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# THE OXYRADICAL-SCAVENGING ACTIVITY OF AZELAIC ACID IN BIOLOGICAL SYSTEMS

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We have previously shown that azelaic acid, a $C_9$ dicarboxylic acid, as disodium salt ($C_9$2Na) is capable of inhibiting significantly the hydroxylation of aromatic compounds and the peroxidation of arachidonic acid due to reactive hydroxyl radicals ($HO^{\cdot}$). In this paper we have investigated the ability of $C_9$2Na to inhibit the oxyradical induced toxicity towards two tumoral cell lines (Raji and IRE1) and normal human fibroblasts (HF). Oxyradicals were generated either by the addition of polyphenols to the medium, or by direct irradiation of phosphate buffered-saline in which cells were incubated from 15 min prior to incubation in normal medium. The effects of $C_9$2Na were compared with those obtained by mannitol (MAN), superoxide dismutase (SOD) and catalase (CAT). $C_9$2Na, MAN, SOD and CAT significantly decreased the polyphenol toxicity towards cell lines cultured up to 24 h. After 48 h of incubation the above compounds lost the capability of protecting cells from polyphenol toxicity. This suggests that the toxic role of oxyradicals ($O_2^{\cdot-}$, $H_2O_2$, $HO^{\cdot}$) persists for about 24 h and, subsequently other toxic mechanisms must be involved, which are not affected by oxyradical scavengers. SOD and CAT did not show any protective effect on UV induced cytotoxicity, while both $C_9$2Na and MAN were capable of reducing significantly the UV damage towards cell lines, even after 48 h incubation. This can be explained by the fact that UV cytotoxicity depends mainly on the generation of $HO^{\cdot}$, that can be "scavenged" by $C_9$2Na or MAN, but not by SOD or CAT. $C_9$2Na and MAN were not significantly degraded in the period during which they afford protection against $HO^{\cdot}$.

KEY WORDS: Azelaic acid, oxyradicals, polyphenols, UV.

## 1. INTRODUCTION

We have previously shown that azelaic acid, a C9 dicarboxylic acid, as disodium salt ($C_9$2Na) is capable of inhibiting significantly the hydroxylation of aromatic compounds such as tyrosine and toluene and the peroxidation of arachidonic acid (C20 : 4 $n$ − 6) due to reactive hydroxyl radicals ($HO^{\cdot}$) produced by Fenton or Photo Fenton-type reactions.[1] While active on hydroxyl radicals, azelaic acid has no activity on superoxide radicals ($O_2^{\cdot-}$).[1]

From these observations it seems likely that $C_9$2Na can be added to the list of well known radical scavengers such as thiourea, ethanol, dimethyl-sulphoxide, benzoate, mannitol, etc., also called radical interceptors,[2] which, as well as azelaic acid, are capable of affecting the generation of hydroxyl radicals but not that of superoxide anions.

In this context, it is important to underline that the above mentioned substances, including azelaic acid, are not specific $HO^{\cdot}$ scavengers, but rather drugs which can be oxidized, though at different degrees of difficulty, by $HO^{\cdot}$ giving rise to new

radicals. For example, $HO^{\cdot}$ react with mannitol (MAN) or dimethylsulphoxide (DMSO) or sodium azelate ($C_9$2Na) producing respectively:

$$HO^{\cdot} + \begin{cases} MAN \rightarrow MAN^{\cdot} + H_2O & (2) \\ (CH_3)_2SO \rightarrow CH_3S(OH)O + \dot{C}H_3 & (2) \\ R\text{-}CH_2COO^- Na^+ \rightarrow R\text{-}CH_2\dot{C}OO + HO^- & (1). \end{cases}$$

Hydroxyl radicals have been implicated in a number of biological phenomena, generally from the viewpoint of their cytotoxic actions. For example, cellular damage induced by ionizing radiation and the destruction of microorganisms by phagocytes have been attributed, in part, to the action of $HO^{\cdot}$.[2,3,4,5]

The presence of $HO^{\cdot}$ in biological systems is generally inferred from some oxidative properties and from the blocking action of $HO^{\cdot}$ scavengers.[4] This paper will deal with the capability of azelaic acid of acting as $HO^{\cdot}$ scavenger in cell cultures, where oxyradicals were generated either by the addition of reactive molecules such as poly-phenols to the medium,[5] or by direct UV-irradiation of phosphate buffer (PBS) in which cells were incubated for 15 min. prior to cultivation in normal medium. The effects of $C_9$2Na were compared with those obtained by both the well known $HO^{\cdot}$ scavenger mannitol (MAN) and the oxyradical scavenger enzymes superoxide dismutase (SOD) and catalase (CAT).

