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Original Paper

Melanogenesis-targeted Anti-melanoma Pro-drug Development: Effect of Side-chain Variations on the Cytotoxicity of Tyrosinase-generated Ortho-quinones in a Model Screening System*

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A set of 26 substituted phenols, 10 of which were synthesised in our laboratories, were tested for their rate of oxidation by mushroom tyrosinase *in vitro* as determined by oximetry and spectrophotometry and for their cytotoxic action in a model system. With one exception (4-hydroxybenzoic acid) all the agents tested were oxidised to the corresponding *ortho*-quinones. The maximum rates of oxidation varied between 15.1 ± 0.59 nmoles oxygen consumed per minute (4-(2-thioethylthio)phenol) and 372.9 ± 5.61 nmoles O_2/min. (4-(2-Hydroxyethylthio)phenol) in a reaction system comprising 300 units tyrosinase and 200 μM substrate. The rates of generation of quinone were in close agreement with these oximetric data. Some anomalies in oxygen stoichiometry were observed due to reoxidation of reaction products. Four categories of compounds were tested: those known to undergo side-chain cyclisation (such as tyrosine) (Group A), alkylphenols of increasing chain length with or without terminal hydroxyl groups (Group B), compounds with charged or bulky side-chains (Group C) and agents with oxy-, thio- and selenyl-ether side-chains (Groups D, E and F). In the majority of cases, the cytotoxicity, measured by the reduction of thymidine incorporation in cells exposed for 30 min to the agent in the presence of tyrosinase, reflected the rate of oxidation and is ascribed to the toxic action of the derived *ortho*-quinone. Tyrosinase-dependent cytotoxicity was absent in cyclising (Group A) and in Group C compounds. Toxicity, expressed by comparison with 4-hydroxyanisole (4HA) (IC_{50} = 11.7 μM), ranged between 0.36 (4-hydroxybenzyl alcohol) and 1.07 (3-(4-hydroxyphenyl)propanol) for Group B compounds, and be-tween 0.83 (4-ethoxyphenol) and 2.08 (4-(2-hydroxyethylthio)phenol) for groups D, E and F. Addition of glutathione to the toxicity assay system abrogated the cytotoxic action and, on the basis of spectrophotometric data, this is ascribed to the prevention of cellular thiol depletion by the *ortho*-quinone products of tyrosinase oxidation of the phenolic substrates. The lack of toxicity of the group C compounds may be due to the inability of their derived quinones to gain access to the cells. Addition of catalase or deferoxamine to the incubation medium was without effect on tyrosinase-dependent toxicity. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: melanogenesis, melanoma, tyrosinase, pro-drug, ortho-quinones, thiols, glutathione, cytotoxicity

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INTRODUCTION

THE FORMATION of melanin is a complex process, the first step of which is the oxidation of the amino acid L-tyrosine

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to the corresponding *ortho*-quinone (dopa-quinone). In humans, this process uniquely occurs in melanocytes which contain the enzyme tyrosinase which catalyses this oxidation. Quinones are chemically reactive compounds that are potentially harmful, but in melanocytes the normal process of melanogenesis is not usually associated with significant toxicity due to the compartmentation of the reaction within membrane-limited organelles (melanosomes) and because of the rapid cyclisation of the intermediate quinone. The discovery that certain substituted phenols have a depigmenting action due to their ability to act as substrates for tyrosinase [1–3], resulting in the generation of cytotoxic quinones [4, 5], has led to the examination of this system as a possible targeted antimelanoma therapeutic strategy in the case of disseminated melanoma [6–8]. For such a chemotherapeutic agent to be useful, the prodrug must evade hepatic metabolism and other potentially toxic reactions [9–13], reach the tumour tissue and enter the malignant melanocytes, evade alternative cellular metabolism, enter melanosomes and be oxidised by tyrosinase to generate significant amounts of the quinone product. Moreover, the quinone must be able to re-enter the melanocyte cytosol and to initiate damaging reactions which kill the cell. There are likely, therefore, to be many structural requirements that need to be optimised, including those that determine the oxidation rate by tyrosinase, the reactivity of the quinone, and its access to the sensitive target moieties in the cell.

We have examined some of these factors for a range of substituted phenol substrates using an *in vitro* cytotoxicity assay. We have compared the cytotoxicity with the oxidation rate of a number of phenols, and present data which suggest that the major cytotoxic action is due to depletion of thiols and that access to intracellular sites is necessary for this cytotoxic action to be exerted.

MATERIALS AND METHODS

Chemicals

With the exception of the agents synthesised by us, as indicated in Table 1, the materials tested were obtained from Aldrich/Sigma, Gillingham, Dorset, U.K. and were used as received. p-Cresol was purchased from BDH Laboratory Supplies, Poole, Dorset, U.K., 4-hydroxyanisole (4HA) was obtained from Koch-Light Ltd and was recrystallised from water. The synthesis of methyl- and propylselenyl phenols are described herein. The remaining compounds were synthesised as previously described [14], purified by flash chromatography and purity checked by NMR (nuclear magnetic resonance).

