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POTENCY, SELECTIVITY AND CELL CYCLE DEPENDENCE OF CATECHOLS IN HUMAN TUMOUR CELLS *IN VITRO*

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Abstract—Enhancement of the potency and melanoma-selectivity of redox agents was sought by two different approaches. In screening a series of catechols, derivatives of moderate half-life (dopa, dopamine, noradrenaline, 3,4-dihydroxybenzylamine, 3,4-dihydroxyphenylacetic acid; $t_{1/2}$ 12–33 hr) had significant toxicity (D_{37} 20–30 μM) and selectivity for melanoma cells compared with HeLa. Less stable catechols (5-hydroxy- and 6-hydroxydopamine; $t_{1/2}$ 4 and 5 hr respectively) were toxic but lacked selectivity whereas more stable derivatives (4-hydroxyanisole, 2,3-dihydroxybenzoic acid; $t_{1/2} > 72$ hr) were less potent ($D_{37} > 100$ μM) and had poor selectivity. Gossypol, a complex catechol derivative, exhibited significant toxicity (D_{37} 7.7 μM) but little selectivity. Enzymes capable of reacting with components of the culture medium and known to continuously generate hydrogen peroxide (glucose-6-oxidase) or superoxide ion (xanthine oxidase) exhibited a similar degree of selectivity as dopa, indicating that active oxygen species are more important mediators of catechol toxicity than quinones. Rhodamine 123, a cationic dye preferentially taken up by some tumour cells, was accumulated equally by melanoma and HeLa yet had a similar selectivity to that of dopa. In the second approach, the potency of dopa was found to be greatly enhanced during early S phase. This phenomenon, found with cells synchronised both by mitotic shake off and by 24 hr accumulation in G_1S in the presence of 5 mM hydroxyurea, occurred during a period in which the proportion of cells in S phase cells was low. These results indicate that human cells are extremely sensitive to extracellular active oxygen species during a relatively short period in early *S* phase, and selective killing of asynchronous melanoma cells therefore requires agents capable of sustaining a redox effect for at least one cell cycle.

A variety of redox-active agents including catechols exhibit antitumour activity *in vitro* and *in vivo* [1, 2], some human melanoma cell lines being particularly sensitive [3, 4]. Studies with D-amino acids indicated that the initial redox events occurred outside of the cell [5]. Intracellular effects of these agents include inhibition of ribonucleotide reductase [6] and DNA polymerase [7], inhibition of DNA synthesis with consequent accumulation of cells in the G_1 phase of the cell cycle [2], and formation of DNA strand breaks [4]. The events primarily responsible for cell death and melanoma selectivity remain undefined. One possibility is that catechols are oxidized to quinones, which react covalently with intracellular SH enzymes such as DNA polymerase [7–9]. Another mechanism involves the reaction of active oxygen species produced by extracellular autooxidation of catechols [5, 10]. This question is important to resolve because of implications for development of more effective drugs for *in vivo* treatment.

The effectiveness of such agents *in vivo* is limited by reduced potency, possibly due to low oxygen tension and high cell density, with consequent increased capacity for scavenging active oxygen radicals [5]. We have therefore screened a range of catechol derivatives in an attempt to establish relationships between structure, potency and selectivity for human melanoma cells in culture. Other agents were included for the purpose of gaining information on the mechanism of action of killing: rhodamine 123, an inhibitor of oxidative phosphorylation [11] and found to be selectively retained by carcinoma cells [12]; glucose-6-oxidase, a convenient source of extracellular H_2O_2 through reaction with glucose in the culture medium [13]; and xanthine oxidase, which generates superoxide ion *in situ* [14]. The question of whether redox toxicity is phase specific was also investigated and found to be related to the above results.

MATERIALS AND METHODS

MM474F fibroblasts were isolated from a culture of a primary melanoma from the scalp of a 35-year old male. The origins of Hela-S_3 and other human melanoma cell lines have been described [4]. Cell cultures were maintained at 37° in 5% CO_2/5% O_2/90% N_2. Tissue culture medium RPMI 1640 (Commonwealth Serum Laboratories, Melbourne, Australia) containing 10% (v/v) foetal calf serum, streptomycin (100 μg/ml) and penicillin (100 i.u./ml), 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (3 mM). Routine assays of cell lines for *Mycoplasma* using agar culture were negative. Cell survival was determined as previously described [4] by treating duplicate cultures (3×10^3 per 16 mm well) seeded 24 hr previously. After continuous

