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MECHANISM OF ANTITUMORAL ACTIVITY OF CATECHOLS IN CULTURE

MAURO PICARDO, SIRO PASSI, MARCELLA NAZZARO-PORRO, AODAN BREATHNACH,* CLAUDIA ZOMPETTA,† ALBERTO FAGGIONI† and PATRICK RILEY‡

Istituto dermatologico San Gallicano, Rome, Italy, * St. Mary's Hospital Medical School, London, U.K., † Istituto Patologia Generale, Universita' di Roma, Rome, Italy, and ‡ Department of Biochemical Pathology, University College, London, U.K.

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Abstract—Cell lines Raji and K 562, lacking tyrosinase, and two melanotic human melanoma cell lines (IRE 1 and IRE 2), were exposed to concentrations from 5×10^{-3} M to 10^{-5} M of different phenols which are substrates of tyrosinase, i.e. l-dopa, dopamine, hydroquinone, terbutylcatechol, and of phenols which are not substrates of the tyrosinase, i.e. resorcinol, butylated hydroxyanisole and hydroquinone dimethyl ether. Cultures were carried out in the presence or in the absence of oxygen radical scavenger enzymes superoxide dismutase, catalase and peroxidase. The stability of each substance in culture medium was assayed by high performance liquid chromatography (HPLC).

Results showed that: (1) catechols which are substrates of tyrosinase decompose fully after 24 hr in medium; (2) they are equally toxic for melanoma and non-melanoma cell lines; (3) their toxicity increases when they are preincubated in medium for 24 hr and 48 hr before addition of cells; (4) their toxicity is significantly reduced by addition of scavenger enzymes; (5) on the contrary, phenols not substrates of tyrosinase are stable in medium and their toxicity is not reduced by scavenger enzymes.

It is concluded that tyrosinase does not play a major role in catechol toxicity *in vitro*, which is probably due to some products of catechol decomposition, especially oxygen radicals, acting outside the cells.

L-Dopa and several of its analogues, i.e. L-dopa methyl ester, L-glutamic acid dihydroxyanilides, 3,4-dihydroxybenzyalamine, *N*-acetyl dopamine, have been shown to be selectively toxic to mouse and human melanoma cells *in vitro*, and to be active against human and mouse melanoma *in vivo* [1–12]. It has been postulated by Wick [5] that the mechanism of action involved the oxidation of the above catechols, possibly through intracellular conversion to the corresponding orthoquinones, a reaction mediated by the presence of tyrosinase within melanoma cells. The orthoquinones, in turn, would interact with DNA polymerase, which is known to be a sulphydryl-dependent enzyme. The consequent inactivation of DNA polymerase could lead to inhibition of cellular growth and ultimately cell death.

In line with this position, Wick organised clinical trials on the chemiotherapeutic effect of L-dopa and dopamine on human melanoma, and, whereas finding some beneficial effects, reported toxic side-effects involving the neural and cardiovascular systems [9].

Although the above theory is very attractive, the mechanism of catechol toxicity and its apparent specific melanotoxicity *in vitro*, remain to be clarified. There are certain properties of catechols that are difficult to reconcile with the proposal that they are specifically metabolised by melanogenic cells. For example, catechols, because of their polarity, may not enter as such into the cells in sufficient concentration to be toxic. Moreover, catechols are very unstable drugs in slightly alkaline solutions (pH 7.2–7.4) such as serum or culture medium, and therefore, misleading conclusions might be drawn from experiments designed to investigate their effects *in vitro*.

Recently Parson [13] demonstrated that the initial events of L-dopa toxicity for melanoma cells *in vitro* occurred outside the cells, and was due to the formation of a stable toxic product, probably melanin, which did not strongly inhibit DNA polymerase. This clearly suggests that, whatever the mechanism of toxicity, it is unlikely to have been due to uptake of L-dopa itself in sufficient quantity. Contemporaneously we have reached similar conclusions with cultures of erythroleukemia and lymphoma-derived cell lines exposed to different diphenols including L-dopa [14].

These considerations have led us to test the stability in culture medium of different catechols, L-dopa, dopamine, hydroquinone and terbutylcatechol, which are substrates of tyrosinase [15], and their toxicity on different cell lines, containing, or not, tyrosinase. We have also done similar tests with a selected monophenol (butylated hydroxyanisole), a diphenol (resorcinol) and a phenol derivative (hydroquinone dimethyl ether), which are not substrates of tyrosinase [15]. In addition we have explored the possibility that scavenger enzymes, such as superoxide dismutase, peroxidase and catalase added to cultures, might affect the stability and toxicity of the above drugs.

