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Differential cytotoxicity of daunomycin in tumour cells is related to glutathione-dependent hydrogen peroxide metabolism

Argante BOZZI, Irene MAVELLI, Bruno MONDOVI, Roberto STROM and Giuseppe ROTILIO
Institutes of Biological Chemistry and Applied Biochemistry, CNR Centre for Molecular Biology, University of Rome, Rome, Italy

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Addition of 0.5 mM-daunomycin, a quinone anti-cancer drug, causes severe inhibition of respiration in Ehrlich ascites cells, whereas Yoshida ascites cells were almost as resistant as rat hepatocytes. An inverse relationship appears to exist in the two types of tumour cells (which are both catalase-deficient) between the extent of cellular damage brought about by intracellular formation of superoxide anion occurring on reaction with O_2 of the drug free radical and the efficiency of the glutathione-mediated H_2O_2-detoxifying system.

The activities of glutathione peroxidase and of the enzymes involved in the biosynthesis and in the reduction of glutathione are well correlated, in different strains of tumour cells, with the cytotoxic effect exerted on these cells by externally produced H_2O_2 (Bozzi *et al.*, 1979). None of the tumour cells tested had any catalase activity, and their content of superoxide dismutase was significantly lower than that of the 'normal' control cells, such as erythrocytes and liver cells (Bozzi *et al.*, 1979). In spite of that, no protection against extracellular sources of O_2^- was afforded by addition of superoxide dismutase or of $OH^\cdot$ radical scavengers (Bozzi *et al.*, 1979). Since daunomycin (daunorubicin), an anthracyclin antibiotic, has been reported to produce O_2 radicals by redox cycling in the presence of purified microsomal fraction ('microsomes') (Handa & Sato, 1975), of microsomal enzymes (Goodman & Hochstein, 1977) or of submitochondrial particles (Thayer, 1977), it seemed of interest to investigate whether the extent of its cytotoxicity on tumour cells is correlated with the rate of their reductive activation on the one hand and to the activity of protecting enzymes on the other. It has indeed been demonstrated (Bartoli & Galeotti, 1979) that maximally deviated tumours, such as the ascites cells used in our experiments, have a lower content of cytochrome *P*-450 activity and a lower rate of production of superoxide anion in their microsomal membranes than normal liver cells. On the other hand they also have, in their cytoplasm, different superoxide dismutase/glutathione peroxidase activity ratios (Bozzi *et al.*, 1976). Since O_2^- is the primary product of the interaction of the reduced drug with O_2 (Misra & Fridovich, 1972), this ratio should modulate the cytotoxic effect of intracellularly redox-cycling drugs; superoxide dismutase will accelerate formation of H_2O_2, which, in the absence of catalase, will be metabolized via glutathione peroxidase. Tumour cells with different superoxide dismutase/glutathione peroxidase ratios would thus represent, with reference to the complete enzyme pattern of liver cells, a good model to test the validity of these assumptions. To this aim the response of two types of tumour cells and of hepatocytes to daunomycin was investigated.

Materials and methods

Ehrlich ascites and Yoshida ascites tumour cells, provided by the Regina Elena Institute for Cancer Research, Rome, Italy, were usually suspended in phosphate-free Ringer solution containing 50 mM-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonic acid), pH 7.3. Cell viability was always more than 90%.

Isolated rat liver cells were prepared by the method of Moldéus *et al.* (1978) and resuspended in Tes/Ringer solution. After each incubation, the pH of the cell suspension was checked, to verify that the effects observed were not due to acidification of the medium. Daunomycin was a kind gift from Professor F. Arcamone (Farmitalia, Milan, Italy). All other chemicals were reagent grade. O_2 consumption, control of cell viability and determinations of glutathione (reduced and oxidized) were carried out as previously described (Bozzi *et al.*, 1979). Cell permeation by daunomycin was

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evaluated by sedimenting the cells, at various times after addition of the drug, in haematocrit tubes, and by measuring at 575 nm the absorption of the drug derivative formed when a measured height of pellet was disrupted by sonication and dissolved in 5 vol. of 1 M-NaOH.