Table 1. *The effect of side-chain structure on the comparative rate of oxidation (COR) and the comparative cytotoxicity index (CCI). COR is based on the maximum rate of tyrosinase-catalysed oxidation of* 4*-hydroxyanisole (i.e.* 1 *unit* = 98.6 *nmoles oxygen utilisation per min). CCI is based on the tyrosinase-dependent cytoxicity of* 4*-hydroxyanisole (i.e.* 1 *unit* = IC_{50} *of* 11.7 μ*M). The tyrosinase-independent cytotoxicity (TIC) shows the normalised cytotoxicity of the compound at the highest concentration used* (10^{-4} *M) in the presence of heat-inactivated tyrosinase*

Group	Compound number	R	(source)		COR	CCI	TIC
A	1	—$CH_2CH(NH_2)COOH$	(Sigma)		1.06	−0.26*	0.07
	107	—$CH_2CH_2NH_2$	(Sigma)		0.44	−0.01*	0.32
	122	—$CH_2CH(NH_2)COOCH_3$	(Aldrich)		1.36	0.15*	0.05
B	130	—CH_3	(BDH)		1.54	0.96*	0.05
	86	—CH_2OH	(Aldrich)		0.29	0.36*	0.21
	97	—CH_2CH_2OH	(Aldrich)		0.97	0.73*	0.33
	89	—$CH_2CH_2OH_2OH$	(Aldrich)		1.31	1.07	0.07
	88	—$CH_2CH_2COCH_3$	(Aldrich)		2.05	0.51	−0.05
C	85	—$COOH$	(Aldrich)		0.00	−0.07*	−0.06
	87	—CH_2COOH	(Aldrich)		0.33	0.19*	0.14
	82	—CH_2CH_2COOH	(Aldrich)		0.66	−0.02*	0.19
	92	—CH_2CH_2COON (—$COCH_2CH_2CO$—)	(Aldrich)		0.78	−0.13*	0.21
	100	—$CH_2CH_2N(CH_3)_3^+$	(Aldrich)		0.79	0.06*	0.84
D	2	—OCH_3	(K-L)	[I]	1.00	1.00	−0.07
	22	—OCH_2CH_3	(CJC)	[II]	0.65	0.83	−0.02
	23	—$OCH_2CH_2CH_3$	(CJC)	[III]	0.77	1.51	−0.09
	57	—$OCH_2CH{=}CH_2$	(CJC)	[XI]	0.66	0.98*	−0.11
	5	—$OCH_2CH(CH_3)_2$	(CJC)	[V]	1.18	1.55	0.14
	27	—$OCH_2CH_2CH_2CH_3$	(Aldrich)	[IV]	0.64	0.96	−0.07
	65	—OCH_2CH_2OH	(CJC)	[VIII]	1.07	1.00	−0.03
E	66	—SCH_3	(Aldrich)	[XIII]	0.47	1.55	0.00
	63	—SCH_2CH_2SH	(CJC)	[XVI]	0.15	0.95*	0.03
	62	—SCH_2CH_2OH	(CJC)	[XV]	3.78	2.08	0.03
	75	—$SCH_2CH_2CH_3$	(CJC)	[XIV]	0.83	0.89	0.03
F	73	—$SeCH_3$	(CJC)		0.64	1.44	0.15
	74	—$SeCH_2CH_2CH_3$	(CJC)		1.66	0.88	−0.06

[ ], numerals in brackets refer to the compound identification number used in Naish-Byfield and coworkers [14]; *estimated as mean inhibition at two fixed concentrations (see text for details); BDH, British Drug Houses Ltd; K-L, Koch-Light Ltd; (CJC), Synthesised by C. J. Cooksey, Dept. Chemistry, UCL.

The synthesis of selenylphenols was carried out as follows: 4-bromophenol was protected by O-*tert*-butyldimethylsilylation and converted to the Grignard reagent by 1,2-dibromoethane-assisted reaction with magnesium. The addition of selenium followed by aerial oxidation gave a mixture of O-*tert*-butyldimethylsilyloxyphenyl di- and polyselenides. Borohydride reduction gave a selenide anion which was alkylated with an alkylhalide. Deprotection of the hydroxyl group with potassium fluoride completed the synthesis (Figure 1). We believe that this is a general method for the synthesis of 4-alkylselenylphenols.

*4-Bromo-O-*tert*-butyldimethylsilylphenol*

Dry tert-butyldimethylsilyl chloride (TBDMSCl) (15.0 g, 0.1 mol) was added to a solution of dry 4-bromophenol (12.1 g, 0.07 mol) and dry imidazole (16.5 g, 0.24 mol) in dry dimethylformamide (DMF) (50 cm^3) and the mixture magnetically stirred (2 d). Water (5 cm^3) and 60/80 petrol (50 cm^3) were then added, followed after 1 h by more water (100 cm^3), the phases were separated and the aqueous phase extracted twice with 1:1 60/80 petrol-diethylether (100 cm^3). The combined organic phases were washed successively with water, aqueous NaOH solution (1 M), water and saturated NaCl solution. After drying ($MgSO_4$), the solution was evaporated and the residue distilled to give a colourless oil, 19.8 g (98%), b.p. 67–68°/0.01 mm. ^1H NMR ($CDCl_3$, 60 MHz), δ 7.13d (2 H), 6.25d (2H), 0.93 s (9 H), 0.16s (6 H). ^{13}C NMR ($CDCl_3$, 100 MHz), δ 154.78, 132.26, 121.68, 113.60, 25.61, 18.16, –4.50.

*Bis(4-*tert*-butyldimethylsilyloxyphenyl) diselenide*

A mixture of 4-bromo-O-*tert*-butyldimethylsilylphenol (23.8 g, 0.083 mol) and 1,2-dibromoethane (9.0 g, 0.048 mol) in diethylether (50 cm^3) was added dropwise to a stirred suspension of magnesium metal (7.0 g, 0.29 mol) in diethylether (50 cm^3) under nitrogen. When the reaction commenced more diethylether (100 cm^3) was added and the rate of addition regulated to maintain gentle reflux. After the end of the addition the mixture was refluxed for 5 h. After cooling, the solution was decanted from excess magnesium metal and precipitated selenium (7.0 g, 0.091 mol) added over a period of 15 min. Stirring was continued for 12 h, then, with ice-cooling, HCl (50 cm^3, 2 M) was added and the suspension filtered. The phases of the filtrate were separated and the diethylether phases evaporated to give a viscous orange oil which was dissolved in ethanol (200 cm^3), NaOH (2.0 g) added and air was bubbled through for 24 h. The dark solution was filtered with charcoal and Celite and evaporated to give a deep yellow oil (22.3 g) which was used directly for the next step.