MATERIALS AND METHODS

Cultures. Four established cell lines were used in the present study: the lymphoma-derived cell line Raji (Dr Aragona, University of Rome), the erythroleukemia cell line K 562 (R. Heberman, NCI, Bethesda, MD) and two melanotic cell lines IRE 1 and IRE 2 (Dr P. G. Natali R. Elena Institute, Rome). All cell lines were cultured in RPMI 1640

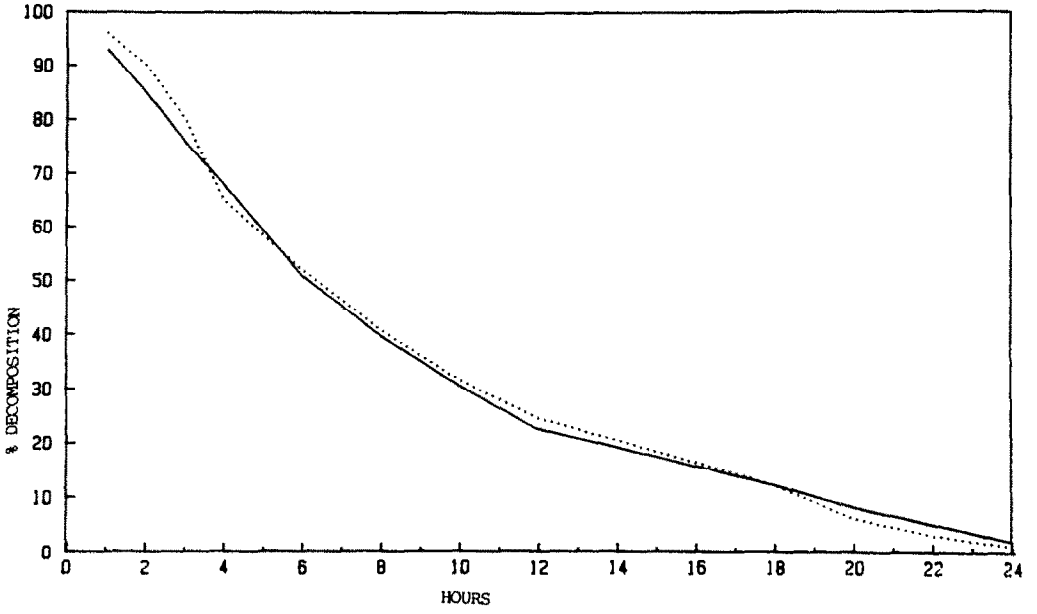

Fig. 1. Rate of decomposition of 0.1 mM L-dopa in complete culture medium at 37° alone and in the presence of IRE 1 cell line: ———, L-dopa alone; ....., L-dopa in the presence of IRE 1 cell line. The presence in culture medium of IRE 1, as well as other cell lines, does not affect in any way the rate of decomposition of L-dopa. After 6 hr of incubation, only 51% catechol remained unchanged and quite fully disappeared after 24 hr. Dopamine decomposition parallels that of L-dopa.

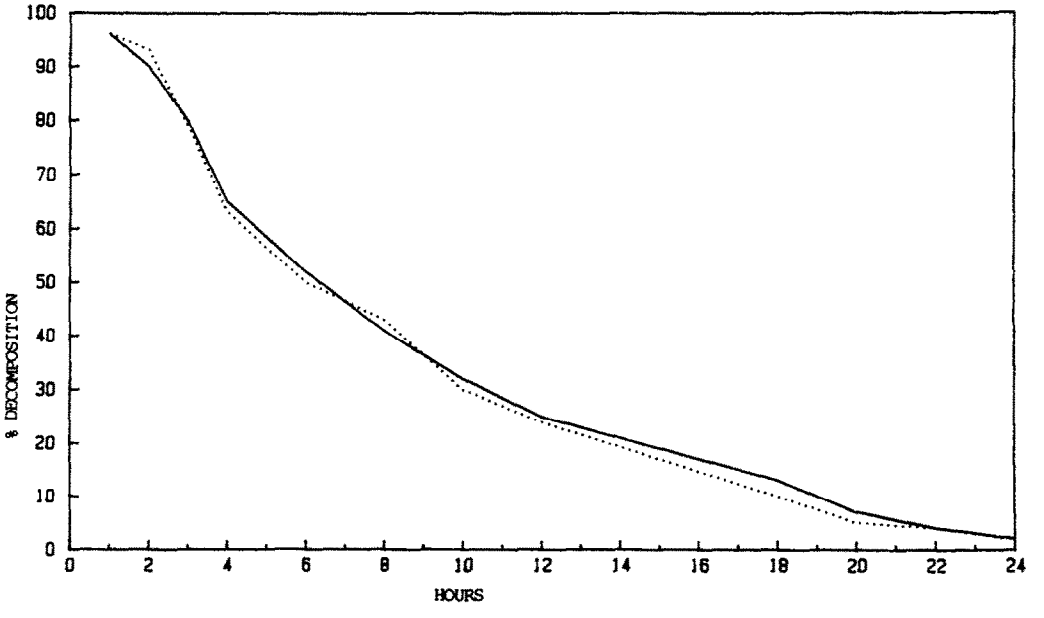

Fig. 2. Rate of decomposition of 0.1 mM hydroquinone in complete culture medium at 37° alone and in the presence of IRE 1 cell line: ———, hydroquinone alone; ....., hydroquinone in presence of IRE 1 cell line. The presence in culture medium of IRE 1 cell line, as well as of other cell lines, does not affect in any way the rate of decomposition of hydroquinone. Terbutylcatechol decomposition parallels that of hydroquinone.