## 2. MATERIALS AND METHODS

Azelaic acid (99% pure) was purchased from Fluka AG and was dissolved in NaOH (Merck AG). Mannitol (MAN), superoxide dismutase (SOD) from human erythrocytes (approx. 3,000 units per mg protein) and catalase (CAT) from bovine liver (approx. 50,000 units per mg protein) were obtained from Sigma Chem. Corp.

### 2.1. *Cell cultures*

Three established cell lines were used in the present study: the lymphoma derived cell line Raji, the melanotic human melanoma cell line IRE1 and normal human fibroblasts (HF). They were cultured in RPMI 1640 (Gibco) with 10% fetal calf serum (FCS) (Gibco) and antibiotics.[6,7]

### 2.2. *Phenols*

L-Dihydroxyphenylalanine (DOPA), Dopamine (DPM) and 6-hydroxydopamine (6-OH DPM) (Sigma Chem. Co.) were used immediately after being dissolved in deionized water. Their stability in the medium at 37°C up to 24 h, in the absence or presence of the different cell lines or oxyradical scavengers, was evaluated by HPLC on a RP 18 column.[6,7] We have previously shown, that polyphenols undergo a rapid decomposition in culture medium and, in general, in buffers at physiological pH, giving rise to oxyradicals.[6,7] $5 \times 10^4$ cells of the three cell lines were exposed to DOPA, DPM and 6-OH DPM, and viability evaluated by $^3$H thymidine (Amersham, spec. act. 2 Ci/mole at 5 $\mu$Ci/ml) after 24 and 48 h of incubation in the absence or presence of $C_9$2Na (from 0.1 mM to 10 mM), or MAN (from 0.1 mM to 10 mM) or superoxide dismutase (SOD 100 UI/ml) or catalase (CAT 100 UI/ml).

### 2.3. *UV irradiation*

5 ml phosphate buffered-saline (PBS, Gibco) pH 7.4 were exposed for different periods to a sunlamp (Solinka 650, Wipasan) with an energy output in the UV region of 0.6 mW/cm$^2$ at a target distance of 20 cm. Raji, IRE1 and HF ($10^6$) cells were added to irradiated buffer and incubated for 15 min in the absence or presence of $C_9$2Na or MAN, or SOD or CAT. Following this period, cells were washed three times with PBS and cultured in normal medium. The percent survival of cells was evaluated after 24 and 48 h of incubation.



### 2.4. *Analysis of $C_9$2Na and MAN*

Some experiments were undertaken to verify whether 1 mM $C_9$2Na or 1 mM MAN underwent degradation after 24 h or 48 h of incubation in the presence of cells and oxyradical generating systems. Culture medium was separated from the cells which were washed two times with 1 ml of PBS. The washings were added to culture medium (pool). In the case of $C_9$2Na, the pool was acidified to pH 2–3 with 1M HCl and then extracted three times with 5 ml of ethyl acetate. 100 $\mu$g of $C_{10}$2Na were added as internal standard to the pool before acidification. The combined ethyl acetate phases were evaporated under $N_2$. The extract was treated with 100 $\mu$l dry pyridine and then directly silylated with 100 $\mu$l of N, O-bis-(trimethylsilyl)trifluroacetamide (Fluka AG) containing 1% trimethyl-chlorsilane (Fluka AG) as catalyst. After 30 min at 80°C, the solution was ready for the analysis. In the case of MAN, the pool was lyophilized after addition of 100 $\mu$g of meso-erythritol as internal standard, and then 1 ml of acetic anhydride (Fluka AG) plus 0.1 ml of 1-methylimidazole (Fluka AG) as catalyst were added. After 10 min at room temperature, 10 ml of water were added to decompose the excess of acetic anhydride. Mannitol and meso-erythritol acetates were extracted three times with 2 ml dichloromethane. The excess of dichloromethane was evaporated under $N_2$. Analyses of $C_9$2Na and $C_{10}$2Na as TMS-derivatives, and mannitol and meso-erthritol as alditol acetates were performed by computerized capillary direct gas-chromatography-mass spectrometry (GC-MS, HP 5890 gas chromatograph plus HP 5970 mass selective detector plus HP 5997 Chem Station) on extracted ion profiles of the compounds. Selected ions were: 201.10 and 317.20 for C9.2TMS, 201.10 and 331.20 for $C_{10}$.2TMS, 145.05 and 217.10 for meso-erytrytol acetate, 139.05 and 261.20 for mannitol acetate. The compounds were quantified by comparison with peak areas of selected ions of reference standards. Chromatographic conditions were the same for both TMS derivatives and alditol acetates. Column: Ultra 1 (cross-linked methyl-silicone gum phase, 50 m × 0.2 mm × 0.33 $\mu$m, HP); injection (1 $\mu$l): splitless; oven temperature: 60°C to 300°C at 100°C/min and hold; injector temperature: 250°C; transfer line temperature: 280°C; carrier gas: helium (0.5 ml/min); electron multiplier voltage: 2200 emV.