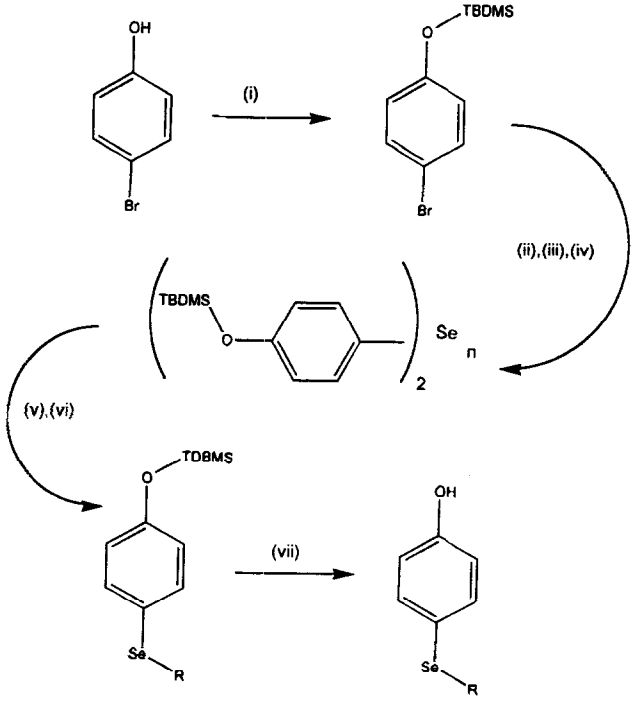

Figure 1. Schematic representation of 4-alkyl selenylphenol synthesis. Reagents: (i) TBDMSCl, imidazole, DMF; (ii) Mg, diethylether; (iii) Se; (iv) air; (v) Na borohydride; (vi) RX; (vii) KF, methanol.

*4-Methylselenyl-O-*tert*-butyldimethylsilylphenol*

The crude diselenide (20.0 g) was diluted with ethanol (200 cm^3) and with magnetic stirring under nitrogen, solid $NaBH_4$ (a total of 16.0 g) was added in portions over 3 h, giving a colourless solution. Excess iodomethane (50 cm^3) was added and the mixture heated at 55°C for 1 h. Water (250 cm^3) was added and the mixture extracted with 1:1 diethylether 80/100 petrol (3 × 100 cm^3). The combined organic phases were washed with water, HCl (1 M), dried ($MgSO_4$), evaporated and the residue distilled to give a colourless oil, b.p. 76–80°/0.01 mm. ^1H NMR ($CDCl_3$, 60 MHz), δ 7.12d (2 H), 6.53d (2 H), 2.21s (3 H, J_{SeH} 9.6), 0.96s (9H), 0.19 s (6H).

4-Methylselenyl-phenol

4-Methylselenyl-O-*tert*-butyldimethylsilylphenol (1.0 g) was dissolved in methanol (15 cm^3) and KF (0.2 g) added. After 30 min the methanol was evaporated to give a white solid which was purified by flash chromatography on silica gel eluting with chloroform—60/80 petrol (3:7) followed by chloroform—60/80 petrol-methyl acetate (3:6:2) to give a pale brown solid (530 mg, 85%). Recrystallisation from 1:1 chloroform—60/80 petrol gave white plates, m.p. 77–78°, ^1H NMR ($CDCl_3$, 400 MHz) δ 7.38d (2H, J 8.7), 6.76d (2 H, J 8.7), 4.77 brs (1 H), 2.31 s (3H, J_{SeH} 11.0), ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.83, 133.77, 121.60, 116.45, 8.76, ^{77}Se NMR ($CDCl_3$) showed a single peak (proton decoupled) and the proton coupled INEPT spectrum showed a 1:1:-1:-1 quartet (J_{SeH} 11.5) of triplets (J_{SeH} 4.1). The EI-MS molecular ion at m/z 188, for ^{80}Se, and the base peak (m/z 173, M^+-CH_3) both showed the expected pattern for the selenium isotopes. MS accurate mass calculated for C_7H_8OSe = 187.9738, measured = 187.9766. Elemental analysis calculated = C 44.94, H 4.31, found C 45.20, H 4.18.

4-Propylselenyl-O-*tert*-butyldimethylsilylphenol was prepared in a similar manner, using 1-bromopropane in place of iodomethane, giving a colourless oil, b.p. 82–88°/0.01 mm, ^1H NMR ($CDCl_3$, 60 MHz) δ 7.09d (2 H), 6.46d (2 H), 2.99t (2 H), 1.55sex (2 H), 0.96s + t (12 H), 0.19s (6 H); ^{13}C NMR ($CDCl_3$, 100 MHz, multiplicity determined by APT) δ 154.84s, 135.45d, 120.04s, 115.20d, 31.20t, 23.26t, 14.17q.

Deprotection gave 4-propylselenylphenol as a pale brown oil, ^{1}H NMR ($CDCl_3$, 200 MHz), δ 7.17d (2 H), 6.54d (2 H), 6.90 s (1 H), 2.71t (2 H), 1.55sex (2 H), 0.93 t (3 H).

Other chemicals

Glutathione (reduced form, free acid, GSH), catalase (CAT) from bovine liver, and deferoxamine mesylate (DFO) were obtained from Aldrich/Sigma, Gillingham, Dorset, U.K. All reagents were made up in distilled water, filter-sterilised and used as indicated below.