medium (Gibco) with 10% fetal calf serum (Gibco) and penicillin (100 U/ml) and streptomicin (100 μg/ml). Every three days the medium was removed and the cells subcultured. For the experiments 2–3 × 10^4 cells/well were seeded on 96 well/plates (Flow) (the suspended cell lines Raji and K 562) or in 24 well plates (Flow) (IRE 1 and IRE 2). Cells were cultured for 2, 4, 6, 8, 12, 20, 24 and 48 hr at 37° in 5% CO_2/air in the absence or presence of different phenols. Cultures were pulsed from 1 to 4 hr with ^{3}H thymidine (3HT) (Amersham, spec. act. 2 Ci/mole at 5 μCi/ml). The suspended cell lines were harvested by using cell harvester (Titertek) and the radioactivity counted by a β counter [16]. In the case of cultured melanoma cell lines, cells were washed and precipitated with 10% trichloroacetic acid for 4 hr at 4°. The precipitate was washed with saline, followed by addition of 1 N NaOH let stand at 4° for 24 hr. Scintillation fluid (Unisolve) was added and the sample counted.

Phenols. Hydroquinone (HQ), L-dopa (DP), dopamine (DPA), terbutylcatechol (TBC), resorcinol (RES), butylated hydroxyanisole (BHA) and hydroquinone dimethyl ether (HQDME) (all purchased from Fluka) were used at concentrations ranging from 5 × 10^{-3} M to 10^{-5} M. The toxicity was also tested in the presence of 100 U/ml superoxide dismutase (SOD) (Sigma), and/or 1U/ml peroxidase (PER) (Sigma) and/or 1000 U/ml catalase (CAT) (Sigma).

Stability of phenols. The stability of phenols in complete medium at 37° for 48 hr in the absence or presence of the different cell lines and scavenger enzymes was evaluated by high performance liquid chromatography (HPLC) on RP 18, 5 μm column [15].

Effect of preincubation of phenols on cell survival. Experiments were performed in order to investigate the effect of the preincubation of phenols on cell survival. The different cell lines were incubated 24 and 48 hr after the previous addition of phenols to culture medium, and the toxicity evaluated as described above.

RESULTS

Stability of phenols

Figures 1 and 2 show the rate of decomposition of 0.1 mM L-dopa (Fig. 1) and 0.1 mM hydroquinone (Fig 2.) in culture medium at 37° alone and in the presence of the IRE 1 cell line. The presence of IRE 1, as well as of other cell lines, did not affect the rate of decomposition of the two catechols. Dopamine decomposition exactly paralleled that of L-dopa, while terbutylcatechol behaved in the same way as hydroquinone.

Peroxidase and, to a lesser extent catalase and superoxide dismutase, both alone and in the presence of cells, accelerated the decomposition of L-dopa to a similar degree (Fig. 3); the calculated half-life of L-dopa (5–8 hr) was reduced to approximately 4.2 hr ($P < 0.05$). The other catechols behaved in the same way. The gradual disappearance with time of the catechols never gave rise to quinone formation detectable by HPLC. Butylated hydroxyanisole, resorcinol and hydroquinone dimethyl ether were more stable: only 10–15% became decomposed after 48 hr of incubation both in the absence or in the presence of cell lines.