### 2.5. *Statistical analyses*

Statistical significance was determined according to Student's t test.

## 3. RESULTS

Figure 1 depicts the rate of decomposition of 0.1 mM DOPA, 0.1 mM DPM and 0.1 mM 6-OH DPM in culture medium. Different concentration of the three polyphenols

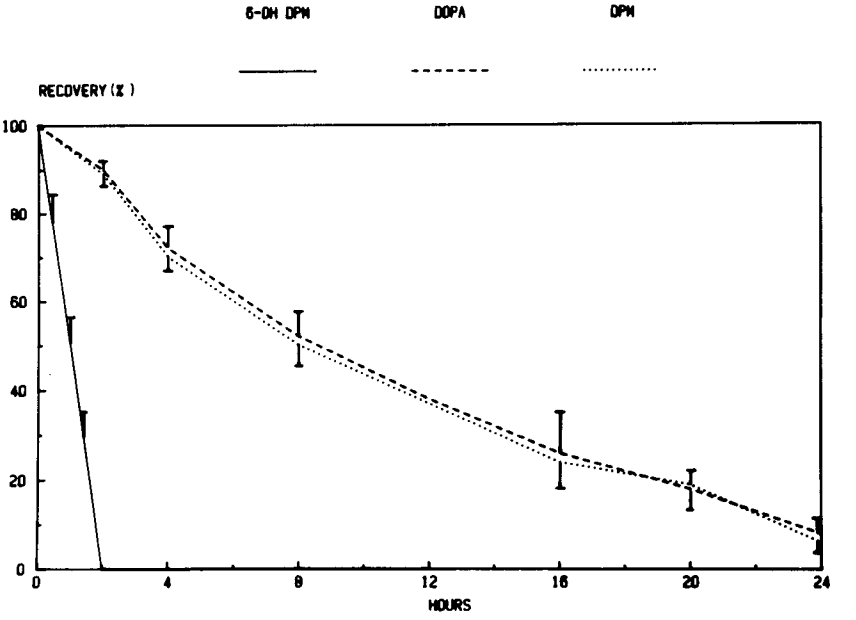


FIGURE 1 Rate of decomposition of 0.1 mM 6-OH DPM, 0.1 mM DPM and 0.1 mM DOPA in culture medium. Each point represents the average ± SD (vertical bars) of five determinations.

showed similar rates of decomposition, which were not affected by the presence either of cell lines or $C_9$2Na or MAN. Paralleling the gradual disappearance with the time of the above drugs, no quinone formation was detectable by HPLC analyses.

Table I shows the percentage survival of Raji, IRE 1 and HF cells cultured for 24 and 48 h in the presence of different concentrations of the three polyphenols. The triphenol 6-OH DPM was more toxic towards the three cell lines than were the dihydric phenols DPM and DOPA, the toxicities of which were similar. In any

TABLE I

Percentage survival of cell lines cultured for 24 h and 48 h in the presence of different concentrations of 6-OH DPM, DPM and DOPA. Each result represent the average ± SD of five experiments performed in quadruplicate.

| Polyphenols | Concentrations (mM) | Cell lines | | | | | |
|---|---|---|---|---|---|---|---|
| | | Raji | IRE1 | HF | Raji | IRE1 | HF |
| | | % survival (24 h) | | | % survival (48 h) | | |
| 6-OH DPM | 0.01 | 53 ± 6 | 52 ± 5 | 90 ± 4 | 38 ± 4 | 41 ± 5 | 88 ± 5 |
| | 0.1 | 15 ± 3 | 12 ± 4 | 53 ± 6 | 8 ± 3 | 7 ± 3 | 35 ± 4 |
| | 1 | – | – | 19 ± 3 | – | – | 5 ± 2 |
| DPM | 0.01 | 84 ± 6 | 81 ± 5 | 96 ± 3 | 62 ± 5 | 58 ± 6 | 84 ± 3 |
| | 0.1 | 50 ± 5 | 49 ± 6 | 86 ± 4 | 38 ± 4 | 36 ± 4 | 75 ± 3 |
| | 1 | 10 ± 4 | 8 ± 4 | 45 ± 4 | – | – | 35 ± 4 |
| 1-DOPA | 0.01 | 82 ± 4 | 80 ± 6 | 97 ± 2 | 60 ± 5 | 59 ± 4 | 80 ± 4 |
| | 0.1 | 52 ± 5 | 50 ± 4 | 84 ± 6 | 36 ± 4 | 34 ± 4 | 72 ± 4 |
| | 1 | 8 ± 6 | 4 ± 2 | 44 ± 4 | – | – | 30 ± 4 |