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Table 1. Toxicity of catechol derivatives in human cell lines

Catechol	$t_{1/2}$ (hr)	D_{37} (μM) MM96L	HeLa	MM96L/HeLa
5-Hydroxydopamine	4	20·†	36	0.55
6-Hydroxydopamine	5	14 ± 3*	25.7	0.57
3,4-Dihydroxyphenylacetic acid	12	34 ± 9	148 ± 3	0.23
Noradrenaline	15	28 ± 9	170 ± 38	0.16
Dopa	18	22 ± 2	80 ± 12	0.28
3,4-Dihydroxybenzylamine	29	27 ± 5	213 ± 21	0.13
Dopamine	33	29 ± 8	126 ± 19	0.23
2,3-Dihydroxybenzoic acid	>72	4200	2900	1.44
Hydroxyanisole	>72	105 ± 17	208	0.5
Gossypol	N.T.	7.7 ± 0.1	12.8 ± 2.5	0.6

* Mean and SD of 2–4 experiments.
† One experiment.

treatment for 6 days, cells were labelled with 2 μCi/ml [^{3}H-methyl]-thymidine (51 Ci/mmole; Radiochemical Centre, Amersham, U.K.) for 2–4 hr, detached with 0.02% trypsin and washed with water onto glass fibre discs for liquid scintillation counting. Dose–response curves were plotted for survival (calculated as % control cpm), determined at five doses for each cell line. Survivals were compared on the basis of the D_{37}, the dose at which survival was 37%. Stock solutions of drugs were freshly prepared in culture medium for each experiment. The action of xanthine oxidase [14] and glucose oxidase [13] was dependent upon substrates endogenous in the culture medium. The half life of these enzymes under culture conditions was >6 hr.

For cell synchrony with HU†, cells were seeded (3×10^3/16 mm well) and immediately treated with 5 mM HU for 24 hr. At various times the cultures were washed four times with medium at 37° and survival determined as described above. Control wells (no HU) were treated simultaneously.

The fact that cells round up and adhere less firmly to a surface during mitosis [15] can be exploited to selectively harvest mitotic cells from asynchronous cultures [16]. In this method, 150 cm^2 flasks containing approximately 2×10^7 cells seeded 24 hr previously were rocked 25 times in fresh medium. Dislodged cells were allowed to attach for two hr in plastic plates (3×10^3/16 mm well) before commencing the experiment, by which time 90% were in G_1 phase as shown by DNA flow cytometry. They were then treated with dopa as described above.

For DNA flow cytometry, parallel cultures (10^5 cells/60 mm plate) were harvested (0.02% trypsin in PBS) and fixed in 25% ice-cold ethanol for at least 1 hr. They were then stained with a mixture of propidium iodide (50 μg/ml), RNase A (1 mg/ml) and Triton X-100 (0.2%) in PBS and analysed with a Becton Dickinson FACS IV (argon ion laser) operated at 488 nm. The proportion of cells in each phase of the cell cycle was calculated from the DNA histogram.

† Abbreviations: PBS, phosphate-buffered saline, pH 7.2; dopa, L-3,4-dihydroxyphenylalanine; HU, hydroxyurea.

RESULTS

Catechol toxicity and selectivity

Nine catechols were tested against a human melanoma (MM96L) and a nonmelanoma (HeLa) cell line, each line having similar size (1500–1700 μm^3) and doubling time (24 hr). As reported previously [4], MM96L was very sensitive to dopa compared with HeLa (Table 1). Noradrenaline, 3,4-dihydroxybenzylamine, dopamine and 3,4-dihydroxyphenylacetic acid were shown to be more selective for MM96L and achieved the same level of toxicity as dopa. 5-Hydroxydopamine, 6-hydroxydopamine and gossypol were slightly more toxic but less selective. Hydroxyanisole and 2,3-dihydroxybenzoic acid were found to be less toxic and had poor selectivity.

The $t_{1/2}$ of these compounds in culture medium at 37° was determined from semilogarithmic plots of the increase in absorbance at 320 nm due to autoxidation. Catechols with $t_{1/2}$ of 10–35 hr (dopa, dopamine, noradrenaline and 3,4-dihydroxybenzylamine) had significant toxicity and selectivity (Table 1). Less stable catechols (5- and 6-hydroxydopamine) had significant toxicity but lacked selectivity whereas the most stable compounds (4-hydroxyanisole and 2,3-dihydroxybenzoic acid) were less potent and had poor selectivity.

