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ENHANCEMENT OF DOXORUBICIN TOXICITY FOLLOWING ACTIVATION BY NADPH CYTOCHROME P450 REDUCTASE

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Abstract—Treatment of MCF-7 cells with doxorubicin in the presence of purified rat NADPH cytochrome P450 reductase (P450red) and NADPH resulted in a marked enhancement of drug cytotoxicity. No potentiation of cell killing was observed when the drug was incubated with P450red and NADPH prior to addition to the cells, implicating the involvement of short-lived species. The increase in doxorubicin toxicity was clearly related to its metabolism by P450red. Of a variety of free radical scavenging agents tested only glutathione was effective in protecting against the toxic metabolites produced by reductive activation. Preliminary experiments also showed that doxorubicin binding to cellular proteins and DNA occurred to a greater extent when cells were treated with the drug and P450red. Our data indicate that oxygen radicals do not play a primary role in cytochrome P450 reductase-mediated doxorubicin cytotoxicity and suggest that, in this case, the mechanism of toxicity involves a reactive species which binds to cellular nucleophiles.

Doxorubicin (Adriamycin®) is firmly established as a major cancer chemotherapeutic agent [1]. Several hypotheses have been proposed to account for the exceptional anti-neoplastic properties of this anthracycline antibiotic. However, none of them has gained uniequivocal experimental substantiation (for review see Ref. 2). One mechanism involves the conversion of doxorubicin to the semiquinone free radical. It has been proposed that this results in either irreversible, probably covalent, binding of the drug to DNA [3–5] and proteins [6, 7] or in redox cycling. In the former case, however, the formation of doxorubicin DNA adducts *in vivo* has not been clearly demonstrated. It has been shown, however, that reduction of the antitumour anthracycline Menagoril *in vitro* results in coupling to 2′-deoxyguanosine [8]. Also preliminary studies using ^{32}P-postlabelling, indicate that Daunorobicin forms DNA adducts *in vitro* if incubated with liver microsomes [9].

In the other mechanism of toxicity, redox cycling of the semiquinone free radical stimulates production of the superoxide anion radical, hydrogen peroxide and ultimately hydroxyl radicals [10]. This process is implicated in both the cardiotoxic and cytotoxic effects of doxorubicin and is substantiated by the reports on doxorubicin-induced lipid peroxidation (for review see Ref. 11) and oxygen-mediated DNA damage [12–14].

The doxorubicin semiquinone free radical has been shown to be generated spontaneously at physiological pH [15] but its formation is greatly enhanced by the enzymes catalysing one-electron reduction. In this regard, the ability of NADPH cytochrome P450 reductase, a flavoprotein component of the endoplasmic reticulum of liver and other organs, has been extensively studied (for reviews see Refs 10 and 16). The metabolic activation of the drug by NADPH cytochrome P450 reductase or microsomes has been shown to promote redox cycling as well as alkylation of nucleophiles *in vitro*. However, the drug concentrations studied were usually much higher than those achievable in the clinic. The experiments reported in this paper were carried out to examine the influence of one-electron reduction on doxorubicin cytotoxicity under biologically more relevant concentrations and to clarify the relative importance of drug-induced redox cycling and alkylation in its cytotoxic effects.

MATERIALS AND METHODS

Materials. 14-[^{14}C]Doxorubicin hydrochloride (14-[^{14}C]Adriamycin hydrochloride) was purchased from Amersham International, dissolved in water to give a specific activity of 0.5 mCi/mmol and kept frozen at −20°. Rat hepatic NADPH cytochrome P450 reductase (P450red‡) was isolated as described previously [17, 18]; in this paper 1 unit of activity is defined as the amount of enzyme required to reduce 1 nmole of cytochrome *c* per min. Superoxide dismutase (3000 U/mg) was obtained from Sigma. The media and supplements used in tissue culture were from Gibco. All other chemicals were purchased from Sigma or BDH and were of tissue culture grade as available.

Cell culture, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. MCF-7

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‡ Abbreviations: MCF-7 cells, human breast carcinoma cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P450red, rat hepatic NADPH cytochrome P450 reductase; PBS, phosphate-buffered saline (0.17 M NaCl, 0.27 mM KCl, 8.1 mM Na_2HPO_4, 0.27 mM KH_2PO_4, pH 7.2); SDS, sodium dodecyl sulphate.

cells (a human mammary carcinoma cell line) were maintained in exponential growth in monolayer culture in RPMI 1640 medium supplemented with 10% foetal calf serum and penicillin–streptomycin. For MTT assays, $7–9 \times 10^3$ cells were plated in 0.18 mL of culture medium in 96-well microtiter plates (Costar) and grown for 24 hr prior to the experiments. The treatment solutions consisting of doxorubicin at different concentrations, radical scavenger when used, NADPH and P450red were prepared just before treatment in serum-free culture medium and immediately added to the cells in a volume of 0.02 mL. The final concentrations of NADPH and the enzyme were 1 mM and 250 U/mL, respectively, unless otherwise indicated. The cells were incubated with the treatment solutions for 3 hr at 37°. The culture medium was then replaced with 0.2 mL of fresh medium and the cells were allowed to grow for 72 hr (approximately two doubling times). The cell survival was assessed using the Microtiter Tetrazolium (MTT) chemosensitivity assay [19]. The concentration of the reduced form of MTT serving as a measure of cell viability in MTT assay was determined spectrophotometrically at 540 nm using a Bio-Rad Model 7550 enzyme immunoassay plate reader.