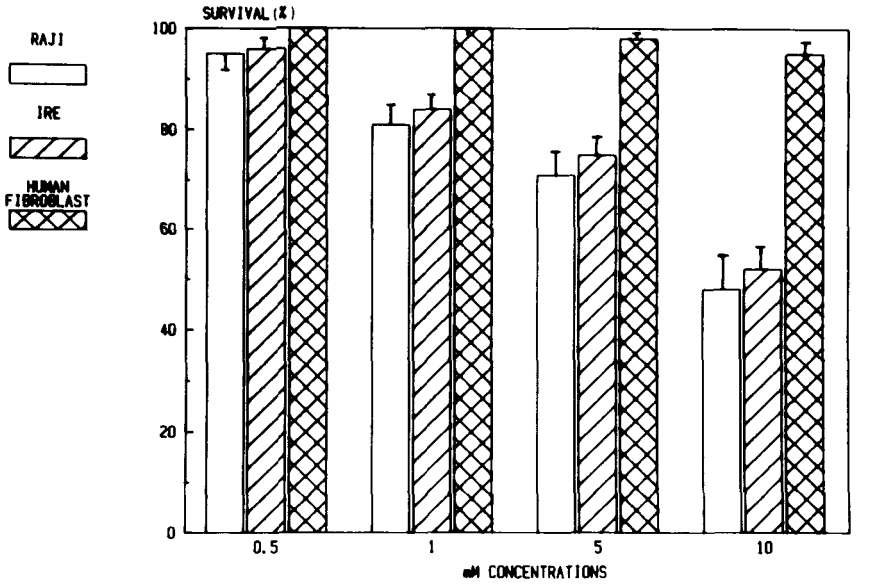


FIGURE 2 Toxicity of $C_9$2Na to cell lines after 24 h incubation. After 48 h the toxicity increases 10–20% (data not shown). Each point represents the average ± SD (vertical bars) of four experiments performed in quadruplicate.

case, HF cells were more resistant than the two tumoral cell lines to the toxic effects of the polyphenols.

The toxic effect toward cell lines after 24 h treatment with different concentrations of $C_9$2Na is shown in Figure 2. It is interesting to note that normal HF were much more resistant to the toxic effect of $C_9$2Na than Raji or IRE1 tumoral cell lines. Unlike $C_9$2Na, MAN and the oxyradical scavenger enzymes SOD and CAT, at all concentrations used, did not affect the percent survival of the cells (data not shown).

The presence in the medium of oxyradical scavengers decreased polyphenol toxicities on all cell lines after 24 h incubation (Table II). The protective effect after 24 h was more striking with 0.1 mM DPM or 0.1 mM DOPA than with similar concentrations of 6-OH DPM, probably because of the higher cell toxicity of the latter. The percentage survival of the tumoral cell lines (Raji and IRE1) was higher with 1 mM $C_9$2Na than with 5 mM or 10 mM $C_9$2Na, probably because of the intrinsic toxicity of the drug at these concentrations towards the two cell lines (Figure 2). By contrast, the higher the concentrations of MAN, which, at these concentrations, was not toxic for the cells, the higher the protective effect (Table II).

After 24 h 10 mM $C_9$2Na produced a complete protection against polyphenol toxicity on normal HF, which were more resistant than tumoral cell lines to the toxic effects of oxyradicals or $C_9$2Na (Table II).

After 48 h incubation, $C_9$2Na, MAN, SOD and CAT, lost the capability of protecting cells from polyphenol toxicity (Table II). For $C_9$2Na and MAN, the lack of activity was not attributable to their decomposition. In fact, GC-MS analyses showed that $C_9$2Na and MAN were not significantly degraded in this period (48 h)

TABLE II

Effect of oxyradical scavengers on the percentage survival of Raji, IRE 1 and human fibroblast (HF) cell lines cultured for 24 h and 48 h in the presence of 0.1 mM 6-OH DPM or 0.1 mM DPM. Each results represents the average ± SD of four experiments performed in quadruplicate. Experiments with DOPA are not reported because the results are similar to those of DPM.

