H_2O, redox reactions, self-polymerization, etc. This can be expressed as:

$$\Delta dQ/dt = k_{SH}\,[Q]\,[BSA] = r_{SH}\,[BSA] \qquad (1)$$

The pseudo-first order rate parameter $r_{SH} = k_{SH}\,[Q]$ allows us to 'rank order' the various hydroxybenzene derivatives according to their empirically observed rates of reaction of their generated quinones with BSA in the absence of direct kinetic information as to k_{SH}, the second order reaction rate constant, and $[Q](t)$ (see discussion).

Results

Depigmentation of C57Bl mice

Preliminary results indicate that both subcutaneous and i.d. injection of MMEH or tBC causes depigmentation

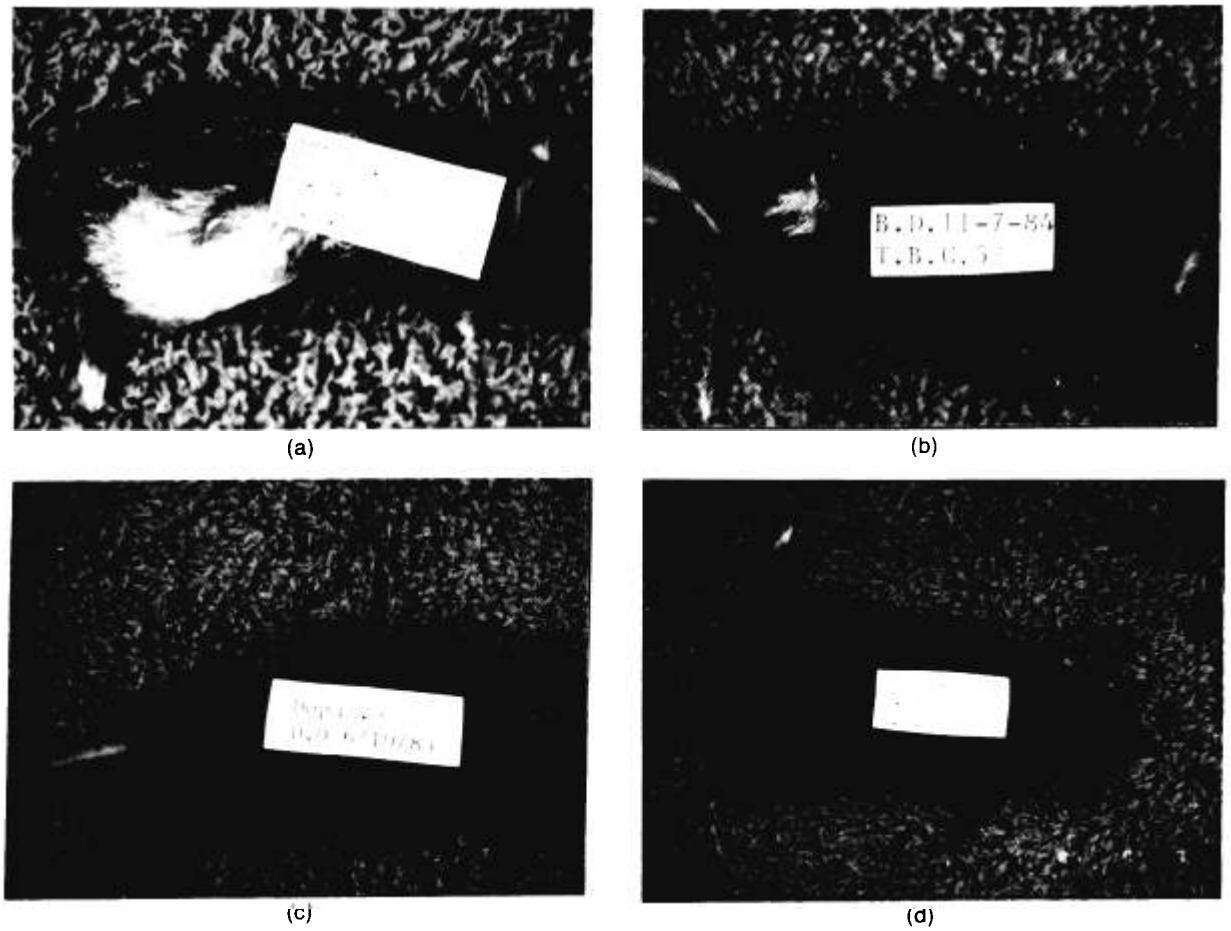

Figure 1. a Depigmentation of C57Bl mouse hair by subcutaneous injection of 0.5% MMEH in physiological saline. Note that depigmentation is delocalized to areas removed from site of injection. **b** Depigmentation of C57Bl mouse hair by subcutaneous injection of 0.5% tBC in physiological saline. Note that depigmentation is essentially localized to area of injection. **c** Lack of depigmentation of C57Bl mouse hair by subcutaneous injection of 0.5% L-DOPA in physiological saline. **d** Lack of depigmentation of C57Bl mouse hair by subcutaneous injection of 0.5% DOPAC in physiological saline. Identical results were obtained in all cases of intradermal injection (see 'Materials and methods' for additional details). Control animals injected with physiological saline showed no necrosis, morbidity, mortality, or depigmentation.

Table 1. Depigmentation of C57Bl mice by hydroxybenzene depigmenters

	No. depigmented/total no.[a]			
Concentration (%)	tBC	MMEH	DOPA	DOPAC
1.0	10/10	10/10	0/10	0/10
0.5	22/35	10/10	0/10	0/10
0.25	34/44	5/9	ND	ND
0.125	49/60	8/10	ND	ND
0.063	18/44	10/10	ND	ND
0.032	7/38	7/9	ND	ND
0.016	3/33	1/3	ND	ND

[a] Total results for both subcutaneous and intradermal injection regimens (see text).
ND = not determined.

in C57Bl mice, whereas injection of DOPA or DOPAC does not (Figure 1a–d; Table 1). The extent of depigmentation is greatest under conditions where anagen hair growth and tyrosinase activity are maximized. Depigmentation caused by MMEH tends to be distributed into areas removed from the injection site, while that due to tBC tends to be more localized. Irritation, necrosis, and systemic toxicity are noticeable for tBC at a concentration of 1.0%, but not for the other compounds. Using five mice for each experiment, depigmentation was noted in all animals for i.d. injection of 0.1% MMEHQ and 0.39% tBCQ (Figures 2 and 3). The ability of these quinones to effect depigmentation was comparable to that of the parent hydroxybenzenes.