Cells

Cytotoxicity assays were made on an established line of epithelial cells derived from rat hepatocytes (CNCM-I 221) essentially as previously described (14). Briefly, the cultures were propagated in growth medium consisting of Eagle's minimum essential medium (MEM) with Earle's salts buffered with 20 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (Hepes) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), bicarbonate (7.5%), and fetal bovine serum 10% (all obtained from Imperial Laboratories Ltd, Andover, U.K.). Cells were grown in loosely-capped polystyrene flasks (75 cm^2 growth area) at 37° in a 2% CO_2 moist atmosphere and subcultured regularly on a weekly basis by trypsinisation and seeded at a dilution of 10^3 cells/ml.

For experiments cells were harvested by trypsinisation, resuspended in growth medium and inoculated at 5×10^4 cells/well into the central 8 wells of 24-well dishes (Falcon 3047, Marathon Laboratory Supplies, London, U.K.) and allowed to settle for 48 h prior to exposure. Cells were washed once with Dulbecco's Phosphate Buffered Saline (PBS) pH 7.4 (Imperial Labs) and incubated in 1 ml PBS. After 30 min the medium was removed for spectrophotometry and the cells washed twice with PBS. Growth medium was replaced to which ^{3}H-TdR (methyl [^{3}H]thymidine, specific activity 37 MBq/ml, Amersham International Ltd, Amersham, U.K.) was added in a final concentration of 1 μCi/ml. At the end of the labelling period (30 mins), the cells were fixed with 5% TCA and washed with PBS. The plates were dried in a stream of warm air and the monolayer digested overnight in 1 M NaOH (250 μl/well). Aliquots (100 μl) of the digestate were transferred to scintillator mixture (Ultima Gold, Canberra Packard, Pangbourne, Berks, U.K.) and counted in a Beckman scintillation counter. The residual digestate was bulked for each set of quadruplicate wells and the extinction at 280 nm recorded on a Unicam spectrophotometer (PU 8600) as an estimate of the total cell numbers. Results were presented as c.p.m. ± standard deviation for each set of wells. Cytotoxicity was expressed as the percentage inhibition of tritiated thymidine incorporation. Tyrosinase-dependent toxicity was calculated as the ratio of net inhibition in the presence of active enzyme to that in the presence of heat-inactivated tyrosinase. IC_{50} values were obtained from the best linear fit of the data on tyrosinase-dependent inhibition. Where insufficient points were available (i.e. <3 separate concentrations of the compound tested) a mean activity ratio was calculated as previously described, using an in-house computer programme [14].

In experiments where cells were exposed to 4HA in the presence of additional GSH or antioxidant enzymes the additions were made to the medium prior to the test agent. The final concentrations used were as follows: glutathione (100 μM), catalase (160 μg/ml), and DFO (100 μM). At these concentrations the reagents were shown to have no effect on tyrosinase activity by the oximetry assay.

Oximetry

Oximetry was carried out using a Clark electrode and monitored by an Oxygen meter (Yellow Springs Co. Inc., U.S.A., model 5300). Two types of chamber were used. For most experiments a water-jacketed reaction chamber was employed, the temperature being homeostatically maintained by circulating water from a 30°C thermostatically controlled water bath. Some experiments were conducted at room temperature in a modified spectrophotometer cuvette with a side arm fitted to permit the oxygen electrode tip to contact the sample. In both cases the sample fluid was stirred vigorously with a small electronic stirrer (Rank Bros., model 200).

Spectrophotometry

Spectra were recorded using a Hewlett-Packard diode array spectrometer (Model 8452A) controlled by standard H-P software.

Calculations and statistics

Oxidation rate calculations were made from triplicate assays using the measured maximum slopes of oxygen utilisation adjusted for temperature and the volume of the reaction vessel. Comparative oxidation rates (COR) were calculated from the mean rates of oxygen utilisation compared with the rate for 4HA (which was included as a standard in each set of estimations). In the case of combined oximetry and spectrophotometry the rate of quinone generation was calculated using the kinetics programme of the spectrophotometer. For 4HA oxidation the rate of quinone formation, using the extinction coefficient of the 420 nm absorption peak (ε = 1720) [15], was found to be the same as the rate of oxygen utilisation on a molar basis, as expected. All means were expressed with standard deviations and probability calculations were made by the two-tailed Student's *t*-test with 5% significance limits.

RESULTS

The compounds tested in this series of experiments are listed in Table 1 which gives the laboratory identification number and the nature of the side-chain (R). All the compounds were 4-substituted phenols. Table 1 also summarizes the oximetry data, expressed as the comparative oxidation rate (COR), and the comparative cytotoxicity index (CCI) using 4HA as the standard (=1.0) in each case.

Oximetry

The COR was calculated as the ratio of the rate of oxygen utilisation by the compound in the presence of tyrosinase and that recorded for 4HA (compound 2) in the same conditions. The rate of oxygen utilisation by 4HA (mean = 98.6 nmoles per min) varied slightly between experiments (coefficient of variation <7%) so 4HA controls were always included. The rates of oxygen consumption determined are given in Table 2. These values are the maximum rates (R_{max}) calculated from the slopes of the recorded oximeter traces. Table 2 also shows the calculated stoichiometry of oxygen utilisation based on the total oxygen uptake at the end of the reaction. The theoretical stoichiometry for the

Table 2. Oximetry data: the maximum rate of reaction (R_{max}) is in nmoles oxygen utilised per min measured for a concentration of 200 μM substrate in the presence of 300 units/ml tyrosinase. SD = standard deviation. Oxygen stoichiometry is given as moles oxygen per mole substrate