Toxic effect of phenols

For each phenol tested toxicity increased with the

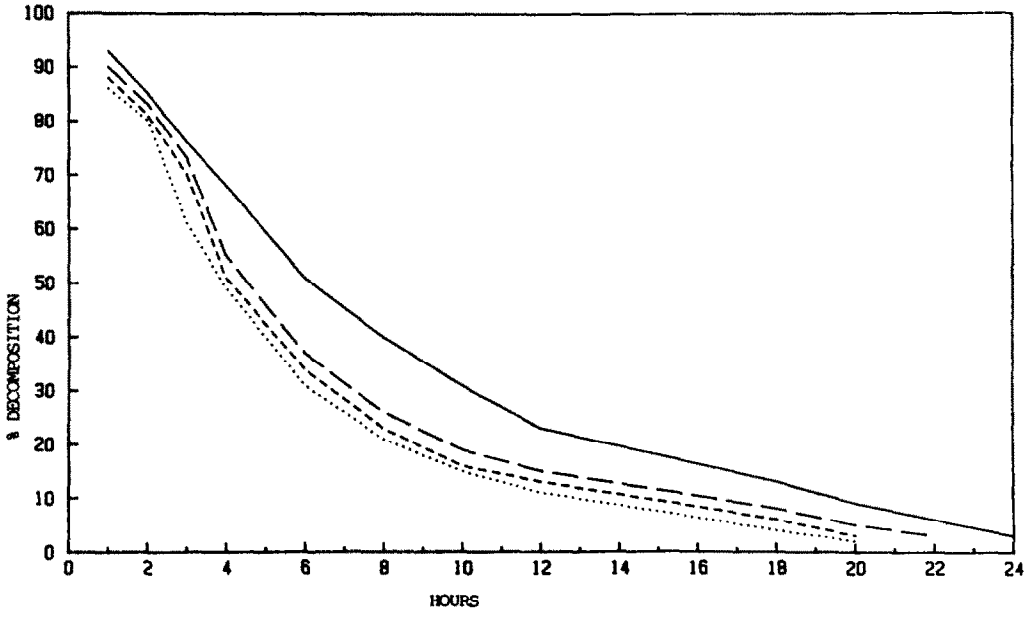

Fig. 3. Rate of decomposition of 0.1 mM L-dopa in complete culture medium at 37° in the absence or in the presence of scavenger enzymes SOD, CAT and PER: ———, L-dopa alone; ———, L-dopa plus SOD; – – – –, L-dopa plus CAT; ....., L-dopa plus PER. The presence in the medium of cells does not affect in any way the effect of scavenger enzymes, which was significant between the 4th and 12th hour. SOD, CAT and PER similarly affect the decomposition of dopamine, hydroquinone and terbutylcatechol.

Table 1. Effect of phenols on percentage survival of different cell lines after 24 hr culture

Phenol	Concentrations (mM)	Cell lines			
		Raji	K 562	IRE 1	IRE 2
L-Dopa	0.1	55 ± 6	58 ± 5	60 ± 8	59 ± 6
	0.5	30 ± 5	33 ± 4	25 ± 4	26 ± 3
Dopamine	0.1	48 ± 4	51 ± 5	46 ± 3	47 ± 5
	0.5	25 ± 3	26 ± 4	22 ± 3	24 ± 5
Hydroquinone	0.01	65 ± 7	69 ± 6	62 ± 7	68 ± 8
	0.1	20 ± 4	20 ± 3	20 ± 2	24 ± 4
Terbutyl catechol	0.1	15 ± 2	19 ± 3	16 ± 2	18 ± 3
Resorcinol	1.0	63 ± 4	68 ± 6	90 ± 6	88 ± 4
Hydroquinone dimethylether	0.1	70 ± 4	76 ± 8	80 ± 8	81 ± 6
Butylated hydroxyanisole	0.5	47 ± 3	59 ± 5	43 ± 6	42 ± 4

Each result represents the mean ± SD of five different experiments with each experiment performed in quadruplicate.

duration of exposure to the agent up to 48 hr. Table 1 summarises the effect of different concentrations of phenols on the percentage survival of the four tumoral cell lines after 24 hr of incubation in culture medium. No significant variation in the level of survival were found among the different cell lines for each drug tested with the exception of resorcinol, a metadiphenol, which was more toxic for Raji and K 562 than for IRE 1 and IRE 2 cell lines ($P < 0.05$). TBC and HQ were the most toxic diphenols. It is interesting to note that BHA and HQDME, not substrates of tyrosinase [15], also showed significant level of toxicity, though not as high as the dihydric phenols. Figures 4 and 5 show survival (%) of Raji and IRE 1 cell lines cultured for a period up to 48 hr in the presence of 0.1 and 1.0 mM L-dopa (Fig. 4) and 0.01 and 0.1 mM HQ (Fig. 5). Again it is evident that toxicity increased with the duration of phenol treatment up to 48 hr.

The effect of phenol preincubation upon cell viability was also determined. Figure 6 displays the decreased survival of Raji and IRE 1 cell lines cultured for 24 hr in the presence of 0.1 mM L-dopa or 0.1 mM HQ preincubated in the medium for 24 and 48 hr when the two diphenols had fully disappeared from the medium (see Fig. 1). It is clear that preincubation of both drugs significantly affected cell survival ($P < 0.01$), and by comparison with Figs 4 and 5, that it affected it to a greater degree than with non-preincubated drugs. The same behaviour was shown by dopamine and terbutylcatechol.

Preincubation, however, did not significantly affect the toxicity of resorcinol, butylated hydroxyanisole or hydroquinone dimethyl ether.