| Polyphenols | Oxyradical scavengers | Concentrations (mM) | Cell lines | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Raji | IRE1 % survival (24 h) | HF | Raji | IRE1 % survival (48 h) | HF |
| 6-OH DPM | – | – | 15 ± 3 | 12 ± 4 | 53 ± 6 | 8 ± 3 | 7 ± 3 | 38 ± 4 |
| 0.1 mM | | 0.5 | 18 ± 4 | 15 ± 4 | 54 ± 5 | 10 ± 4 | 9 ± 3 | 39 ± 5 |
| | C9 2Na | 1 | 45 ± 5* | 47 ± 6* | 69 ± 5* | 12 ± 4 | 12 ± 5 | 42 ± 5 |
| | | 5 | 37 ± 4* | 38 ± 3 | 84 ± 7* | 6 ± 2 | 4 ± 1 | 41 ± 4 |
| | | 10 | 12 ± 5 | 8 ± 3 | 95 ± 4* | – | – | 52 ± 5* |
| | | 0.5 | 20 ± 4 | 18 ± 3 | 57 ± 6 | 9 ± 3 | 8 ± 3 | 39 ± 6 |
| | MAN | 1 | 42 ± 6* | 41 ± 5* | 66 ± 4* | 10 ± 4 | 9 ± 4 | 38 ± 4 |
| | | 5 | 75 ± 5* | 78 ± 7* | 81 ± 6* | 12 ± 4 | 11 ± 5 | 42 ± 5 |
| | | 10 | 92 ± 6* | 89 ± * | 96 ± 3* | 15 ± 5** | 14 ± 4** | 48 ± 5** |
| | SOD | 100 UI/ml | 74 ± 6* | 71 ± 4* | 94 ± 5* | 11 ± 4 | 10 ± 4 | 39 ± 5 |
| | CAT | 100 UI/ml | 68 ±4* | 72 ± 6* | 92 ± 6* | 8 ± 4 | 9 ± 3 | 36 ± 4 |
| DPM | – | – | 50 ± 5 | 49 ± 6 | 86 ± 4 | 38 ± 4 | 37 ± 6 | 75 ± 5 |
| 0.1 mM | | 0.5 | 67 ± 4* | 64 ± 5* | 93 ± 4* | 37 ± 5 | 35 ± 4 | 76 ± 6 |
| | C9 2Na | 1 | 88 ± 6* | 85 ± 4* | 96 ± 3* | 41 ± 4 | 40 ± 4 | 78 ± 4 |
| | | 5 | 80 ± 3* | 81 ± 4* | 99 ± 2* | 26 ± 8** | 29 ± 5** | 84 ± 4** |
| | | 10 | 47 ± 4 | 43 ± 5* | 99 ± 3* | 15 ± 3* | 13 ± 3* | 88 ± 3* |
| | | 0.5 | 69 ± 3 | 66 ± 5 | 95 ± 3* | 38 ± 4 | 36 ± 4 | 78 ± 3 |
| | | 1 | 89 ± 4* | 86 ± 4* | 96 ± 4* | 39 ± 3 | 40 ± 3 | 79 ± 4 |
| | | 5 | 94 ± 4* | 93 ± 3* | 99 ± 2* | 44 ± 3 | 42 ± 4 | 83 ± 4** |
| | | 10 | 98 ± 2* | 96 ± 4* | 99 ± 2* | 50 ± 6** | 48 ± 3** | 86 ± 5** |
| | SOD | 100 UI/ml | 88 ± 6* | 81 ± 4* | 95 ± 4* | 40 ± 4 | 42 ± 5 | 77 ± 5 |
| | CAT | 100 UI/ml | 82 ± 5* | 79 ± 6* | 94 ± 3* | 37 ± 6 | 40 ± 4 | 78 ± 6 |

*$p < 0.01$, **$p < 0.05$ against the values of controls.

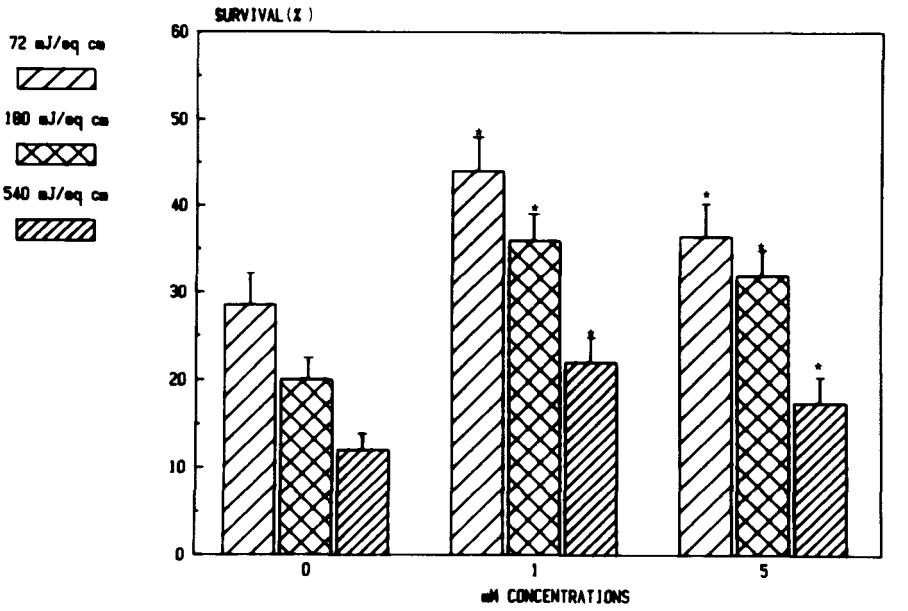


FIGURE 3 Protective effect of $C_9$2Na on the toxcity towards Raji cells after 24 h incubation, following increasing amounts (72, 180, 540 mJ $cm^{-2}$) of total UV dose on PBS. For further details see text. Each point represents the average $\pm$ SD (vertical bars) of four experiments performed in quadruplicate. $^*p < 0.01$ tested against values of controls.

during which they were incubated with polyphenols or exposed to oxyradicals generated by UV irradiation of PBS.