Group	Compound number	Name	R_{max}	SD	Oxygen stoichiometry
A	1	L-tyrosine	104.9	2.93	1.43
	107	Tyramine	43.8	1.18	1.30
	122	L-tyrosine methyl ester	133.9	2.86	1.63
B	130	p-cresol	151.7	3.78	0.74
	86	4-hydroxybenzyl alcohol	28.4	2.60	0.98
	97	4-hydroxyphenethyl alcohol	96.1	2.35	1.18
	89	3-(4-hydroxyphenyl)propanol	128.7	1.18	1.20
	88	4-(4-hydroxyphenyl)butanone	202.2	17.6	1.38
C	85	4-hydroxybenzoic acid	0.0	0	0.03
	87	4-hydroxyphenylacetic acid	32.7	1.15	1.15
	82	3-(4-hydroxyphenyl)propionic acid	65.3	6.30	0.96
	92	3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinamide	76.6	2.66	1.11
	100	3-(4-hydroxyphenyl)propionyl-trimethylammonium	77.4	4.0	1.04
D	2	4-hydroxyanisole	98.6	0.92	1.00
	22	4-ethoxyphenol	64.1	1.17	0.73
	23	4-propoxyphenol	76.3	1.34	0.63
	57	2-propenyloxy-4-hydroxybenzene	65.3	1.18	0.82
	5	4-iso-butoxyphenol	116.2	5.82	0.89
	27	4-n-butoxyphenol	63.5	4.11	0.41
	65	4-(2-hydroxyethoxy)phenol	105.6	1.48	0.82
E	66	4-(methylmercapto)phenol	45.9	0.64	0.81
	63	4-(2-thioethylthio)phenol	15.1	0.59	0.18
	62	4-(2-hydroxyethylthio)phenol	372.9	5.61	1.20
	75	4-(butylthio)phenol	82.0	2.33	0.73
F	73	4-(methylselenyl)phenol	63.4	1.96	0.87
	74	4-(propylselenyl)phenol	114.4	0.74	0.65

conversion of a phenol to the corresponding *ortho*-quinone is 1.0 i.e. one mole of oxygen is required to convert one mole of phenol to one mole of quinone according to the reaction shown in Figure 2. Some of the variation of the stoichiometry compared with the expected ratio is due to subsidiary reactions. For example, in the case of L-tyrosine (compound 1), cyclisation of dopaquinone results in the effective disproportionation of dopa quinone to dopa and dopachrome. Since dopa is readily oxidised by tyrosinase with the reduction of 1/2 mole of oxygen per mole of catechol (Figure 2), the stoichiometry of oxygen utilisation is theoretically increased to 1.5 moles oxygen per mole of tyrosine oxidised. These considerations also apply to tyramine (cpd 107) and tyrosine methyl ester (cpd. 122). This is reflected by the values recorded for the stoichiometry (see Table 2), and the secondary reactions may also contribute to the overall rate of oxygen utilisation recorded for these substrates. This cyclisation phenomenon does not appear to occur in the case of the ether side-chains (group D, E and F compounds) for which stoichiometry values of 1.0 or less were recorded. We believe that in these cases the maximum rates obtained by oximetry are good estimates of the relative rate of quinone formation by the primary tyrosinase-catalysed oxidation of the corresponding substrates and this conclusion is consistent with the rate of accumulation of the quinones measured by UV-visible spectrophotometry. Oxygen stoichiometry greater than unity was observed in the case of compounds with side-chains bearing terminal hydroxyl groups. These included Group B compounds (97, 89) and 4-(2-hydroxyethylthio)phenol (compound 62) suggesting that some cyclisation takes place through the side-chain hydroxyl group. The butanone-substituted compound (cpd 88) also appears to cyclise. It would have been anticipated that the thiol analogue (compound 63), being a strong nucleophile, would readily cyclise and thus exhibit a raised oxygen stoichiometry but this compound was found to be a poor substrate for tyrosinase.

Spectrophotometry

Examination of the difference between the spectra recorded for the substrate plus tyrosinase reaction system *in vitro* and those obtained for the corresponding system in the presence of cells (i.e. in the supernatant of the cytotoxicity

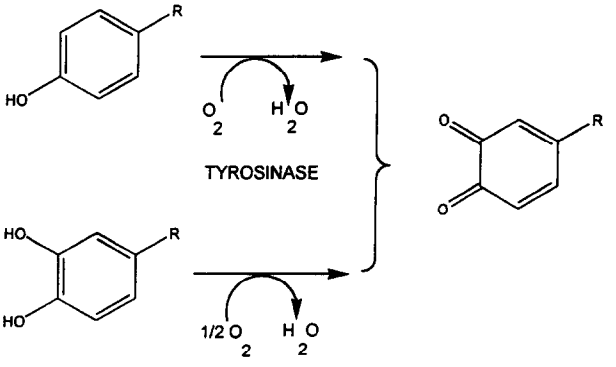

Figure 2. Tyrosinase-catalysed oxidation of phenolic substrate to corresponding *ortho*-quinone utilising one mole of oxygen per mole of substrate oxidised; and oxidation of catecholic substrate utilising half a mole of oxygen per mole of substrate.

assay) demonstrated the formation of a product with spectral characteristics resembling those of the product formed between anisyl *ortho*-quinone and glutathione. Since, in both cases, tyrosinase is present, it is probable that the product of the reductive addition will be reoxidised to the corresponding quinone by reaction with the quinone product of the enzyme reaction, with the catechol formed by this redox exchange being reoxidised by tyrosinase. Thus the spectra are likely to be of the quinones of the GSH addition products. In the case of anisyl orthoquinone, this product has a pronounced absorption peak near 320 nm and a component absorbing in the visible spectrum near 500 nm (Figure 3). Table 3 lists the wavelengths of the absorption maxima found in the cytotoxicity assay supernatant of the agents tested compared with the absorption peaks of the corresponding *ortho*-quinones. In all cases where the agent exhibited significant cytotoxicity, there was spectroscopic evidence of the formation of an adduct. In the case of Group B compounds, some of the peaks present in the cell supernatant differed from those of the *in vitro* GSH adduct, especially at the longer wavelengths (e.g. for compound 86 the uppermost peaks are at 490 nm and 368 nm, respectively) which may indicate more complex metabolic interactions. However, in several cases (e.g. group D compounds) there was close spectral correspondence with the GSH adduct formed *in vitro*, although there was evidence of slightly red-shifted peaks in the cell supernatants and the precise nature of the products will need to be established by chromatographic identification. By contrast, no such shift in the quinone spectrum occurred in the case of non-cytotoxic agents (group C) and the supernatant spectra correspond to those of the corresponding quinones. The cyclizing compounds of group A gave rise to stable quinones exemplified by dopachrome with an absorption peak near 520 nm which are capable of reaction with GSH *in vitro*, but absent in the supernatant spectrum (see Table 3).