Uptake of doxorubicin by MCF-7 cells. MCF-7 cells were incubated with 10 μM [^{14}C]doxorubicin for 3 hr at 37° in the presence or absence of 250 U/mL P450red and 1 mM NADPH. The cells used to determine background doxorubicin uptake were exposed for 2 min at 4° on the culture medium containing the labelled drug. After incubation, cells were rinsed several times with (phosphate-buffered saline) PBS. Triplicate cell suspensions (about 2×10^6 cells each) were finally spun down, resuspended in 0.3 mL of 0.5 M perchloric acid, 2.2% (w/v) sodium pyrophosphate and hydrolysed at 70° for 1 hr. The hydrolysates were mixed with Triton-based scintillation fluid and were counted in a Packard TRI-CARB liquid scintillation analyser.

HPLC of doxorubicin metabolites. MCF-7 cells (2×10^6) were treated with 10 μM drug in the presence or absence of 250 U/mL P450red and 1 mM NADPH for 3 hr at 37°. The cells were harvested, washed thoroughly with PBS and then resuspended in 0.5 mL of 10 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA and, 0.15 M KCl prior to sonication on ice for 3 × 3 sec. Cell debris was removed by centrifugation and the resulting supernatants extracted with 5 mL of chloroform: propan-2-ol (2:1). The organic phase was then evaporated and redissolved in 0.2 mL of 50% (v/v) MeOH in water. Culture media from incubated cells were submitted to HPLC without additional preparations. Analysis of doxorubicin metabolites by HPLC was performed using a Hewlett–Packard Model 1090 essentially as described by Cummings [20]. A μ-Bondapak C_{18} (Waters) column with a mobile phase of 5 mM phosphoric acid, pH 3, in propan-2-ol (76:24) was used. Elution was isocratic at a flow rate of 1 mL/min. Peaks were detected by fluorescence using 480 nm (excitation) and 560 nm (emission). In addition, a multi-diode array spectrophotometric detector was used to identify metabolites with different absorbance characteristics to the parent drug.

Reduction of doxorubicin by P450red in cell-free system. Measurements of the rate of doxorubicin reduction by P450red in cell-free systems were carried out essentially under the same conditions as those used in the cytotoxicity assays. A mixture consisting of different concentrations of doxorubicin, 0.5 mM NADPH and 250 U P450red in 1 mL PBS was incubated in a quartz cuvette at 37°. The reduction of doxorubicin was followed spectrophotometrically (Shimadzu Model MPS-2000) at 480 nm and the oxidation of NADPH, measured in parallel samples, at 340 nm. The decrease of absorption at both wavelengths was linear for at least 15 min.

Lipid peroxidation. Replicate samples containing $10^6–10^7$ MCF-7 cells suspended in a final volume of 1 mL of RPMI 1640 medium without Phenol red were used. The cells were mixed with appropriate concentrations of doxorubicin in the presence or absence of NADPH (1 mM) and 250 U P450red and incubated at 37° for 3 hr. Lipid peroxidation was assessed by measurement of malondialdehyde formation using the modified method of Gavino *et al.* [21]. Cell suspensions were mixed with 0.5 mL 20% trichloroacetic acid, followed by the addition of 1 mL of 0.67% (w/v) thiobarbituric acid supplemented with 0.05 mg/mL butylated hydroxytoluene and heated for 30 min in a boiling water bath. The mixtures were then centrifuged to remove particulate material and the absorbance of the supernatants measured at 535 nm. Malondaldehyde concentration was quantitated using a standard curve generated with 1,1,3,3,-tetraethoxypropane.

Protein and DNA binding. MCF-7 cells (approximately 4×10^7) were incubated with [^{14}C]doxorubicin for 3 hr at 37° in the presence or absence of 250 U/mL P450red and 1 mM NADPH. The cells were harvested, rinsed thoroughly with PBS and resuspended in 2 mL of a solution containing 0.5% sodium dodecyl sulphate (SDS), 150 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 8.0. This solution was then treated with ribonuclease A (10 μg/mL) for 1 hr at 37°. The cell lysates were extracted with 2 mL of chloroform: isoamyl alcohol (24:1) and the layers were separated by centrifugation. In order to measure protein-bound doxorubicin, the interphase from the chloroform: isoamyl alcohol extraction was extracted three times with 5 mL of the following sequence of solvents: twice EtOH, 70% EtOH, acetone:chloroform (4:1), acetone:EtOH (5:1), ethylacetate:EtOH (5:1) and *n*-butanol to remove unbound drug. The final protein pellet was dissolved in 1 mL of 0.1 M NaOH and protein concentration and radioactivity in this solution were measured.