Light- and electron-microscopic examination of biopsy specimens taken from depigmented areas of mice

injected with tBC or MMEH at moderate concentrations (Figure 4a–c) show selective cytotoxicity to melanocytes as early as 2 h post-injection, with little damage to surrounding keratinocytes. Biopsies from depigmented areas show negative DOPA staining, while surrounding hair follicles are DOPA-positive. The earliest observed signs of damage at the EM level are a 'halo' of clear cytoplasm surrounding the melanosome, disturbance of other protoplasmic organelles, i.e. endoplasmic reticulum, Golgi apparatus, ribosomes, and mitochondria, and vacuolization in protoplasmic sap, especially in the area of the melanosome. Later changes include alterations in melanocyte morphology, nuclear damage, death and disintegration; these occur as early as 24 h post-injection. Melanocyte damage was not observed with DOPA or DOPAC.

Reactivity of hydroxybenzenes

tBC, MMEH, DOPA and DOPAC are all facile substrates of mushroom tyrosinase. Only the first two compounds form 1,2-benzoquinone derivatives which are stable enough to be detected by conventional spectroscopy, and only the first two compounds depigment C57Bl follicular melanocytes. Dopaquinone rapidly undergoes cyclization to form dopachrome. DOPAC forms a species which has an absorption maximum at 349 nm, with no detectable maximum at ca 400 nm, expected for the 1,2-quinone. The 'stable' compound is formed presumably through an unstable 1,2-benzoquinone intermediate, since DOPAC can be converted to 5-S-L-cysteinyl-DOPAC in the presence of tyrosinase and excess L-cysteine.[10]

DOPA, MMEH, tBC and DOPAC are all substrates of highly purified mouse melanoma tyrosinase (Table 2). As might be expected, the latter three compounds are somewhat sluggish in comparison to DOPA.

Figure 2. Depigmentation of C57Bl mouse hair by intradermal injection of 0.1% MMEHQ in 80% ethanol. Note delocalization of depigmentation. Control animals injected with 80% ethanol showed some necrosis, but no morbidity, mortality or depigmentation.