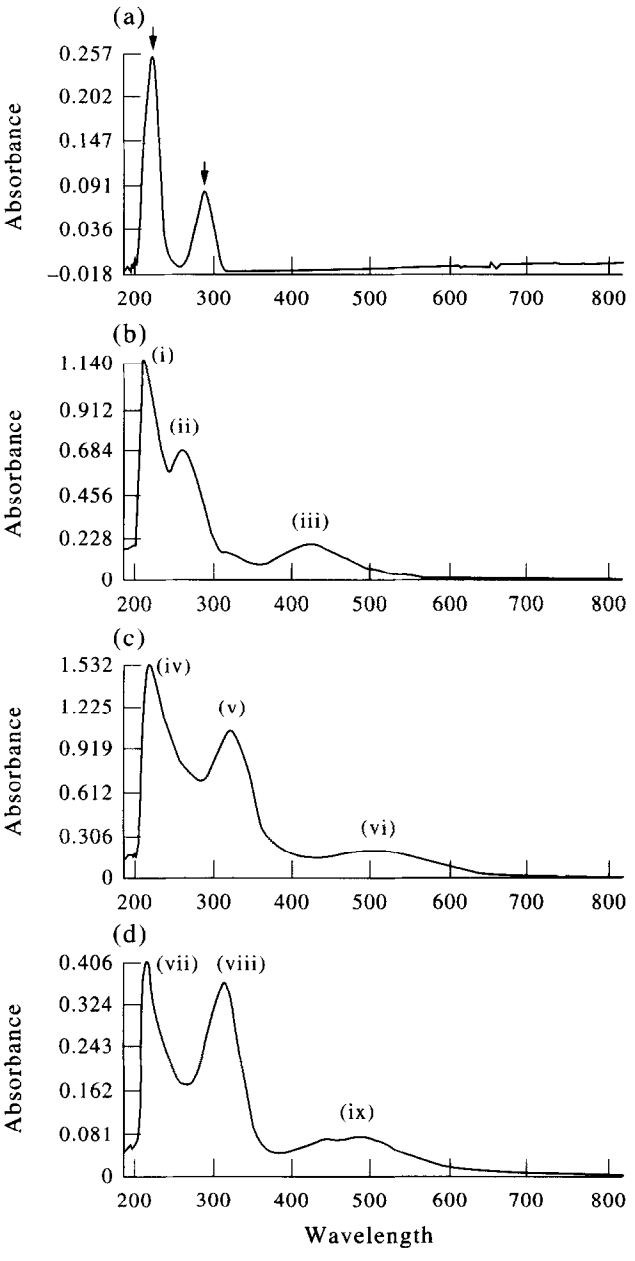

Figure 3. Absorption spectra of (a) 4-HA; (b) the tyrosinase oxidation product (anisyl-3,4-quinone); (c) the GSH addition product generated *in vitro* by GSH addition; and (d) the products in the reaction system in the presence of cells. The quinone absorption peaks at 206, 264 and 420 nm are indicated as (i), (ii), (iii) respectively; the peaks after *in vitro* GSH addition are marked (iv), (v), (vi); and the supernatant peaks (vii), (viii), (ix) (see Table 3).

Cytotoxicity

The cytotoxicity of the agents tested is summarised in Table 1. The IC_{50} values were determined from the best linear fit of at least three data points plotted as the tyrosinase-dependent percentage inhibition of thymidine incorporation against the logarithm of the concentration. From these data, the comparative cytotoxicity index (CCI) was calculated by comparison with the IC_{50} for the lead compound, 4-hydroxyanisole (11.7 μM). In some instances, where IC_{50} values were not obtained, the CCI is based on the ratio of the average percentage inhibition produced by the test compound compared with that produced by 4HA at two fixed concentrations (50 and 100 μM final concentration). Table 1 also shows the tyrosinase-independent cytotoxicity (TIC) of the compounds for comparison. In most cases these values, obtained from data on cells exposed to the agents in the presence of heat-inactivated tyrosinase, are near zero or negative indicating lack of toxicity or slight stimulation of thymidine incorporation.