The aqueous, DNA-containing phase was treated with 0.25 mg/mL proteinase K for 4 hr at 37°, then DNA was isolated by a phenol extraction procedure. DNA (about 50 μg per sample) was digested with 2.5 U of DNase I, 1.23 mU of spleen exonuclease (PD II), 0.06 U of snake venom exonuclease (PD I) and 2.5 U of alkaline phosphatase in 40 mM Tris buffer, pH 7.4, 10 mM $MgCl_2$ for 2 hr at 37°. Enzymes were removed by passing the digests through Amicon YMT-30 filters. Nucleosides were then separated by HPLC on Spherisorb 5 μM ODS 4.6 × 250 mm

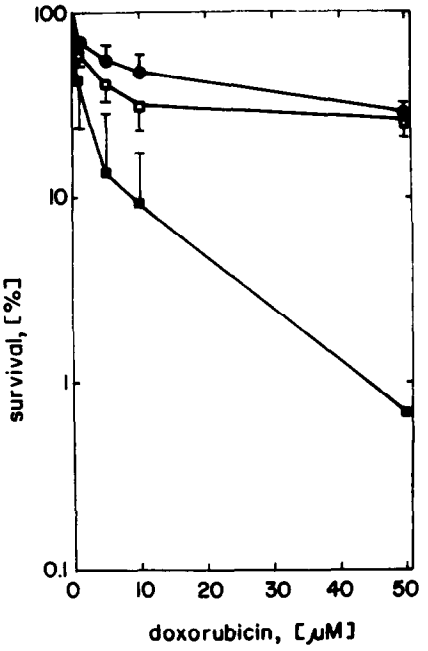

Fig. 1. Effect of P450red on doxorubicin toxicity. MCF-7 cells were treated with either the drug alone (□) or together with 250 U/mL P450red and 1 mM NADPH (■) or with the drug incubated for 1 hr with 2500 U/mL P450red and 1 mM NADPH prior to cell treatment (●). The results are means ± SD of 3–10 independent experiments carried out in triplicate. Other details are given in the Materials and Methods.

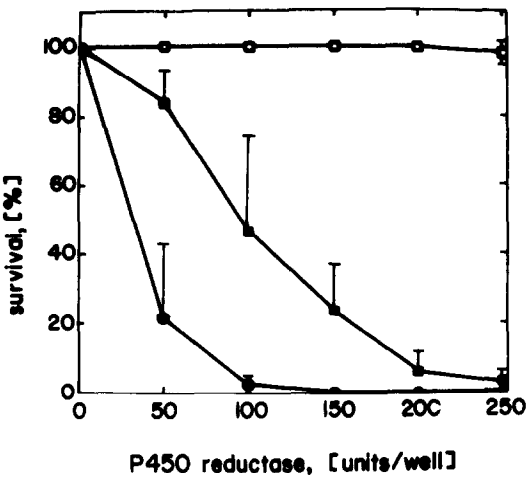

Fig. 2 Effect of P450red concentration on doxorubicin cytotoxicity. MCF-7 cells were treated with 1 μM (□), 10 μM (■) or 50 μM (●) doxorubicin for 3 hr in the presence of NADPH (1 mM) and indicated doses of P450red. The results are means ± SD of 3 independent experiments carried out in triplicate. Percentage survival is calculated relatively to cultures incubated in the presence of doxorubicin alone. The survival at these doses being 59.2 ± 7.3, 31.2 ± 8.1 and 25.5 ± 4.9%, respectively.

column (Jones Chromatography Ltd). Elution was performed with a gradient of MeOH in 50 mM sodium phosphate, pH 3.5. The MeOH gradient was as follows: 10% for the initial 10 min, increasing to 75% in 20 min, maintained 75% for the next 10 min, decreased to 10% in 5 min and 10% for the final 15 min of the run. The amount of nucleosides injected on the column was monitored with a UV detector. For the detection of doxorubicin–nucleoside adducts 2-mL fractions were collected from each elution, mixed with Opti-Fluor high flash-point liquid scintillation cocktail (Packard) and counted in a Packard TRI-CARB liquid scintillation analyser.

RESULTS

Influence of P450red on doxorubicin toxicity in MCF-7 cells

Treatment of MCF-7 cells with doxorubicin in the presence of exogenously added purified rat P450red resulted in a marked enhancement of drug cytotoxicity (Fig. 1). Neither P450red nor NADPH alone affected the drug potency (data not shown). The loss of cell viability due to the enzyme and the cofactor added together varied from experiment to experiment but usually did not exceed 10%. When the drug was incubated with P450red for 1 hr at 37° prior to application to the cells, no enhancement of cell killing was seen. Indeed, toxicity was slightly reduced relative to incubations with doxorubicin alone (Fig. 1). The potentiation of the cytotoxicity was dependent on both drug dose and the amount of P450red (Fig. 2). Below a doxorubicin concentration of 1 μM and a reductase concentration of 250 U/mL, no significant effect on cell viability could be demonstrated.