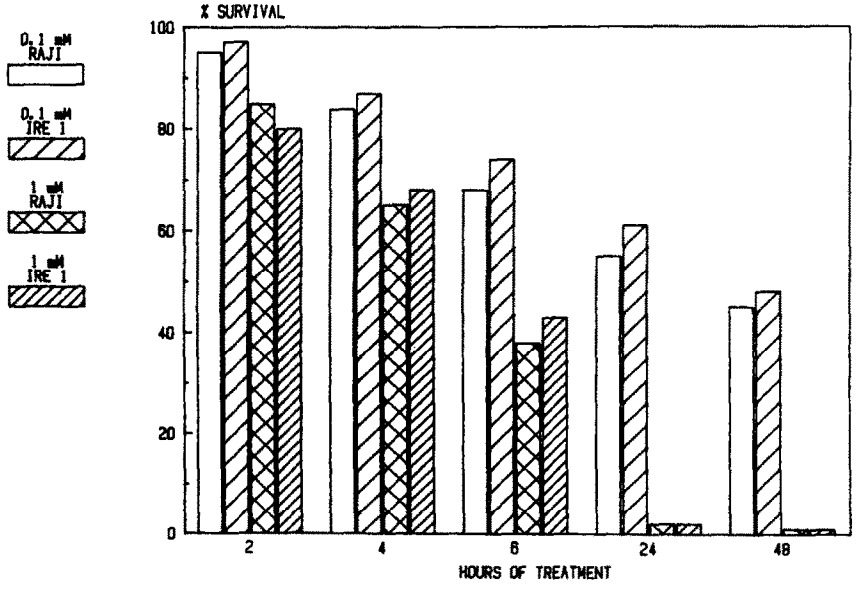

Fig. 4. Percentage of survival of Raji and IRE 1 cell lines cultured for periods up to 48 hr in the presence of 0.1 mM and 1.0 mM L-dopa. Each result represents the mean of five experiments with each experiment performed in quadruplicate. All SD < 10%.

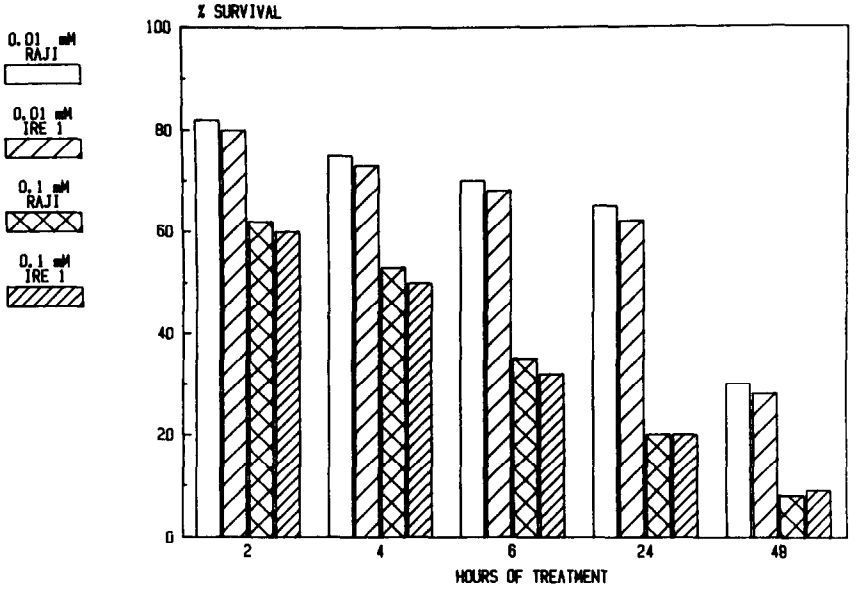

Fig. 5. Percentage of survival of Raji and IRE 1 cell lines cultured for periods up to 48 hr in the presence of 0.01 mM and 0.1 mM hydroquinone. Each result represents the mean of five experiments with each experiment performed in quadruplicate. All SD < 10%.

The addition to the medium of oxygen radical scavenger enzymes, such as SOD, CAT or PER, did not show any significant effect on the toxicity of phenols which are not substrates of tyrosinase and which are stable drugs (Fig. 7). On the contrary, they greatly reduced ($P < 0.01$) the adverse effect of catechols which are substrates of tyrosinase and unstable drugs. The effect was partly additive when these scavenger enzymes were used together in association (Figs 8 and 9). The same behaviour was shown by DPA and TBC.

The protective effect of scavenger enzymes remained unaltered after 24 hr of preincubation, but was considerably reduced after 48 hr (Table 2).

DISCUSSION

This study has clearly demonstrated that the four catechols which are substrates of tyrosinase and which were used in the experiments (DP, DPA, HQ and TBC) undergo complete decomposition within 20 hr of incubation alone in complete culture medium