The tabulated values show that tyrosinase-dependent cytotoxicity is in some cases related to chain length, e.g. for the series of alcohols in Group B (methyl, ethyl, propyl) the CCI values increase in the order 0.36, 0.73, 1.07 i.e. approximately by 0.36 per CH_2 group. This parallels the rate of oxidation. The trend is not so clear in the case of the ether substituted side-chains (groups D, E and F) and solubility criteria may be important in these cases. Compounds with potentially cyclising amino groups in the side chain e.g. (tyrosine and tyramine) (group A) were found to be non-toxic as were other agents with a net charge on the side-chain e.g. carboxylic acids and trimethylammonium functional groups (group C). Bulky side groups appeared also to diminish cytotoxicity (e.g. N-succinimide, cpd 92). There was no evidence that these non-toxic agents failed to generate quinones. As shown in Table 1 the oxidation rate of the majority of the non-toxic derivatives is rapid, an exception

Table 3. Absorption peaks of quinones and adducts

Group	Compound number	Quinone absorption peaks			After GSH addition			Supernatant peaks		
		(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)	(ix)
A	1	220	276 (306)	522	212	366	520	212	264	320
	107	222	276 (302)	522	214	270 (346)	512	210	268	360
	122	218	336 (400)	490	212	296	398	210	260	340
B	130	210	272	322	222	262 (320)	476	218	284	488
	86	212	318	418	224	262	368	216	280 (306)	490
	97	210	274	342	222	268 (370)	476	216	288	498
	89	208	276	340	216	266 (312)	482	216	276 (340)	476
	88	206	280	328	226	272	370	220	290	376
C	85*	—	—	—	—	—	—	—	—	—
	87	212	280 (328)	420	214	272	360	226	280 (310)	420
	82	212	272	480	212	272	342	218	272	482
	92	210	264	490	214	262	342	218	262	492
	100	208	268	486	210	270	346	212	272	480
D	2	206	264	420	212	304	434	216	312	476
	22	206	268	420	212	306	426	216 (282)	312	460
	23	210	276	424	212	306	424	212	312	464
	57	210	264	422	212	306	434	216	312	492
	5	208	268	420	212	304	426	212	312	480
	27	206	256	420	212	310	444	216	312	476
	65	208	298	452	210	294	488	216	304	476
E	66	208 (250)	326	462	218	334	483	220	356	490
	63	208 (228)	254	482	212	316	480	232 (258)	306	480
	62	210	356	494	214	338	500	216 (250)	336	480
	75	208	328	464	212	342	480	210 (280)	340	440
F	73	216	350	486	216	320	356	210 (226)	264	352
	74	216	352	490	214	310	368	230	362	420

The table shows the major absorption peaks (in nm) of the quinone products of tyrosinase action (i, ii, iii); the corresponding peaks when glutathione is added (incubation time 4 h at 24°C) *in vitro* (iv, v, vi) and the peaks detected in the supernatant when cells were present for 30 min at 37°C followed by standing for 1 h at 24°C (vii, viii, ix). The values in parentheses represent subsidiary peak wavelengths. The asterisk (*) indicates that no quinone was formed in the case of compound 85.

being 4-hydroxybenzoic acid (cpd 85) which is not oxidised by mushroom tyrosinase.

Modification of cytotoxicity

The evidence that cytotoxicity is associated with the formation of a thiol adduct resembling an addition product with glutathione suggested the possibility that the toxic action is the result of depletion of cellular GSH. Experiments were, therefore, made to distinguish between toxicity due to the generation of a redox active product, which could act by being a source of reactive oxygen species by redox cycling, and that resulting directly from depletion of cellular glutathione. In these experiments, the effect on cytotoxicity of addition of GSH, catalase or deferoxamine was tested in the 4HA system. The results are shown in Figure 4 and indicate that supplementation of the medium with GSH in which cells were exposed abolishes cytotoxicity whereas the other agents tested were without effect.

DISCUSSION

Group A compounds include tyrosine (cpd 1), tyrosine methyl ester (cpd. 122) and tyramine (cpd 107) which undergo cyclisation by nucleophilic attack on the ring by the amino group in the side-chain. Since this is a reductive addition, it forms the basis of the disproportionation of dopaquinone resulting in the formation of dopachrome and dopa, which is reoxidised by tyrosinase with the consequent increase in the stoichiometry of oxygen utilisation to 1.5.

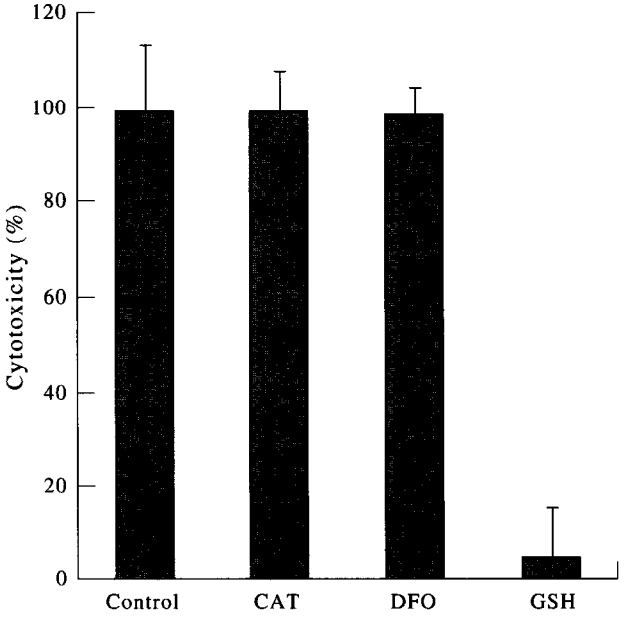

Figure 4. Cytotoxicity of 4HA in the presence of tyrosinase to show the effect of adding catalase (CAT), deferoxamine (DFO) and GSH to the incubation medium. The histogram shows the means and standard deviations.

The lack of cytotoxicity manifested by these compounds is ascribable to the relative unreactivity of dopachrome which may be inherent in its stabilization by the quinone-imine tautomer.

Group B includes p-cresol (cpd 130) and compounds with terminal hydroxyl groups. These are more rapidly oxidised, and are equivalently more toxic, with increasing chain length. Their oxygen utilisation stoichiometry suggests that with chain lengths of 2 or more corresponding quinones are able to form ring compounds, the quinones of which (unlike dopachrome) remain active since tautomeric stabilisation cannot occur. The situation is not so clear in the case of the carbonyl end group (compound 88): it is possible that this may cyclise through its hydrate.