In the course of the above experiments, we noticed that in cultures in which the enhancement of cytotoxicity was demonstrated, a change of the colour of the culture medium had occurred from bright orange due to doxorubicin to purple red. The HPLC analysis demonstrated that in the absence of P450red the culture medium contained mainly the intact drug (Fig. 3A). However, when the enzyme was present, a metabolite was detected with virtually the same retention time as the parent compound but displaying a distinct absorption spectrum from doxorubicin with much reduced absorption in the visible region (Fig. 3B). The identity of the observed species is unknown.

In order to establish whether the enhancement of doxorubicin cytotoxicity in the presence of P450red was due to damage to cellular membranes, we examined the intracellular drug concentration as well as metabolite composition. The possibility of P450red being taken up by the cells was ruled out as Western blot analysis showed that in cell lysates no rat protein could be detected and they contained only human protein cross-reacting with anti-rat antibody (data not shown). Intracellular concentration of doxorubicin, measured using an isotopically labelled form of the drug, did not seem to be influenced by the presence of the enzyme. For

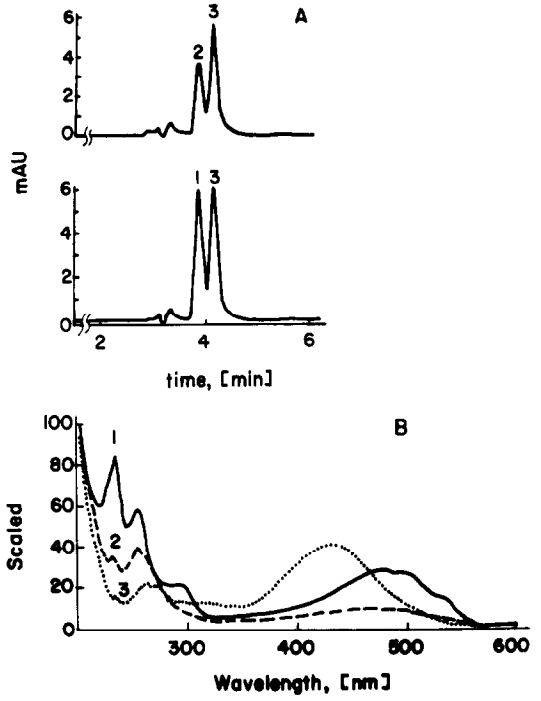

Fig. 3. Chromatograms of culture media taken from MCF-7 cells after 3 hr incubation with doxorubicin (10 μM). Panel A: bottom chromatogram, cells treated with the drug alone; top chromatogram, cells treated with the drug in the presence of P450red (250 U/mL) and NADPH (1 mM). Panel B: spectra of the detected peaks recorded with a multi-diode array spectrophotometric detector. Peaks 1 and 2 correspond to intact doxorubicin (solid line) and its presumptive metabolite (broken line), respectively. Peak 3 is Phenol red (dotted line), a dye present in culture medium.

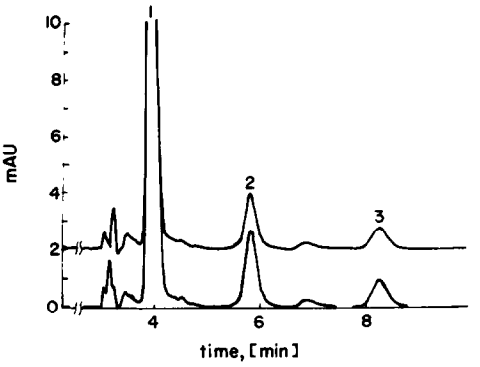

Fig. 4. HPLC separation of doxorubicin metabolites extracted from MCF-7 cells. The cells were treated for 3 hr with 10 μM doxorubicin alone (bottom chromatogram) or in the presence of 250 U/mL P450red and 1 mM NADPH (top chromatogram). Peak 1 corresponds to intact drug, peaks 2 and 3 to drug-related metabolites.

Table 1. Comparison of reduction of doxorubicin by P450red and NADPH consumption in cell-free system

DOX(μM)	DOX reduction (nmol/min)	NADPH consumption (μmol/min)
0	—	0.23 ± 0.11
1.0	NO	0.95 ± 0.23
10	0.65 ± 0.06	3.35 ± 1.77
50	12.33 ± 2.81	14.85 ± 8.02

Doxorubicin was incubated with P450red (250 U/mL) and NADPH (0.5 mM) at 37°. The decrease in absorbance was followed spectrophotometrically at 480 nm to determine the rate of doxorubicin reduction or at 340 nm to measure NADPH consumption. Extinction coefficients of 11,030 for doxorubicin and 6200 for NADPH were used in calculations. These rates were not corrected for the small absorption of the reaction product at 480 nm.