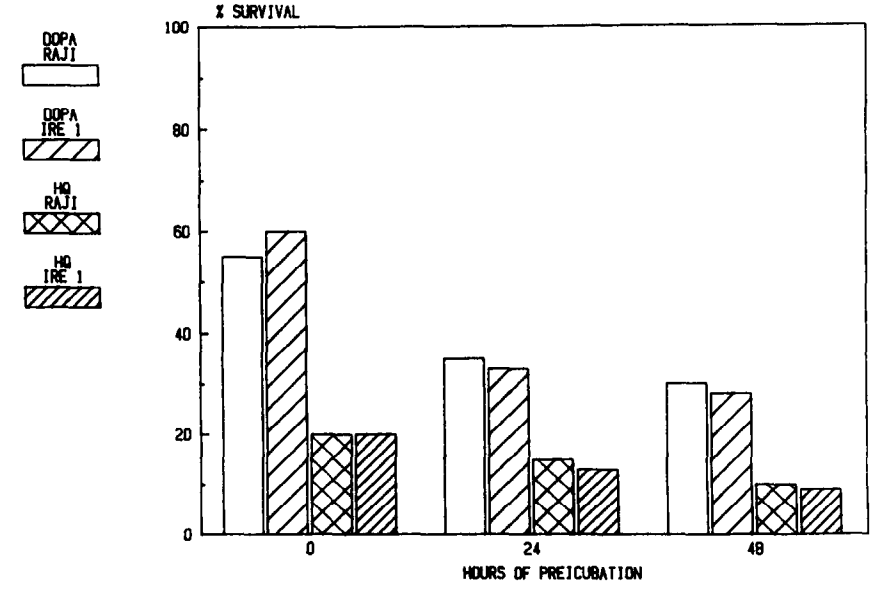

Fig. 6. Effect of 0.1 mM L-dopa and 0.1 mM hydroquinone preincubated in the medium for 24 and 48 hr on Raji and IRE 1 cell lines; survival after 24 hr. Each result represents the mean of five experiments with each experiment performed in quadruplicate. All SD < 10%. DP and TBC show the same effect.

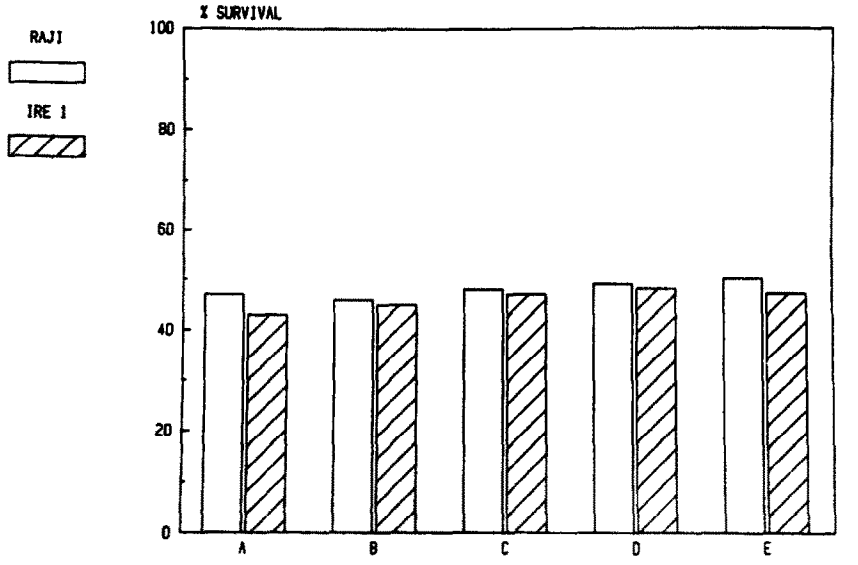

Fig. 7. Effect of the addition of oxygen radical scavenger enzymes on the toxicity of 0.5 mM BHA on Raji and IRE 1 cell lines cultured for 24 hr. Each result represents the mean of five experiments with each experiment performed in quadruplicate. All SD < 10%. RES and HQDME show the same effect. (A) 0.5 mM BHA; (B) A plus PER; (C) A plus SOD; (D) A plus CAT; (E) A plus SOD plus CAT plus PER.

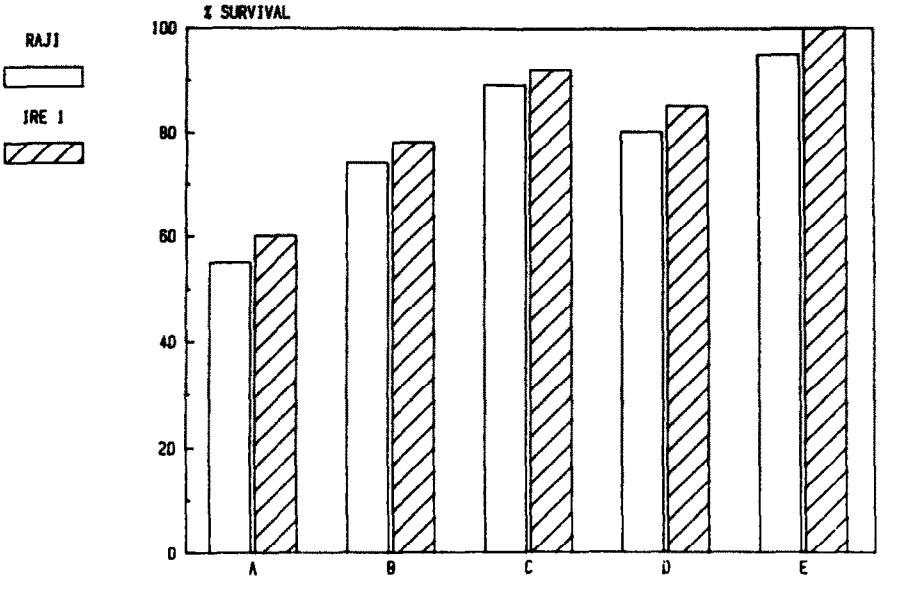

Fig. 8. Effect of the addition of oxygen radical scavenger enzymes on the toxicity of 0.1 mM L-dopa on Raji and IRE 1 cell lines cultured for 24 hr. Each result represents the mean of five experiments with each experiment performed in quadruplicate. All SD < 10%. (A) 0.1 mM L-dopa; (B) A plus PER; (C) A plus SOD; (D) A plus CAT; (E) A plus SOD plus CAT plus PER.