It is well known that UV irradiation of aqueous systems leads to time dependent formation of oxyradicals.[5] Figure 3 shows that the UV-induced toxicity towards Raji cells increased with the UV dose and that $C_9$2Na, was able to reduce significantly the toxicity after 24 h of incubation. As in the case of polyphenol toxicity, $C_9$2Na afforded higher protection at 1 mM than at 5 mM concentration (Figure 3). IRE1 cells behaved in the same way as Raji cells. SOD and CAT did not show any significant effect on UV induced toxicity, while the protective effect of MAN increased up to 5 mM concentration (Figure 4).

As regard HF, since they were more resistant to UV induced toxicity than Raji and IRE1 tumoral cell lines, the total UV dose was increased to 900 mJ/$cm^2$.

Figure 5 depicts the protective effect of different concentration of $C_9$2Na and MAN on the toxicities produced by 540 mJ/$cm^2$ and 900 mJ/$cm^2$ total UV doses. The positive effects of $C_9$2Na were significantly higher than those of MAN at all concentrations used. After 48 h incubation, UV induced toxicity towards the three cell lines increased slightly (10–20%), but, the percent protection of $C_9$2NA or MAN was the same (data not shown).

## 4. DISCUSSION

The process of autoxidation of polyphenols in solution at physiological pH results in the generation of oxyradicals, semiquinones and quinones by the

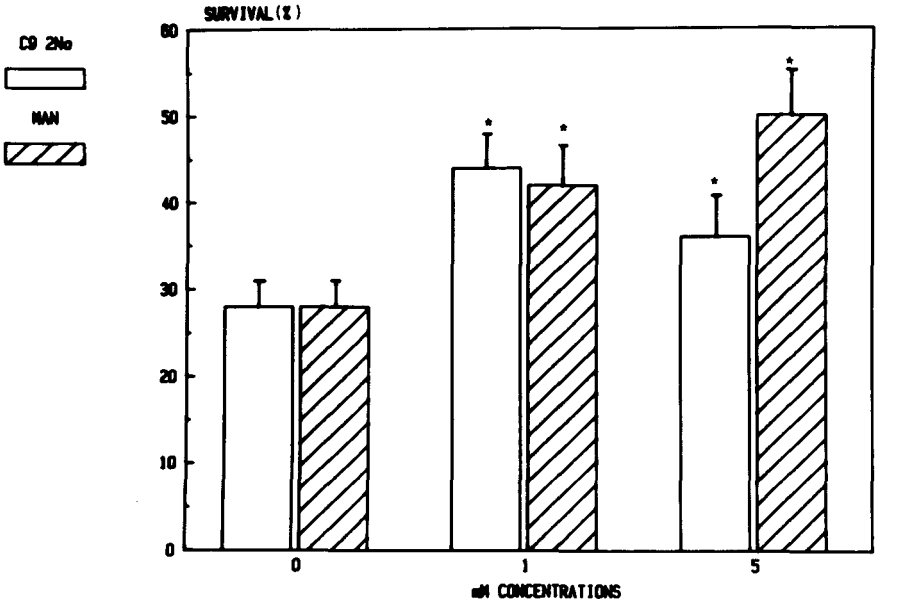


FIGURE 4 Protective effect of different concentrations of $C_9$2Na and MAN on the toxicity towards Raji cells, after 24 h incubation, following 72 mJ cm$^{-2}$ total UV doses. For further details see text. Each point represents the average ± SD (vertical bars) of four experiments performed in quadruplicate. *$p < 0.01$: tested against values of controls.

reactions:[3,6–8]

(1) $O_2 + e^- \rightarrow O_2^{\cdot-}$,

(2) $QH_2 + O_2 \rightarrow Q + H_2O_2$

(3) $QH_2 + Q \rightarrow 2\dot{Q}H$

(4) $\dot{Q}H + O_2 \rightarrow Q + O_2^{\cdot-} + H^+$

(5) $H_2O_2 + O_2^{\cdot-} \rightarrow HO^{\cdot} + HO^- + O_2$

where $O_2^{\cdot-}$ represent superoxide anion radicals, $H_2O_2$ hydrogen peroxide, $HO^{\cdot}$ hydroxyl radicals, $QH_2$ polyphenols, Q quinones and $\dot{Q}H$ semiquinones.

Generally, the capacity for autoxidation depends upon the number and steric position of hydroxy groups, the electronic properties and position of possible substituents on the benzene ring and, consequently, the polarity.[9] Thus the triphenol 6-OH DPM is much more easily and rapidly oxidizable in culture medium than the two orthodiphenols DOPA and DPM (2 h against 24 h) (Figure 1) and is more toxic towards the three cell lines used in our study, Raji, IRE1 and HF (Table I, II).