Results are means ± SD of 3 experiments.

NO, not observed within 0.5 hr incubation at 37°.

10 μM doxorubicin it amounted to 0.072 ± 0.013 and 0.085 ± 0.045 nmol/10^6 cells in incubations with and without P450red, respectively. The doxorubicin related compounds extracted from cell lysates were analysed using HPLC. Chromatographic profiles obtained were similar for the cells incubated with or without P450red and exhibited three main peaks (Fig. 4). The peak designated 1, constituting about 75% of total, was identified as intact doxorubicin. Metabolites eluting in peaks 2 and 3 displayed spectral properties characteristic for doxorubicin chromophore (data not shown). Their retention times indicate that they are more hydrophobic than the parent drug and probably correspond to doxorubicin 7-hydroxyaglycone and doxorubicin 7-deoxyaglycone. These metabolites were not detected in culture medium and may suggest that they were either formed intracellularly or are not found in the medium due to their dilution.

Reduction of doxorubicin by P450red in vitro

The potentiation of doxorubicin cytotoxicity in the presence of P450red could be explained by the generation of oxygen free radicals due to redox cycling, by alkylation of cellular macromolecules by drug metabolites or by a combination of both of these mechanisms. In order to establish the participation of these reaction pathways we carried out incubations of doxorubicin with P450red and NADPH *in vitro* and compared doxorubicin-induced NADPH oxidation with doxorubicin reduction monitored at 480 nm (Table 1). The incubation conditions were similar to those used in cytotoxicity tests. At a drug concentration of 1 μM, a concentration at which no reductase-mediated potentiation of toxicity was seen, no doxorubicin reduction, monitored at 480 nm, was seen. At higher doses, metabolism occurred which increased with increasing doxorubicin concentration. NADPH consumption was observed in all incubations, including those which did not contain doxorubicin

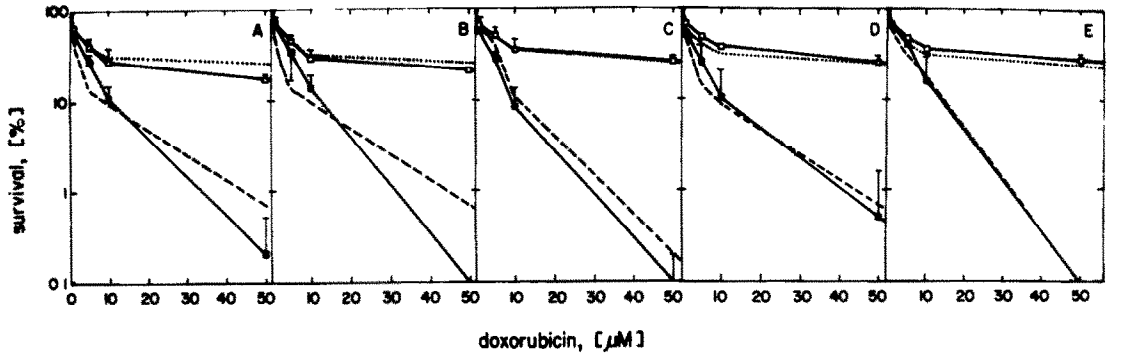

Fig. 5. Effect of free radical scavengers and antioxidants on P450red-mediated cytotoxicity of doxorubicin; (A) cells preincubated with 25 μM α-tocopherol before drug treatment; (B) 25 μM α-tocopherol present during treatment; (C) 50 μg/mL superoxide dismutase; (D) 1 mM mannitol; (E) 1 mM 5,5-dimethyl-1-pyrrolidine *N*-oxide. Survival was determined by MTT assay after 3 hr exposure of MCF-7 cells on doxorubicin and a free radical scavenger in the presence (■) or absence (□) of P450red (250 U/mL), NADPH (1 mM) as described in the Materials and Methods. Cells treated with the enzyme, NADPH and the scavengers were used as a control. The data presented are means ± SD of 3 independent experiments. Survival curves obtained in parallel experiments for doxorubicin treatments with (broken line) and without (dotted line) P450red in the absence of scavengers are given for comparison.

(Table 1). The rate of NADPH oxidation increased with drug dose but was not linearly correlated with it. Since doxorubicin metabolism as well as redox cycling (indicated by excessive NADPH consumption) took place concurrently at drug concentrations where P450red-mediated enhancement of cell killing was observed, both of the mechanisms described above could play a role in doxorubicin toxicity.