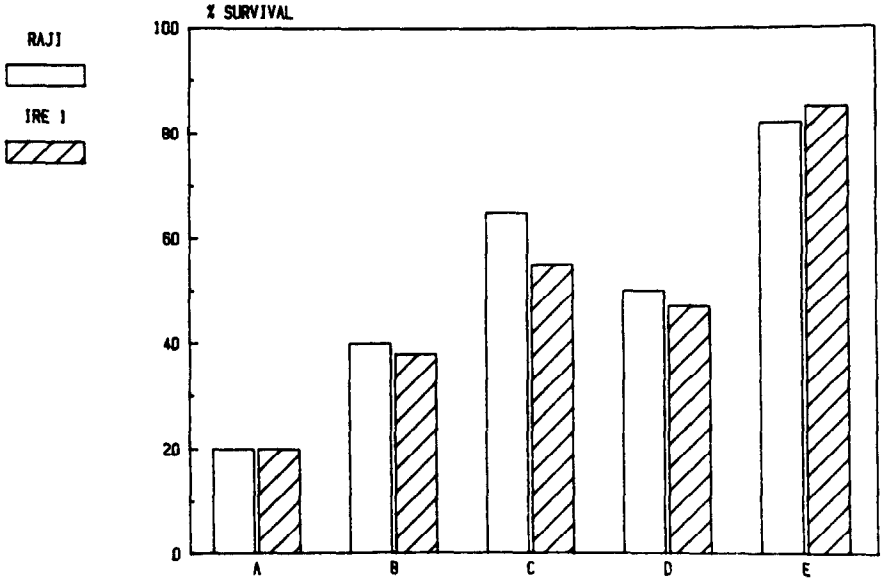

Fig. 9. Effect of the addition of oxygen radical scavenger enzymes on the toxicity of 0.1 mM HQ on Raji and IRE 1 cell lines cultured for 24 hr. Each result represents the mean of five experiments with each experiment performed in quadruplicate. All SD < 10%. (A) 0.1 mM HQ; (B) A plus PER; (C) A plus SOD; (D) A plus CAT; (E) A plus SOD plus CAT plus PER.

at a concentration of 0.1 mM. The rate of decomposition was increased by the addition to the medium of oxygen radical scavenger enzymes, SOD, CAT, PER, but not by the presence of cell lines. On the contrary, the stability of the phenols which are not substrates for tyrosinase, such as RES, BHA and HQDME, was only slightly affected by incubation, and was unresponsive to the presence of scavenger enzymes and/or cell lines.

Catechols which are substrates for tyrosinase (DP, DPA, HQ and TBC) did not show any specific toxicity against melanoma cell lines containing tyrosinase as compared with the other cell lines lacking tyrosinase (Table 1); yet, their adverse effect on all cell lines was significantly modified by the presence in the medium of SOD, CAT and PER, both individually and in association. Phenols which are not substrates for tyrosinase (BHA, RES, HQDME) also exhibited significant levels of toxicity for all cell lines tested, but in this case, the presence of scavenger enzymes had no effect.

The above results suggest that tyrosinase does not play a significant role in the mechanism of toxicity of catechols in culture.

In the case of catechol substrates of tyrosinase, it is clear that the major component of toxicity is not due to the uptake of these compounds as such, since the preincubation experiments showed that after

Table 2. Effect of oxygen radical scavenger enzymes on percentage survival of Raji and IRE 1 cell lines in the presence of 0.1 mM L-dopa or 0.1 mM HQ preincubated in the medium for 24 and 48 hr

Enzyme	Preincubation (hr)	L-Dopa		Hydroquinone	
		Raji	IRE 1	Raji	IRE 1
None	0	55 ± 6	60 ± 5	20 ± 2	20 ± 4
(control)	24	36 ± 4	34 ± 3	17 ± 3	15 ± 4
	48	30 ± 3	28 ± 4	11 ± 3	10 ± 3
Superoxide	0	90 ± 4*	92 ± 3*	65 ± 7*	55 ± 8*
dismutase	24	70 ± 7*	71 ± 4*	60 ± 6*	58 ± 9*
	48	36 ± 6	36 ± 7	20 ± 6	18 ± 5
	0	75 ± 6*	78 ± 8*	40 ± 5*	38 ± 3*
Peroxide	24	55 ± 6*	54 ± 7*	36 ± 4*	35 ± 4*
	48	34 ± 5	33 ± 6	13 ± 4	12 ± 3
	0	80 ± 4*	85 ± 6*	50 ± 6*	48 ± 7*
Catalase	24	58 ± 5*	61 ± 4*	45 ± 5*	45 ± 3*
	48	35 ± 4	36 ± 8	15 ± 5	16 ± 6

Each result represents the mean + SD of five experiments with each experiment performed in quadruplicate. Scavenger enzymes were added together with cells.