The above results prompted the use of structurally unrelated agents which either enter the cell (rhodamine 123) or generate active oxygen species continuously in the culture medium (xanthine oxidase and glucose oxidase). Rhodamine 123 was shown to be highly toxic and exhibited specificity for melanoma cells in a similar manner to noradrenaline (Fig. 1). 2-Deoxyglucose, which enhances the toxicity of rhodamine 123 by inhibiting glycolysis [17, 18], exhibited a small degree of synergism in the present study (Table 2). Two hour treatments with rhodamine 123 with or without 2-deoxyglucose did not prove toxic to either MM96L or HeLa at the same dose range as used for the continuous treatments. Analysed by flow cytometry and fluorescence activation at 488 nm, the uptake of 10 μM rhodamine 123 after 1 hr treatment was similar in MM96L and HeLa cells (peak channels for fluorescence were 141 and 129 respectively).

Table 2. Toxicity of rhodamine 123 and oxidases to human tumour cells

Cell Line	D_{37} (μM)		D_{37} (units/ml × 10^{-3})	
	Rhodamine 123	Rhodamine 123 + 2-deoxyglucose*	Xanthine oxidase	Glucose oxidase
MM96L	1.3 ± 0.4(4)†	0.7 ± 0.2(4)	4.4 ± 2.4(3)	1.4 ± 0.2(2)
HeLa	8.9 ± 1.9(2)	6.3 ± 0.6(2)	25.5 ± 0.4(2)	4.4 ± 1.2(2)
MM96L/HeLa	0.15	0.11	0.17	0.32

* 300 μM 2-deoxyglucose, nontoxic when used alone.
† Mean ± SD. Number of experiments in parenthesis.

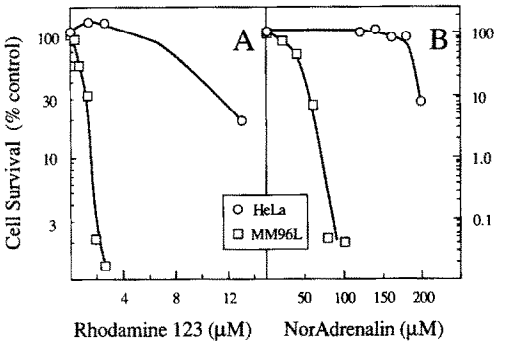

Fig. 1. Dose responses of cell survival comparing a melanoma (MM96L) and nonmelanoma cell line (HeLa): (A) rhodamine 123; (B) noradrenaline.

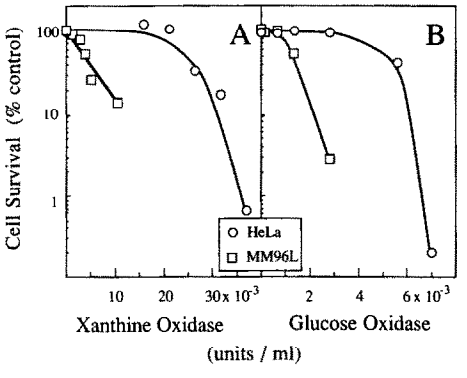

Fig. 2. Dose responses of cell survival using xanthine oxidase (A), and glucose oxidase (B).

Table 3. Comparison of the toxicity of glucose oxidase and dopa in human cells

Cell Line	D_{37}	
	Dopa (μM)	Glucose oxidase (units/ml × 10^3)
Non-melanoma		
HeLa	80.0 ± 12*	4.4 ± 1.2
MM474f	47.3	7.4
Melanoma		
MM96L	22.7 ± 2.3	1.4 ± 0.22
MM418	80.69	8.2
MM138	6.4	0.66 ± 0.27
MM253c1	76.4 ± 16.7	6.09 ± 0.88
MM229	5.22	0.41

* Mean and SD of 2–4 experiments.

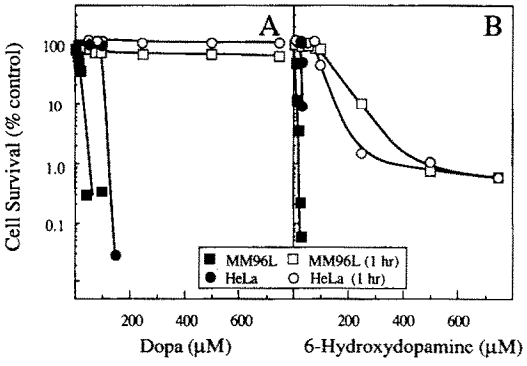

Fig. 3. Comparison of the toxicities of continuous (■, ●) and 1 hr treatments (□, ○) of cells with dopa (A) and 6-hydroxydopamine (B).