Results

Fig. 1 shows the effect of 0.5 mM-daunomycin on the O_2 uptake by Ehrlich ascites cells, by Yoshida ascites cells and by isolated hepatocytes, in the absence or presence of succinate or of glucose as external substrates. It is apparent that, under all conditions and in particular when only endogenous substrates or succinate are available, Ehrlich ascites cells were most sensitive to inhibition by the drug, whereas Yoshida cells behaved rather similarly to hepatocytes, undergoing only a very slight inhibition. In the presence of glucose, addition of daunomycin resulted in a strong initial activation of the O_2 uptake in both types of tumour cells; at longer incubation times, however, the respiration of Ehrlich cells was inhibited by the drug. Drug-uptake experiments showed (Fig. 2) that the drug reached comparable concentrations in the three cell types; in line with this result, the addition of the polyenic antibiotic lucensomycin, which causes a generalized increase of permeability in the cell membrane (Strom *et al.*, 1972), did not alter the overall response pattern.

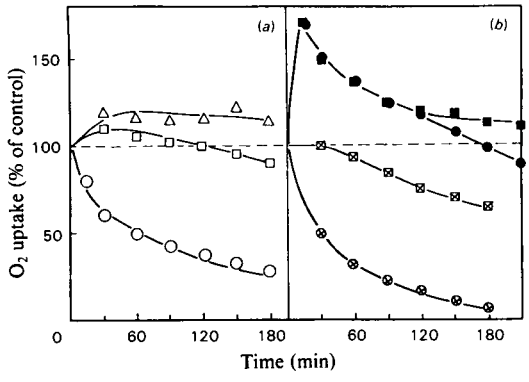

Fig. 1. *O_2 uptake (% of control) by Ehrlich and Yoshida ascites cells and by isolated rat hepatocytes in Tes/Ringer buffer, pH 7.3, in the presence of 0.4 mM-daunomycin*
Cell concentration (selected so as to obtain approximately the same respiration rate): Ehrlich, 12×10^6/ml; Yoshida, 8×10^6/ml; hepatocytes, 4×10^6/ml. (*a*) Rat liver (△), Yoshida (□) and Ehrlich (○) cells with 15 mM-succinate as external substrate; (*b*) Ehrlich (●) and Yoshida (■) cells with 15 mM-glucose as external substrate or Ehrlich (⊗) and Yoshida (⊠) cells with no external substrate.

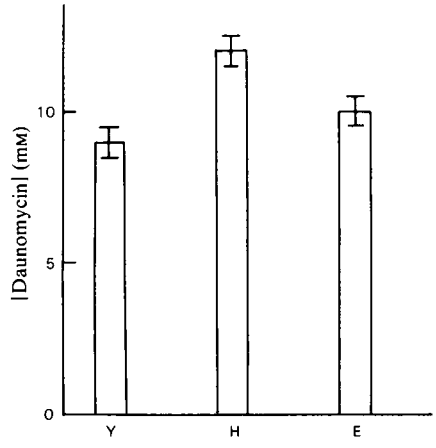

Fig. 2. *Daunomycin uptake of Yoshida ascites cells (Y), isolated rat hepatocytes (H) and Ehrlich ascites cells (E)*
The concentration of the drug in packed cells was measured after 30 min of incubation at 37°C. The experimental conditions are as described in the legend to Fig. 1.