Controversy persists as to whether polyphenol toxicity is mediated by oxyradicals[10,11] or depends on the covalent binding of the quinone oxidation products of polyphenols with sulphydryl or other nucleophilic groups within the cells.[12–14] Graham *et al.*[8] found that 6-OH DPM and 2,4,5-trihydroxyphenylalanine kill C1300 neuroblastoma cells

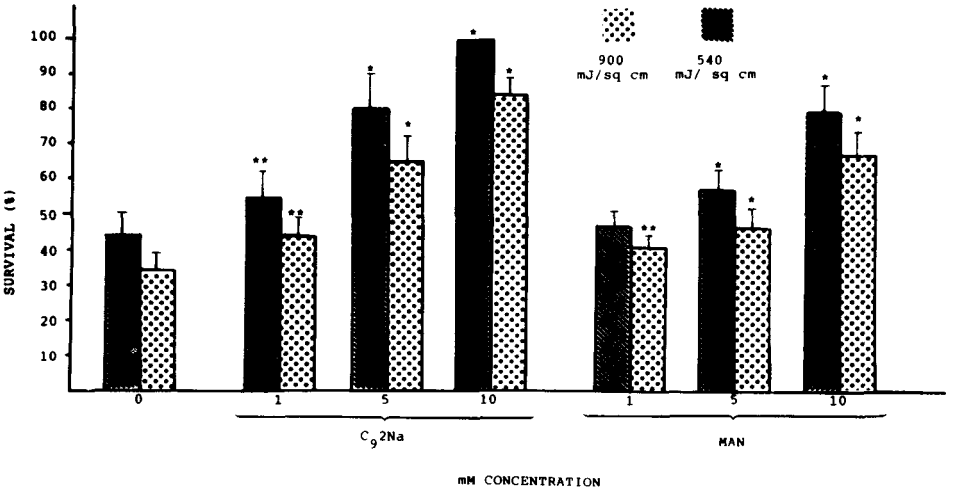


FIGURE 5 Protective effect of different concentrations of $C_9$2Na and MAN on the toxicity toward HF, after 24 h incubation, following 540 mJ $cm^{-2}$ and 900 mJ $cm^{-2}$ total UV doses. For further details see text. Each point represents the average $\pm$ SD (vertical bars) of four experiments performed in quadruplicate. $^{*}p < 0.01$, $^{**}p < 0.05$: tested against values of controls.

in vitro through the production of oxyradicals, while for DPM and DOPA the reaction of quinone oxidation products with nucleophiles also contributes to their cytotoxicity.

We have recently shown that, in cell cultures, the major component of toxicity up to 24 h of polyphenols was due to toxic oxygen species acting outside the cells and not to cellular uptake of these polyphenols as such. In fact the addition of oxyradical scavenger enzymes, *i.e.*, SOD and/or CAT, significantly decreased the adverse effect of these drugs on cell lines.[6,7]

Likewise, the addition to the culture medium of $HO^{\cdot}$ scavengers $C_9$2Na or MAN significantly decreases the toxicity of polyphenols towards cell lines, further indicating the key role played by oxyradicals, in particular $HO^{\cdot}$, in the mechanism of cytotoxicity up to 24 h incubation (Table II). It is worth mentioning that the % survival of tumoral Raji and IRE1 cells is higher with 1 mM than with 5 mM or 10 mM $C_9$2Na (Table II). The contrast is easily explained by considering that the two concentrations of $C_9$2Na exert 25–30% and 45–55% toxicity respectively on these two cell lines (Figure 2). It is surprising, however, that the intrinsic toxicity of $C_9$2Na is not additive or synergistic with that induced by polyphenols (Table II). On the contrary, in the presence of a cell line such as HF, which is more resistant than tumoral cell lines to the toxic effects of oxyradicals or $C_9$2Na, 10 mM of the drug produces a complete protection against polyphenol toxicity (Table II). Similarly, the higher the concentration of MAN, which is not toxic for the cell up to 10 mM concentrations (data not shown), the higher the protective effect towards the three cell lines (Table II).

After 48 h incubation, $C_9$2Na, MAN, SOD and CAT lose the capability to protect cells against polyphenol toxicity. This suggests that the toxic role of oxyradicals persists for about 24 h. Subsequently, other toxic mechanisms must be involved, such as: (a) the disappearance from the medium of essential growth factors which may be destroyed or inactivated by oxyradicals, semiquinones and quinones; (b) the possible

concomitant formation of toxic radical melanoid polymers;[15–16] (c) the reaction of quinones and semiquinones with external nucleophiles, i.e., sulphydryl groups, amino or phenolic hydroxyls, as an alternative to cyclization.[17] It is interesting to underline that no quinone formation was detectable by HPLC analyses during 48 h incubation. This does not mean that quinones are not formed during polyphenol oxidation, but rather that they have too short lifetimes and too high reactivity toward nucleophiles to be measurable by HPLC.