Xanthine oxidase and glucose oxidase both showed selectivity for killing the melanoma cell line MM96L (Table 2), a large shoulder being apparent on the dose response curve of the resistant cell line (Fig. 2). Compared in seven tumour lines and a strain of fibroblasts (Table 3), sensitivity to glucose oxidase correlated with sensitivity to dopa ($r = 0.81$, $P < 0.05$).

The toxicities of brief (1 hr) and prolonged treatment periods were compared using catechols of short and medium $t_{1/2}$. 6-Hydroxydopamine was found to have significant toxicity even with a 1 hr exposure whereas dopa had no effect (Fig. 3).

Cell cycle dependence of catechol toxicity

The sensitivity of different phases of the cell cycle to dopa treatment was investigated with cells synchronised either by blocking in G_1 with the ribonucleotide reductase inhibitor HU [19] or by collecting mitotic cells shaken off from proliferating cultures [15]. MM96L cells synchronised by HU were extremely sensitive two hr after release from the block, then rapidly increased in resistance to killing by dopa (Fig. 4A). Asynchronous, dopa treated cells and synchronised, untreated cells were not affected. DNA flow cytometric analysis of parallel cultures confirmed that HU had synchronised the cells, with subsequent progression from G_1 through S and into G_2 (Fig. 4B). HU pretreatments of 2–6 hr did not sensitise cells to dopa, indicating that the above phenomenon was not due to direct synergism between HU and dopa.

The same pattern of dopa sensitivity in early S phase was shown by HU-synchronised HeLa cells (Figs 4C and 4D), although as expected from the D_{37} values, this line was not as sensitive as MM96L. The same controls were carried out as with MM96L to show that brief pretreatment with HU did not sensitise cells to dopa. Evidence of the phase-specific nature of the phenomenon was also obtained with post-mitotic cells dislodged from untreated cultures. Again, cells were highly sensitive to killing by dopa during a brief period which corresponded to early S phase (Fig. 5), the level of toxicity being much greater than that found with HU-synchronised cells.

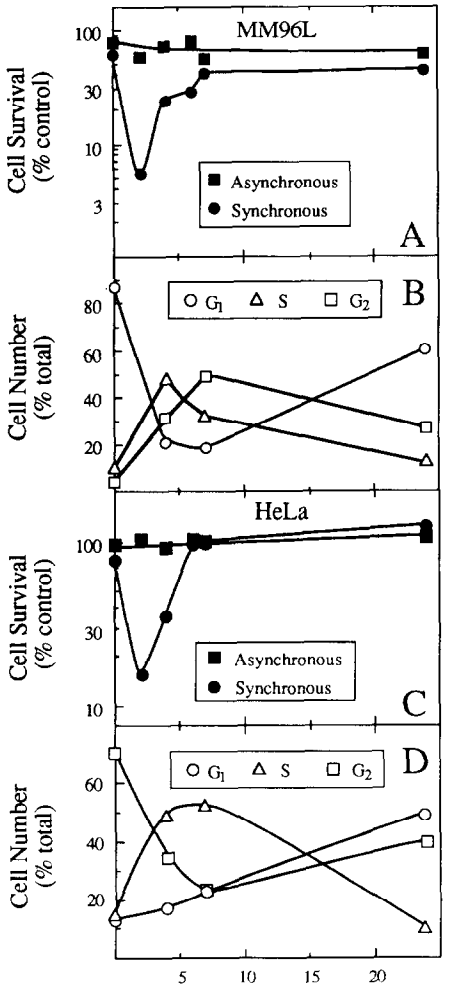

Fig. 4. Dependence of dopa toxicity on cell cycle stage in MM96L (A, B) and HeLa (C, D). Cells were synchronised by 24 hr incubation with 5 mM HU, washed and treated at various times with 5 mM dopa for 1 hr, and cell survival determined after 6 days. (A and C) Sensitivity of cells determined at various times after release from HU block. (B and D) Percentage of cells in each phase of the cycle, after release from the HU block.