Effect of free radical scavengers on the enhancement of doxorubicin toxicity by P450red

In order to determine the role of redox cycling in the enhancement of doxorubicin cytotoxicity by P450red, we investigated the effects of a variety of oxygen free radical scavengers. The compounds chosen were: α-tocopherol, a naturally occurring antioxidant which inhibits the formation of lipid hydroperoxides and has been shown to reduce doxorubicin-induced lipid peroxidation *in vitro* [22]; superoxide dismutase, which can prevent the formation of hydroxyl radicals and has been reported to protect MCF-7 cells against doxorubicin toxicity [23]; mannitol, a hydroxyl radical specific scavenger which inhibits doxorubicin-mediated *in vitro* strand scission of plasmid pBR322 DNA [24] and finally 5,5-dimethyl-1-pyrrolidine *N*-oxide (DMPO), a spin trap forming stable adducts with hydroxyl free radicals which does not bind the doxorubicin semiquinone free radical [25].

Survival curves for all these agents were determined for MCF-7 cells in the absence of P450red and in cultures containing the enzyme and NADPH (Fig. 5). None of the oxygen radical scavengers used prevented the potentiation of doxorubicin cytotoxicity by P450red. They also had no effect on drug-induced cell killing in the absence of the enzyme.

In contrast, the addition of glutathione to the incubations was very effective in protection of MCF-7 cells against P450red-mediated toxicity of doxorubicin (Fig. 6). The gluthathione concentrations were 1 mM (Fig. 6A) and 10 mM (Fig. 6B) which are similar to physiological levels. The protection was observed over a wide range of drug doses and was more effective at the higher glutathione concentration. Interestingly, in the absence of P450red, glutathione was ineffective in preventing cell killing by doxorubicin. Indeed, a slight increase in drug cytotoxicity was reproducibly observed.

Damage to cellular components

Another approach aimed at establishing the role of reactive oxygen species in toxicity of doxorubicin involved measuring the levels of malondialdehyde, a product of lipid peroxidation. The slight elevation in the production of this compound in MCF-7 cells was brought about by both the addition of the drug or P450red and NADPH (Fig. 7). These levels seemed to increase further when the cells were incubated with doxorubicin in the presence of the enzyme, however, the increases were not statistically significant. It is worthy of note that the concentrations of malondialdehyde did not exceed 0.25 nmol/10^7 cells (which was at the limit of detection) even for the drug doses inducing 100% cell kill (50 μM). The highest value obtained was about half the malondialdehyde concentration detected in MCF-7 cells treated with 0.5 mM cumene peroxide and 1 mM $FeCl_2$, a lipid peroxidation promoting system used in these experiments as a positive control.

In view of the lack of convincing evidence that P450red-mediated cytotoxicity of doxorubicin involves oxygen free radicals, we also conducted some preliminary studies to examine whether doxorubicin was metabolized to a product(s) which binds covalently to cellular proteins and DNA. The covalent binding to cellular proteins and DNA was assessed following incubation of MCF-7 cells with

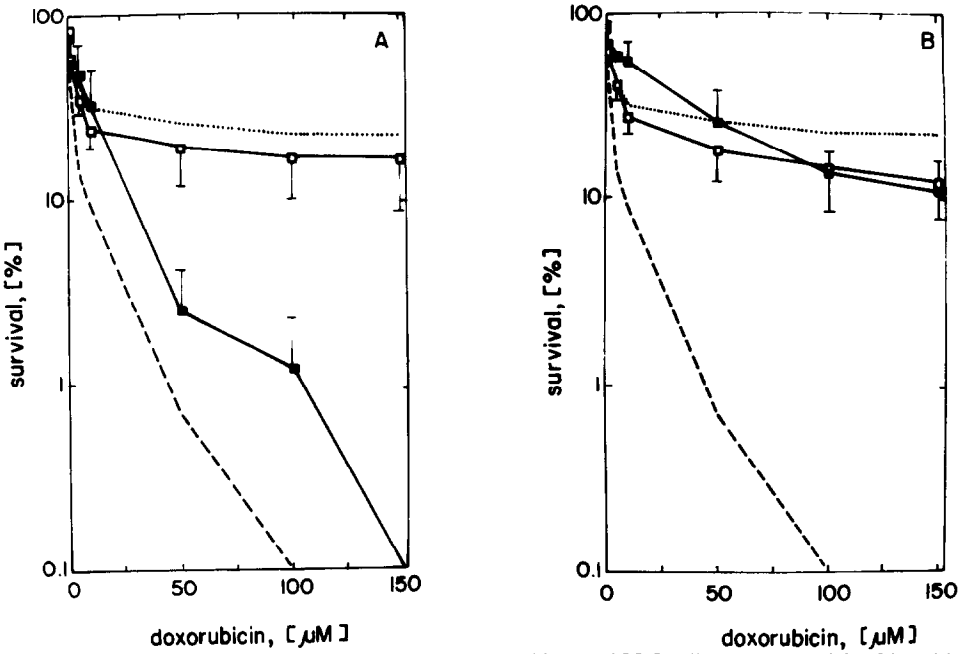

Fig. 6. Modulation of doxorubicin cytotoxicity by glutathione. MCF-7 cells were treated for 3 hr with the drug added together with glutathione in the presence (■) or absence (□) of P450red (250 U/mL) and NADPH (1 mM). In (A) the glutathione concentration was 1 mM and in (B) 10 mM. The results are means ± SD of 7 independent experiments carried out in triplicate. Survival curves obtained for doxorubicin treatments with (broken line) and without (dotted line) P450red in the absence of glutathione are given for comparison.