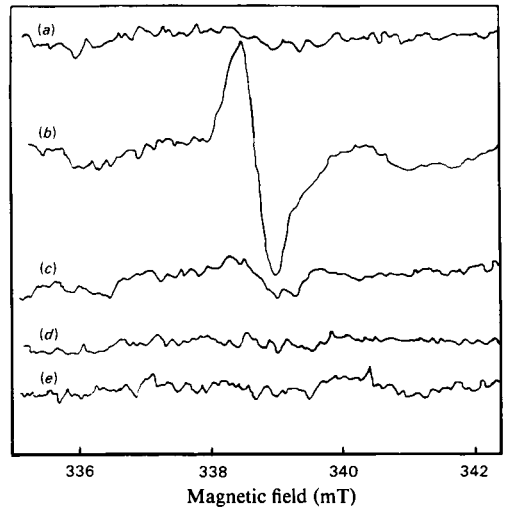

Fig. 3. *Room temperature e.p.r. spectra of Ehrlich ascites cells (13×10^7/ml) in Tes/Ringer buffer, pH 7.3*
(*a*) The cells, 5 min after addition of 1 mM-daunomycin; (*b*) is same as (*a*), but after 45 min incubation at room temperature in the e.p.r. cell; (*c*) is the same as (*b*), after oxygenation by gentle shaking in air; (*d*) is same as (*b*), but without any added drug; (*e*) solution of 1 mM-daunomycin in Tes/Ringer buffer after 45 min incubation at room temperature. E.p.r. conditions: modulation amplitude, 0.5 mT; microwave frequency, 9.52 GHz; microwave power, 50 mW.

E.p.r. experiments were carried out to investigate the formation of drug radicals. In all cases, an e.p.r. signal of the free-radical type developed after some incubation time (Fig. 3; e.g. an experiment with Ehrlich cells). This signal, which is absent from the cell-free supernatant and the shape of which is typical of immobilized radicals, is likely to be the daunomycin semiquinone produced by the reaction of the drug with cellular electron-transporting chains. The increase of this e.p.r. signal with time was clearly dependent on the anaerobiosis that developed in the cells layered in the very thin and flat e.p.r. sample container used for these experiments. In fact (Figs. 3 and 4), the maximum signal height reached by the radical did not decay on standing, but the signal disappeared when the cell suspension was reoxygenated.

To further validate the hypothesis that the observed toxic effects of daunomycin are essentially O_2-dependent, respiration of three cell types was measured after pre-incubation with daunomycin in the presence and absence of air for 2h. Pre-incubation in either N_2 or air atmosphere had practically no effect on the subsequent respiration rate of all types of cells. When the drug was present during pre-incubation, resistant cells showed no inhibition, both in air and N_2. On the contrary, the O_2 consumption of Ehrlich cells pre-incubated with daunomycin in air was 40% of that measured with cells pre-incubated with the drug under N_2.

Table 1 reports the concentrations of reduced and oxidized glutathione found in Yoshida and Ehrlich cells after incubation with daunomycin. Compared with the controls kept at 4°C, only samples incubated with the drug showed a clear decrease of reduced glutathione and an increase of oxidized glutathione. This effect is more evident with Yoshida cells, whereas the reduced glutathione concentrations of Ehrlich cells, which were initially already very low, are only slightly affected by the drug.

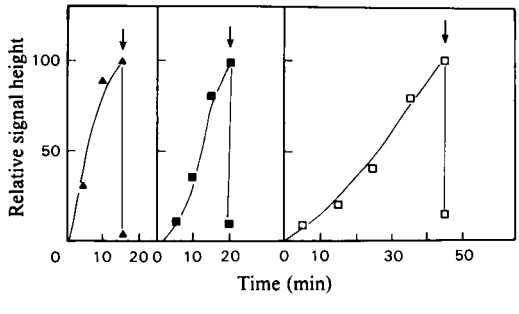

Fig. 4. *Time course of daunomycin e.p.r. signal height, on incubation at room temperature in the e.p.r. cell in the presence of isolated rat hepatocytes (▲; 40×10^6 cells/ml), Yoshida cells (■; 90×10^6 cells/ml) and Ehrlich cells (□; 130×10^6 cells/ml) suspended in Tes/Ringer buffer*
Drug concentration was 1 mM. The arrows indicate the moments at which the mixtures were reoxygenated. E.p.r. conditions were as described in the legend to Fig. 3.

Discussion

The results of the present work support the hypothesis (Rotilio *et al.*, 1973; Bozzi *et al.*, 1976) that the metabolic ratio between formation of H_2O_2 by superoxide dismutase systems and its removal by glutathione peroxidase (plus catalase, if present) is important in determining the resistance of the cells to intracellular oxidative stress. Furthermore, it is confirmed that certain strains of ascites tumour cells are suitable systems to study O_2-mediated cytotoxicity (Bozzi *et al.*, 1979).