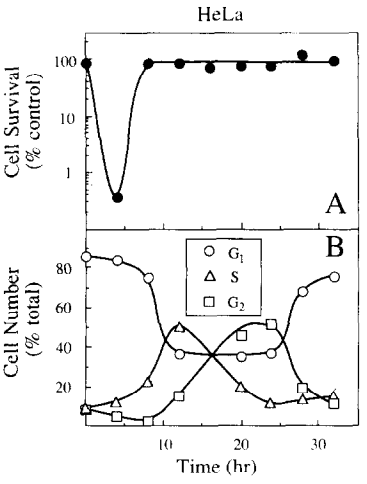

Fig. 5. Cell cycle dependence of dopa toxicity in HeLa cells synchronised by mitotic shake-off. (A) Sensitivity of cells to 5 mM dopa at various times after synchrony. (B) Percentage of cells in each phase of the cycle.

DISCUSSION

This study showed that redox-active catechols are toxic specifically to early S phase cells, a finding which helps to explain the structure–activity relationships of the present series of catechols and which may suggest more rational approaches to devising *in vivo* treatments. The most effective group of catechols had half-lives for autoxidation of 12–33 hr, sufficient to generate oxygen species during several cell cycles. This group included 3,4-dihydroxybenzylamine, a compound predicted to have a longer $t_{1/2}$ than dopa because of inability to cyclise [2] and found in this study to be more selective than dopa. The most stable, and least active, compounds presumably failed to generate sufficient radicals. Hydroxyanisole, a member of the latter group, has been used at high doses to treat melanoma [20] but appears to act differently from catechols because it exhibited less selectivity for the MM96L melanoma line. The hydroxydopamines were the least stable of the series and also had low selectivity for MM96L, due to more effective killing of the nonmelanoma line, HeLa. These compounds also differed from the more stable catechols in being toxic after an exposure period of only 1 hr, suggesting that the radical flux is sufficient to saturate the protection system in HeLa cells. The presence of a saturable resistance mechanism in HeLa cells was demonstrated by a large shoulder on the noradrenaline and enzyme dose responses for cell survival.

The dopa-like selectivity of the radical-generating enzymes, glucose oxidase and xanthine oxidase, lends strong support to the proposal that initial events in catechol toxicity occur outside of the cell [5] and involve active oxygen species, rather than covalent binding of catechol via quinone [7–9]. Hydrogen peroxide added directly to cultures was not selectively toxic to MM96L [5], and was less effective in killing rodent tumour cells *in vitro* than

when generated *in situ* by glucose oxidase [13]. The selectivity of glucose oxidase in the present study may, like that of the medium half-life catechols, be due to release of peroxide over one or more cell cycle periods. Further, the similar effects of glucose oxidase and xanthine oxidase suggest that the toxicity of superoxide results from disproportionation to hydrogen peroxide.

Rhodamine 123 was investigated because its uptake and retention by different cell types can be determined readily by fluorescence [12]. It affects oxidative phosphorylation with consequent inhibition of ATP synthesis and protein synthesis [21] but, unlike dopa [2], did not inhibit DNA synthesis [21]. Potentiation of rhodamine 123 by 2-deoxyglucose, presumably due to further depletion of ATP by inhibition of glycolysis [17, 18], was not observed in this study. When used at cytotoxic levels, rhodamine 123 was found in the cytoplasm as well as in the mitochondria of sensitive carcinoma cells [22]. Thus selectivity for melanoma cells may involve an effect on the cell membrane via depletion of ATP, or some extramitochondrial effect as yet undefined. It will be of interest to determine the localisation of rhodamine 123 in human melanoma cells.

The finding that catechol-sensitive and -resistant human cells are very susceptible to killing by dopa in early S phase is of considerable interest because it provides (a) a basis for selecting agents of suitable redox activity, as discussed above, (b) a convenient experimental tool for obtaining populations of sensitised cells, and (c) working hypotheses for identifying the primary cellular target of redox agents. Incorporation of 5-bromodeoxyuridine into the DNA of synchronised HeLa cells in early S produced a marked decrease in cloning efficiency [21], suggesting that cell survival is highly dependent on nucleotide pool sizes and/or the integrity of newly synthesized DNA in early S phase [23]. The later stages of catechol toxicity may involve such effects, due to inhibition of DNA synthesis [2] and induction of DNA strand breaks [4]. Alternatively, S phase sensitivity could result from an early, membrane-related effect of catechol autoxidation. The cell surface remains constant in area from mitosis until G_1/S and then increases by 30% in mid-S phase and G_2 [24]. Such changes may make the membrane more susceptible to damage, such as oxidation of lipids by active oxygen species. It is of interest that human fibroblasts were found to be sensitive to killing by oxidised lipid when in S phase [25]. Selectivity of redox agents for melanoma cells may thus involve a higher susceptibility to lipid damage.

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