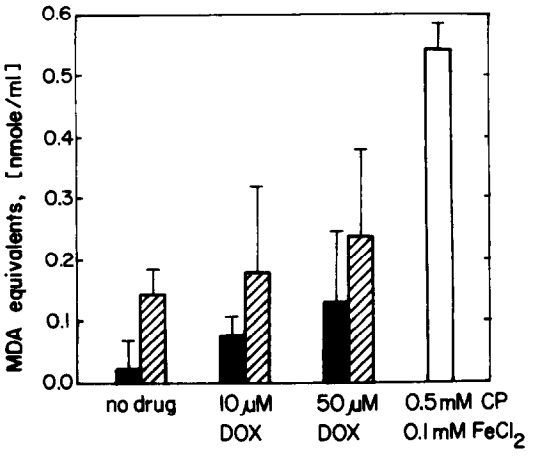

Fig. 7. Effect of P450red on doxorubicin (DOX)-mediated lipid peroxidation in MCF-7 cells. The cells were exposed to the drug for 3 hr in the presence (▨) or absence (■) of the enzyme then lipid peroxidation was assessed by the thiobarbituric assay for malondialdehyde (MDA). The data obtained for MCF-7 cells treated with 0.5 mM cumene peroxide (CP), 1 mM $FeCl_2$ are given for comparison (□). The results are means ± SD of 3 independent experiments carried out in duplicate. Student's *t*-test analysis showed that means of MDA equivalents in cell suspensions incubated with or without P450red were statistically different at levels of significance $P \leq 0.001$ in the samples where no drug was added, $P \leq 0.11$ and $P \leq 0.18$ in samples exposed to 10 and 50 μM doxorubicin, respectively.

the isotopically labelled drug in the presence and absence of the enzyme. The radioactivity associated with proteins was determined after exhaustive organic solvent extraction as described in Materials and Methods. The levels of irreversible, probably covalent binding in incubations with no enzyme added were 0.19 and 1.43 ± 0.46 nmol/mg protein for 10 and 50 μM doxorubicin, respectively. These values rose substantially when P450red was included in incubations and reached the level of 0.36 nmol/mg protein for 10 μM and 4.41 ± 0.95 nmol/mg protein for 50 μM doxorubicin. The above data are results of a single experiment in the case of 10 μM and a mean ± SD of three experiments in the case of 50 μM drug.

The determination of doxorubicin covalent binding to DNA is difficult due to drug intercalation. To avoid this problem, we developed a method for measuring drug–nucleoside adducts following DNA digestion and HPLC separation. Since very little is known about stability of such adducts, the conditions of digestion and HPLC resolution were kept as mild as possible (see Materials and Methods for details). Figure 8 shows the distribution of radioactivity in fractions obtained from MCF-7 cells treated with [^{14}C]doxorubicin with or without P450red. In samples obtained from the cells treated with the drug alone only background levels of the label were detected. In contrast, HPLC resolution of digested DNAs from cells exposed to doxorubicin together with P450red resulted in a peak of radioactivity equivalent to about 0.19 nmoles of the drug per 50 μg DNA. The retention time of the peak corresponds to the

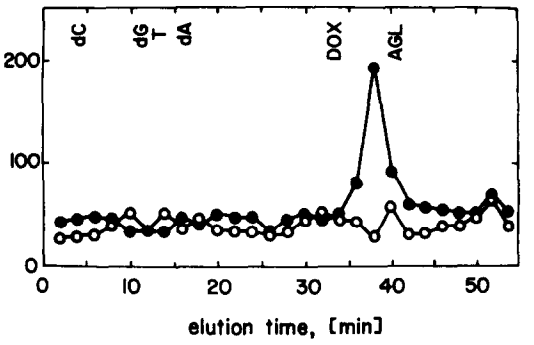

Fig. 8. HPLC separation of doxorubicin–DNA adducts. MCF-7 cells were treated with the isotopically labelled drug (50 μM) for 3 hr in the presence (●) or absence (○) of P450red (250 U/mL) and NADPH (1 mM). After incubation, DNAs were isolated from the cells, digested to nucleosides which were then resolved by HPLC as described in Materials and Methods. For the detection of adducts, 2-mL fractions of the eluates were collected and analysed by liquid scintillation counting. The elution times of the four normal deoxyribonucleosides (dC, dG, T and dA) as well as the expected positions of doxorubicin (DOX) and doxorubicin 7-deoxyaglycone (AGL) are indicated. The points represent means of 3 measurements.

elution position of doxorubicin-related species (Fig. 8), and therefore may represent drug–DNA adducts. Although this interpretation must be taken with caution, it is difficult to explain the observed higher level of radioactivity associated with cellular DNA obtained from incubations containing P450red unless there is an increase in alkylation.