Group C compounds include 4-hydroxybenzoic acid (cpd 85) which is not oxidised by tyrosinase and is therefore non-toxic in this system. It also includes agents with longer side-chains with a terminal —COOH group (compounds 87 and 82), a bulky substituted carboxyl (cpd 92), and a trimethylammonium group (cpd 100). The corresponding quinones are formed by tyrosinase, and are reactive as shown by the data summarised in Table 3, so that the explanation of their non-toxicity may lie with their inability to gain access to the target molecules, possibly by virtue of their net electrical charge or by steric hindrance.

Group D includes all the oxy-ethers tested. All of these compounds were easily oxidised by tyrosinase and, in general, their cytotoxicity reflects the rate of oxidation although some agents (e.g. propoxyphenol, cpd 23) were more toxic than would be anticipated on the basis of oxidation rate alone. This was also true for thioethers (Group E) especially methylthiophenol (cpd 66) and thioethylthiophenol (cpd 63). The latter was found to exhibit clear tyrosinase-dependent toxicity, although it was apparently a poor substrate for the enzyme, possibly by virtue of interference by the terminal thiol group with the copper atoms at the active site. By contrast, the hydroxyethylthio- side-chain (cpd 62) greatly favoured tyrosinase-catalysed oxidation (comparative oxidation rate = 3.78) and this compound was also the most cytotoxic with an IC_{50} of 5.7 μM. Nevertheless the toxicity, on the basis of oxidation rate, would be expected to be greater which may indicate that the corresponding quinone is able to undergo cyclisation, an inference consistent with the oxygen stoichiometry recorded. The difference between the expected and actual toxicity may, therefore, represent the slightly diminished reactivity of the putative cyclic quinone. The selenyl ethers (Group F) exhibited properties closely similar to the corresponding thioethers, although the oxidation rate was higher for the corresponding selenyl compound.

In all instances where cytotoxicity was observed, we have shown the formation of a product in the cellular supernatant with spectroscopic properties similar to those seen in the case of the *in vitro* glutathione addition to the anisyl orthoquinone. We conclude that it is likely that the observed material is derived from the formation of thiol adducts (probably mainly with cellular GSH). Since we do not observe these adducts in the non-toxic examples, we postulate that the thiol addition reactions take place within the cells and that the adducts are able to leak into the incubation medium. Lack of toxicity is, therefore, ascribed to an inability of the tyrosinase-generated quinones to enter the cells.

Clearly, if the mechanism of toxicity involves thiol depletion it will be abrogated by the external addition of thiol (e.g. GSH) in a concentration sufficient to react with all the quinone generated in the system, and we have shown that this is so in the case of the lead compound 4-hydroxyanisole.

Our study has not adduced any evidence in favour of a toxic action which involves the generation of redox-cycling derivatives of the *ortho*-quinones produced by tyrosinase oxidation of the set of compounds tested.

In this study, we have concentrated mainly on compounds that are readily oxidised by tyrosinase. The comparative rates of oxidation are consistent with the proposal that the ease of oxidation by tyrosinase is a function of the electron-donor nature of the side-chain [17]. Oxy-ethers appear to be generally comparable with thio- or selenyl-ethers as substrates for tyrosinase with the notable exception of hydroxyethylthiophenol. The data also indicate that the oxidation rate increases as a function of the chain length for alcohols and for carboxylic acids. A terminal cationic group such as trimethylammonium or a bulky N-succinimide ester group does not inhibit oxidation. All the agents tested except compound 85 were oxidised rapidly enough to be completely oxidised in the exposure period under the conditions of the cytotoxicity test. Despite this, comparison of the comparative oxidation rates and the cytoxocity shows that some of the compounds that give rise to orthoquinones in the presence of tyrosinase are remarkably non-toxic in the test system. The *in vitro* reactivity of these orthoquinones with thiol groups does not account for this discrepancy since the compounds are not significantly less reactive and, indeed, some are more reactive (e.g. compound 87, E.J. Land, Paterson Labs, Manchester) than the quinones found to be cytotoxic. We have shown in other experiments [18] that there is a relationship between the electron withdrawing nature of the side-groups and the rate of reaction with nucleophiles such as cysteine and glutathione. Nevertheless, the propensity of a substrate to be oxidised and the corresponding ability of the derived quinone to react with nucleophiles are not mutually exclusive. Inspection of the structural distinction of those compounds that are less cytotoxic than either their oxidation rate or the reactivity of their quinones would indicate, suggests that the possession of a side-group with a net charge or with a bulky component may hinder access of the quinone to the potential target. If this is correct, it may be because the sensitive target is located in a hydrophobic site, for example, the thiol groups present in ion pumps in the plasma membrane or alternatively that the quinone has to reach the cell interior in order to react with the cellular thiols, for example in the form of GSH. The evidence suggesting that material resembling a GSH addition compound is found in the medium in the case of cells exposed to cytotoxic quinone precursors implies a mechanism of toxicity in which lipophilic quinones gain entry into the cell where they react with soluble thiols resulting in the export of addition products of glutathione. Whether this is the primary toxic mechanism in the model system or whether the leakage of thiol-containing material into the medium is secondary to some primary disruption of the plasma membrane remains to be clarified.

In conclusion, although the model system employed differs in a number of important respects from the *in vivo* arrangement present in melanotic tumour tissue, notably in the location of the tyrosinase, the present study indicates that there are important constraints on the composition of the side-group of potential tyrosinase-activated prodrugs. In particular, we conclude that, in general, the possession of a net charge or a bulky group causing steric restriction of access to, or passage through, the cell membrane prevents cytotoxic action by reactive *ortho*-quinones. The data suggest that from the point of view of melanogenesis-targeted antimelanoma drug design a lipophilic side-chain is an important structural feature of potential melanocytotoxic phenols.

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