* Values were significant at $P < 0.01$ as compared to respective controls.

24 hr the catechols underwent almost complete oxidation, yet cells added at this stage or after 48 hr preincubation were still killed. This suggests that the cytotoxic material is a product of catechol decomposition and support the view of Parson [13] that the initial stage in the mechanism of L-dopa toxicity does not require entry into the cell.

The suggestion that a drug can be toxic by acting outside the cells *in vitro* is not new. Tritton and Yee [17] found, in fact, that the antineoplastic drug adriamycin coupled to an insoluble agarose support, was actively cytotoxic to L-1210 cells in culture, under conditions in which no free adriamycin could enter the cells.

Our data show that the addition of oxygen radical scavenger enzymes to the medium with phenol substrates of tyrosinase, increased the cell survival (Figs 8 and 9), which supports the proposal that the major toxic effect is due to active oxygen species. Graham *et al.* [18] have examined the relative contribution of the generation of the oxygen radicals and the covalent interaction of quinone formed by autoxidation of diphenols. It would appear that in the case of rapidly autoxidizing catechols, the major toxic products are those formed by the reduction of oxygen by the reactions:

(1) $QH_2 + O_2 \rightarrow Q + H_2O_2$;
(2) $QH_2 + Q \rightarrow 2QH^{\cdot}$;
(3) $QH^{\cdot} + O_2 \rightarrow Q + O_2^{\cdot -} + H^+$

These reactions generate hydrogen peroxide (H_2O_2), superoxide anion radicals ($O_2^{\cdot -}$), the semiquinone ($QH^{\cdot}$) and the quinone (Q) from the catechol (QH_2). O_2 and H_2O_2 can also react together in metal-catalysed reactions to form more deleterious oxygen species such as hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1\Delta g\ O_2$) [19–21]. All these products are reactive and able to damage the cells.

It would be possible to postulate that quinones, one of the oxidation products of the catechols, may enter the cells and undergo one electron reduction to semiquinone radicals. These subsequently enter redox cycles with molecular oxygen to produce reactive oxygen species, as described for menadione and other quinonoid antitumor drugs [22–24].

However, the orthoquinones formed by the catechols, are extremely reactive compounds, as compared to antitumor drugs containing quinone nucleus or menadione; just formed, they immediately react with the nucleophiles in the medium [25] giving rise to different species, or bind to the reducing groups of the external cell surface [26].

The studies carried out on the consumption of phenols in complete medium, in fact, never give rise to quinone detectable by HPLC.

The toxic role of oxygen radicals persists for about 24 hr (Table 2), but subsequently other mechanisms must be involved, such as the disappearance from the medium of essential growth factors, which may be destroyed during the autoxidation of catechols and/or the possible concomitant formation of radical polymers.

The latter proposal is a controversial question. According to Pawelek and Lerner [27], the L-dopa sensitive Cloudman S-91 melanoma cells were resistent to synthetic melanin, while Parson [13] demonstrated that both synthetic melanin and melanin formed in the medium by decomposition of L-dopa, were selectively toxic for human melanoma cells.

It might be thought that the lower toxicity of BHA, HQDME and RES, as compared to that of DP, DPA, HQ and TBC, might be due to the fact that drugs of the first group are not substrates of tyrosinase [15]. Certainly they are less easily oxidized in the condition existing in the culture medium, and therefore do not significantly generate oxygen radicals. This is consistent with the results that neither cell survival, nor the rate of decomposition were affected by the presence of scavenger enzymes.

The nature of toxic mechanism of these compounds is not clear, but a primary intracellular action is possible. Because of their relative non-polarity, BHA and HQDME easily enter into the cells and are perhaps more toxic than the diphenol RES, which is more polar.

The problem of the uptake by the cells of the catechols is a very confused one. According to Wick *et al.*, L-dopa toxicity appears to parallel the ability of the cells to incorporate ^3H L-dopa, which in turn is dependent on the presence of tyrosinase activity [3, 28]. Parson, however, found that the uptake of L-dopa was similar in HeLa and MM96 human melanoma cell lines [13]. Because of the instability of L-dopa in the media, neither the method of ^3H uptake, nor the fluorimetric assay give reliable measurements of L-dopa incorporation into the cells. Both these could give rise to misleading measurements and interpretations because they do not discriminate between L-dopa and L-dopa decomposition products, which are able to react with nucleophiles present in the medium and/or bind to nucleophilic groups on the external membrane of the cells.

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