The e.p.r. experiments show that a radical accumulates in the various types of cells when they are incubated with the drug and the O_2 is its main cellular scavenger; direct reaction of drug radicals with cellular components, e.g. nucleic acids (as suggested by Bachur *et al.*, 1978), is therefore unlikely to be a primary event. It appears, moreover, that the products of the reaction between O_2 and daunomycin semiquinone, primarily the superoxide anion and H_2O_2, are more toxic to the Ehrlich ascites cells than to the other two cell types.

The cytotoxic effect of daunomycin on Ehrlich cells results in a strong time-dependent inhibition of

Table 1. *Determination of reduced and oxidized gluthathione in Ehrlich (E) and Yoshida (Y) ascites tumour cells*
Experimental conditions were as in Fig. 1(*a*). Incubation time was 2h. Values are expressed as $\mu g/10^6$ cells (±S.E.M., $n = 4$). Controls kept at 4°C gave values not significantly different from those without drug.

	Glutathione content			
	Without daunomycin		With 0.5 mM-daunomycin	
Redox state of glutathione	E	Y	E	Y
Reduced	1.42 ± 0.02	10 ± 0.04	0.73 ± 0.02	3.5 ± 0.03
Oxidized	0.68 ± 0.02	2.2 ± 0.03	1.61 ± 0.02	5.5 ± 0.03

O_2 consumption (Fig. 1). Although O_2 uptake in the presence of succinate is immediately affected, the addition of glucose strongly activates O_2 consumption in the presence of the drug at early times, the inhibitory effect of daunomycin on cell respiration taking place only at longer incubation times. In Yoshida cells, addition of glucose plus daunomycin also results in an early activation of O_2 uptake, no inhibition occurring, however, later on. This early activation may be explained by an increased microsomal NADPH oxidation in the presence of daunomycin (Goodman & Hochstein, 1977), whereas the late inhibition occurring in Ehrlich cells could reflect leakage of substrates through damaged cell membranes, as it is somewhat counteracted by re-addition of glucose. On the other hand, the inhibition of succinate oxidation points toward a damage produced by daunomycin in Ehrlich cells at the mitochondrial membrane level.

The lack of substantial modification of the overall pattern by lucensomycin, and the similar daunomycin uptake, rule out the possibility that the different response of the three cell types is due to differential permeation of the cell membranes by the drug.

If we assume that H_2O_2 is the final product of intracellular redox processes involving daunomycin, either at the microsomal (Handa & Sato, 1975) or at the mitochondrial (Thayer, 1977) level, its stability would be such as to allow its diffusion out from the site of production, thus causing, on the one hand, a damage to cell and/or mitochondrial membranes and, on the other, interaction with defensive enzymes (Jones *et al.*, 1978). Our results (Table 1) agree with these predictions, since a significant increase of glutathione oxidation is observed on incubation with daunomycin. Moreover, Yoshida cells, which have high glutathione and glutathione peroxidase contents (Bozzi *et al.*, 1979), can oxidize large amounts of glutathione and efficiently counteract the oxidative stress caused by the drug; on the contrary Ehrlich cells, which have low activities of glutathione-related enzymes (Bozzi *et al.*, 1979), show sluggish utilization of the small amounts of reduced glutathione they possess, and, accordingly, are less capable of resisting the oxidant injury.

As a conclusion, these results demonstrate for the first time the formation of a drug radical and its O_2-dependent reaction in intact cells, and relate the extent of cellular damage to the efficiency of H_2O_2 detoxification via the glutathione system. The degree of O_2 uptake inhibition in all three cell types investigated (Fig. 1) is in fact quantitatively compatible with the ratios of activities of glutathione peroxidase to superoxide dismutase, the normalized values of these ratios being 1, 0.6 and 0.3 for liver, Yoshida and Ehrlich cells respectively (Bozzi *et al.*, 1976). It should, however, be kept in mind that cytotoxicity by daunomycin and related compounds is a complex phenomenon that cannot be entirely accounted for by a single molecular mechanism. Nevertheless, it is noteworthy that O_2-dependent effects can be selectively observed under specific experimental conditions.

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