According to Bielski and Gebicki[5] the primary reactive species in irradiated water are hydroxyl radicals, hydrated electrons, and hydrogen radicals in addition to stable products such as molecular hydrogen, hydrogen peroxide and protons. The whole process of formation of primary products can be written in the following conventional way:

$$H_2O \xrightarrow{\sim\sim} HO^{\cdot}, H^{\cdot}, e^{-}_{aq}, H_2, H_2O_2, H_3O^{+} \quad (5).$$

$O_2^{-}$ does not appear among the primary products and $H_2O_2$ concentrations built up by the average radiations doses are usually insufficient to cause extensive oxidation.[5] This agrees with the lack of protective effects of SOD and CAT towards UV induced cytotoxicity (data not shown). On the contrary, the $HO^{\cdot}$ "interceptors" $C_9$2Na and MAN are able to reduce significantly the toxicity (Figures 3–5) which is directly proportional to the period of irradiation (Figure 4).

We preferred to irradiate PBS rather than the culture medium or the cells themselves in order to avoid the possibility of damaging medium active components or essential cellular constituents, which might worsen the damage due to oxyradicals. In this connection, another well known $HO^{\cdot}$ scavenger, DMSO, has been used as a protector of human lymphoblastoid cells against the cytotoxic effects of X-irradiation. DMSO was effective only as a protector from lethal effects of $HO^{\cdot}$, while, at both genetic loci, the induced mutant fractions were not affected by the drug.[18]

As in the case for the addition of polyphenols to cultures of the tumoral Raji and IRE1 cells, and for the same above mentioned reason, the activity of $C_9$2Na peaks at 1 mM concentration, while higher levels of MAN further reduce the UV induced cytotoxicity (Figure 4). In addition, experiments carried out with HF, which are more resistant cells than tumoral ones, show that concentrations of $C_9$2Na in the order of 5–10 mM afford a strong protection against UV damage even at high total UV doses and that such protection is better with $C_9$2Na than with MAN at all concentrations used (Figure 5). After 48 h incubation, the toxicity towards the three cell lines increases slightly (about 10–20%) but, unlike polyphenols, the percent protection of $C_9$2Na or MAN persists unmodified (data not shown). This can be explained by the fact that, under the experimental conditions, UV cytotoxicity depends mainly on the generation of $HO^{\cdot}$ that can be "scavenged" by $C_9$2Na or MAN. On the contrary, in the case of polyphenols, in addition to oxyradicals, other factors, as mentioned above, which are not affected by $C_9$2Na or MAN or SOD or CAT, may play an important cytotoxic role (Table II).

It is important to underline that both $C_9$2Na and MAN are not significantly degraded in the period (48 h) during which they carry out scavenging activity towards $HO^{\cdot}$.

We have stated in the Introduction that the so called $HO^{\cdot}$ interceptors, including $C_9$2Na, can be oxidized by $HO^{\cdot}$ at different degrees of difficulty yielding new radicals. However, we have previously shown that the diacid decomposition is detectable only in the presence of high $HO^{\cdot}$ fluxes.[1] With low concentrations of $HO^{\cdot}$, such as those

generated in culture either by polyphenol autoxidation or by UV irradiation of PBS, the degradation of $C_9$2Na or MAN and the formation of by-products is not quantifiable by our analytical methods: the rate of decomposition may be so slow as to be kinetically insignificant.

In conclusion, the results obtained in this study show that $C_9$2Na is capable of inhibiting oxyradical-mediated toxicity in cell cultures generated by both polyphenol autoxidation and UV irradiation. Its effectiveness is similar to that exhibited by MAN and, in the case of polyphenol oxidation, by SOD and CAT (Table II). It is however, necessary to draw a distinction between the mechanisms of action of these substances. SOD catalyses the dismutation of $O_2^{\cdot-}$ to form $O_2$ and $H_2O_2$ which is reduced to $H_2O$ and $O_2$ by catalase. Both superoxide anion radicals, and hydrogen peroxide (which are the most common species among the radical intermediates formed by chemical and enzymatic processes), are considered to be "sluggish oxidants" and it is likely that they serve as mediators of cellular toxicity related to oxygen.[19,20] By metal-catalyzed reactions (Haber–Weiss cycle), $O_2^{\cdot-}$ and $H_2O_2$ are able to produce hydroxyl radicals which are among the most reactive free radicals in biological systems and their formation must be considered a central event in toxicity.[19–20]

These data, showing the $HO^{\cdot}$ scavenging capacity of $C_9$2Na in culture, may represent a useful basis for a better understanding of its multiple and different biological and therapeutical activities.[21]

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