Figure 3. Depigmentation of C57Bl mouse hair by intradermal injection of 0.39% tBCQ in 80% ethanol. Depigmentation is essentially localized. Control animals injected with 80% ethanol showed some necrosis, but no morbidity, mortality, or depigmentation.

The 1,2-quinone derivatives generated by the mushroom tyrosinase-catalysed oxidation of hydroxybenzenes exhibit widely differing reactivities towards BSA, as judged by the value of rate parameter r_{SH}. Table 2 shows that value of this parameter for tBC ($r_{SH} = 2.02 \times 10^{-2}$/s) is roughly 13-fold greater than for MMEH ($r_{SH} = 1.23 \times 10^{-3}$/s) and DOPAC ($r_{SH} = 1.53 \times 10^{-3}$/s), and 200-fold greater than for DOPA ($r_{SH} = 1.1 \times 10^{-4}$/s).

Discussion

As *in vivo* depigmentation with tBC and MMEH is most striking under conditions of active hair growth and high tyrosinase activity, it is apparent that the *in vivo* depigmenting capability of these compounds is related to tyrosinase-catalysed formation of cytotoxic 1,2-benzoquinone derivative(s), as has been previously noted (for review, see reference 2 and references therein). We have directly demonstrated that i.d. injection of the 1,2-benzoquinones themselves, namely MMEHQ and tBCQ, causes depigmentation which is comparable to that of the parent compounds. However, there was no correlation between *in vivo* depigmentation and the ability of a given compound to act as a substrate for mammalian tyrosinase *per se* (Table 2). This implies that the reaction of formed 1,2-benzoquinones with critical melanocytic targets, most likely -SH groups on such enzymes as DNA polymerase[14] or ribonucleotide reductase,[15] is necessary for *in vivo* follicular depigmentation by MMEH and tBC to occur. Such a reaction would be favoured by the formation of quinones which are stable enough to be isolated, but which react efficiently with -SH groups on skin proteins. The 1,2-benzoquinones formed from MMEH and tBC possess these properties, and are good depigmenters. We also note that catechol, 4-methylca-

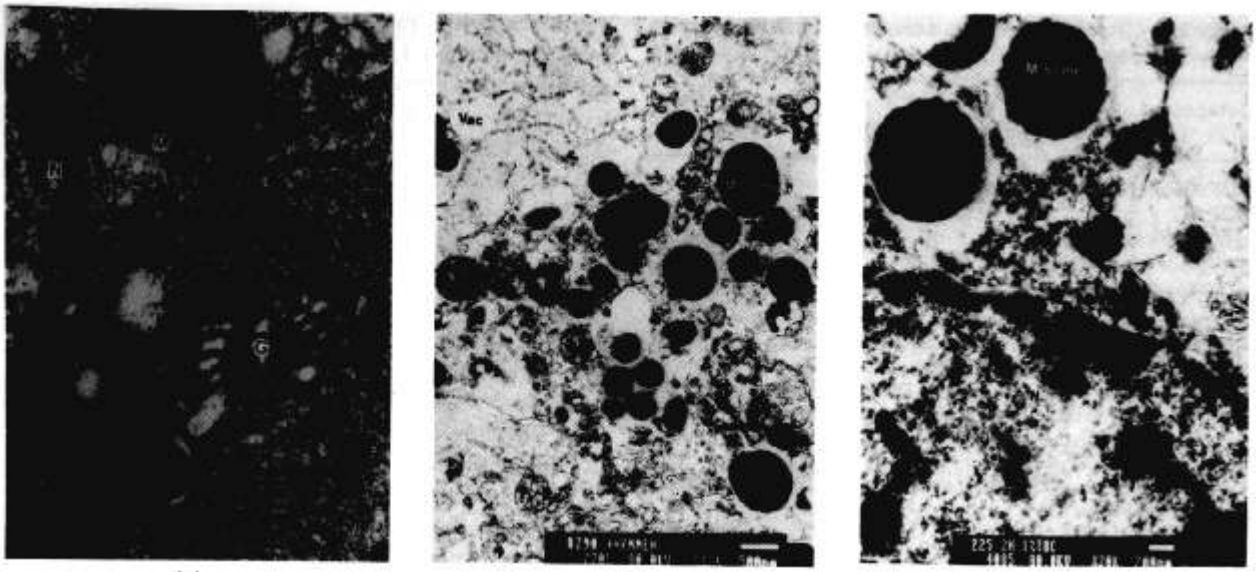

Figure 4. a Electron micrograph of follicular melanocyte from control C57Bl mouse (× 90 000). Note intact melanosomes (M), mitochondria, Golgi apparatus (G), nucleus (N) and nuclear membrane. **b** Electron micrograph of follicular melanocyte from C57Bl mouse 4 h after injection with 1.0% MMEH in physiological saline (× 12 000). Note vacuolization (Vac), including 'halo' around melanosome (M'some), and damaged mitochondria (Mit). **c** Electron micrograph of follicular melanocyte from C57B1 mouse 2 h after injection with 1.% tBC in physiological saline (× 20 000). Note vacuolization, including 'halo' around melanosomes (M'some), and damage to nuclear membrane (Memb) around nucleus (N). For both compounds, subsequent changes include alterations in melanocyte morphology, damage to the nuclear envelope, and melanocyte death (see text).

techol and 4-*t*-butylphenol depigment C57Bl mice, and form isolable 1,2-quinones (unpublished results). Conversely, nondepigmenters DOPA and DOPAC generate quinones which are too unstable to be detected by ordinary absorption spectroscopy. Evidently, DOPA- and DOPAC-quinones are too short-lived and/or their steady-state concentrations too low to react efficiently with critical -SH under physiological conditions *in vivo*, even though the net reactivity of these quinones to -SH may be comparable to that of MMEHQ at high BSA concentrations *in vitro* (Table 3). Interestingly, Jimbow *et al.*[16] observed that 4-S-cysteinylcatechol, a substrate for B-16 melanoma tyrosinase which forms an unstable quinone, does not depigment C57Bl mice *in vivo*.

The parameter $r_{SH} = k_{SH}$ [Q] is a pseudo-first order rate constant which contains the second order rate constant for the reaction between quinone and BSA -SH as well as a quinone concentration term (see equation 1). Both terms contribute to the experimentally observed net reactivities of the quinones with BSA; their individual values may be very different for each compound. For example, pulse radiolysis measurements indicate that dopaquinone reacts with cysteine -SH with $k_{SH} = 3 \times 10^7$/M/s,[17] while MMEHQ reacts with $k_{SH} = 3.5 \times 10^5$/M/s.[5] Comparison of these results with our r_{SH} values implies that [Q](t) is roughly three orders of magnitude lower for dopaquinone than for MMEHQ (3.0×10^{-12} M and 4.4×10^{-9} M, respectively). In view of the rapid self-cyclization of dopaquinone to form (leuko)dopachrome, this result might well have been expected. This is consonant with the idea expressed by Ito *et al.*[18] that the yield of BSA-bound catechol per catechol oxidized depends on a competition between intermolecular -SH addition and intramolecular cyclization. Clearly, other reactions mentioned above may contribute to quinone consumption, too.

Implicit in the determination of r_{SH} values is the assumption that addition of the quinone to the -SH group on BSA is the only important quinone reaction. There are several studies which establish that quinones react much more efficiently with -SH than with other amino acids[5,17,19] or isolated double bonds as in trans-2-butene.[5] As the determination of r_{SH} involves a difference measurement, the quinone reactions which do not directly involve BSA are automatically subtracted out of equation 1. Consequently, the above assumption appears reasonable. It is perhaps relevant in this regard to compare our results with those of Ito *et al.*,[18] who directly measured the covalent binding of a series of catechols (including DOPA, DOPAC and 4-methylcatechol) to BSA by HPLC. Our values of r_{SH} are in good qualitative agreement with their observed quinone reactivity (4-methylcatechol > DOPAC > DOPA), as measured by yield of protein-bound cysteinylcatechol.

The possibility that a major component of *in vivo* cytotoxicity is due to DP autoxidation seems unlikely,

Table 2. Hydroxybenzenes as substrates of B-16 melanoma tyrosinase

Compound	$r_q \times 10^{9a}$ (M/s/unit)
DOPA	4.4
DOPAC	2.0
MMEH	0.85
tBC	2.6

Substrates (300 μm in phosphate buffer, pH 7.4) were reacted with B-16 melanoma (enzyme units: 2 for DOPAC; 12 and 24 for DOPA, tBC; 70 for MMEH; see text). Results are of duplicate or triplicate runs on each compound. The r_q value for the DOPAC sample is uncertain by approximately ± 30% because of the low enzyme activity associated with the B-16 tyrosinase used in this sample. Uncertainty of other r_q values is ≤ ±5%.
[a] Rate of 1,2-quinone formation as judged spectroscopically (see text and reference 13).

since depigmentation does not correlate with ease of autoxidation in our compounds, as judged spectroscopically. However, the participation of hydroxybenzene semiquinone, active oxygen and/or other free radicals subsequent to tyrosinase oxidation cannot be ruled out by these experiments.

tBC was a more localized *in vivo* depigmenter than MMEH, but showed higher non-specific cytotoxicity. These observations may reflect the lower reactivity of MMEH compared to tBC for mammalian tyrosinase, and the much higher reactivity of tBCQ *vis à vis* MMEHQ to protein -SH (Table 3). MMEH is likely to diffuse farther than tBC before ultimately combining with melanocytic -SH; tBCQ is more likely to react randomly with non-melanocytic -SH groups than MMEHQ.

Our results on *in vivo* depigmentation of normal C-57Bl follicular melanocytes by MMEH and tBC are thus consistent with a mechanism involving reaction of tyrosinase-generated 1,2-benzoquinones with skin protein -SH. For this limited series of compounds, it appears from Table 3 that the best prognosticator of a good depigmenter may be the stability of its 1,2-benzoquinone in the absence of -SH.

Table 3. Reactivity of quinone oxidation product to bovine serum albumin

Compound	dQ/dt[b]		ΔdQ/dt[c]	r_{SH}	Stable quinone
	+BSA	−BSA			
DOPA[a]	3.63	0.96	2.67	1.1	yes
DOPA	78.8	78.8	–		
DOPAC	69.9	24.0	45.9	15.3	no
MMEH	41.2	4.2	37.0	12.3	no
tBC	612.5	5.2	607.3	202	yes

To generate quinones, substrates (300 μM in phosphate buffer, pH 7.4) were reacted with 3 μg (18 units)[a] or 30 μg (177 units) mushroom tyrosinase in the presence or absence of 300 μM BSA. MMEH reaction was supplemented with 45 μM L-DOPA as co-factor. r_{SH} is the pseudo-first order rate parameter for reaction of 1,2-quinone with 300 μM BSA/s × 10^4 (see text for further information).

[b] Rate of 1,2-quinone formation in M s × 10^8.
[c] ΔdQ/dt – difference between the two rates in M s^{-1} × 10^8.

That this mechanism may differ from that which would be operative *in vitro* and/or for melanoma cells should be re-emphasized. A number of studies *in vitro* suggest, at least for catechols, the importance of active oxygen intermediates generated by autoxidation of the hydroxybenzene,[7,10,20] which act outside the cell. In the latter systems, tyrosinase does not seem to play a major role, since catechols which are substrates of tyrosinase do not appear to show any specific toxicity against tyrosinase-containing melanoma cells compared to other cell lines which do not contain tyrosinase.[7] There appears to be little[21] or no[22] relationship between *in vitro* cytotoxicity and tyrosinase activity. This may be attributable to relatively small differences in tyrosinase activity among the cell lines studied.[21] On the other hand, Naish-Byfield *et al.*[23] observed tyrosinase-dependent toxicity to non-melanogenic cells in a model *in vitro* system for a series of 30 substituted phenols. They concluded that, for these compounds, reductive ring addition to 1,2-quinone (e.g. to -SH) is a determinant of cytotoxicity and that autoxidation is not important. Moreover, the relative contribution due to cytotoxicity by extracellularly generated active oxygen species is generally different for each hydroxybenzene,[4] and depends, among other things, on hydroxybenzene structure, reactivity, polarity, and accessibility of the drug to critical cell components.[23]

Such mechanistic complexities may give rise to differences in *in vivo* and *in vitro* results. *In vitro* cytotoxicity of our compounds to melanoma cells follows a different 'rank order' than that for *in vivo* depigmentation of C57Bl follicular melanocytes, with tBC ≫ MMEH = DOPA = DOPAC.[1,7,24] These considerations become important when follicular depigmentation is used as a preliminary screen for possible melanoma therapeutic agents (see reference 2 and references therein).

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