DISCUSSION

NADPH cytochrome P450 reductase catalyses one-electron reduction of quinones and has been shown to reduce doxorubicin to its semiquinone free radical form *in vitro* [3, 16, 26]. A principal object of this work has been to establish whether this metabolic pathway influences the cytotoxicity of this drug towards tumor cells. We demonstrated that treatment of MCF-7 cells with doxorubicin in the presence of exogenous P450red and NADPH significantly increased drug cytotoxicity and that the cytotoxic product was a short-lived species.

The drug reduction seemed to take place exclusively outside the cells. Although this could be considered an artificial system, it has been shown that the doxorubicin semiquinone free radical generated intracellularly partitions freely between intra- and extracellular compartments and is found predominantly extracellularly [25, 27]. Closer examination of doxorubicin metabolism by P450red identified a reaction product which had the same retention time on HPLC as the parent compound (Fig. 3A). This suggests that it was of similar chemical structure to doxorubicin and probably still contained the sugar moiety. The identity of this metabolite is unknown. To our knowledge, the production of this metabolite has only been reported once previously [28] in incubations containing purified NADPH cytochrome *c*-(ferrodoxin)oxidoreductase from *Euglena gracilis*. The authors proposed that the product formed was doxorubicin hydroquinone. This does not, however, appear to be the case because the product does not display the characteristic hydroquinone absorption maximum at 410–430 nm [29], also the retention time of the hydroquinone on HPLC under the conditions used is different to that of the doxorubicin [30].

The metabolite formed by P450red during incubation of MCF-7 cells with doxorubicin was stable in culture medium and could be detected after several weeks of storage at −20°. It cannot therefore represent the ultimate toxic species which is short-lived. The stable doxorubicin metabolite could not be detected intracellularly and probably undergoes further metabolism.

Studies to establish the mechanism of reductase-mediated doxorubicin toxicity demonstrated that this was not due to elevated drug accumulation. The increase in cell killing was clearly related to doxorubicin metabolism and depended on the amount of reductase used.

Although under the treatment conditions used in MTT assays, P450red promoted doxorubicin-mediated redox cycling, we failed to obtain evidence that oxygen radicals were part of the mechanism of cell death. Most notably, superoxide dismutase which prevents the generation of the hydroxyl radicals, the most damaging active oxygen species [25] did not provide any protection. In addition, no significant increase in lipid peroxidation, another oxygen radical-mediated reaction, was observed in these experiments. It is important to note that MCF-7 cells appear to be resistant to the oxygen radicals generated by P450red in the absence of the drug, however Chinese hamster ovary cells appear to be very sensitive to reductase-mediated attack [31]. This indicates that the mechanism of doxorubicin cell killing may well depend on the properties of the target cell.

Interestingly, glutathione which is known to affect the efficacy of a variety of antineoplastic agents, protected MCF-7 cells very effectively against doxorubicin activated by P450red. As discussed above, this protection does not appear to be due to the neutralization of oxygen radicals and may indicate a direct conjugation of glutathione with the activated drug.

The above discussion, together with the observation that doxorubicin cytotoxicity was only enhanced at concentrations where doxorubicin metabolism (distinct from redox cycling) was also measured, indicates the toxic mechanism involves covalent binding of doxorubicin metabolites to cellular macromolecules. Support for this possibility comes from recent studies which demonstrated the covalent modification of oligonucleotides by doxorubicin activated *in vitro* by P450red [32]. Our preliminary evidence for irreversible, probably covalent, drug binding of doxorubicin to cellular proteins and DNA also supports this toxic mechanism (see also Ref. 33).

In a number of reports published to date, it has been proposed that the cytotoxic activity of

doxorubicin and other quinone-containing compounds stems from their ability to exchange electrons with molecular oxygen and thereby to promote generation of reactive oxygen free radicals. However, some very recent studies carried out using doxorubicin-resistant tumor cell lines do not support the above hypothesis. For example, it has been shown that MCF-7 cells, 500-fold resistant to the drug, exhibited only 4-fold resistance to superoxide- and hydrogen peroxide-generating systems [34]. Also, no significant difference in radical production by MCF-7 or Chinese hamster ovary cells sensitive and resistant to doxorubicin could be measured [25]. In a further study, comparison of another class of quinone containing compounds, the naphthoquinones, demonstrated that their cytotoxic activity was correlated with their ability to arylate nucleophiles rather than with redox cycling [35].

In this study, we have been unable to demonstrate a major role for doxorubicin-mediated generation of oxygen free radicals in the toxic pathway. Moreover, our data provide preliminary indication that P450red, the enzyme most frequently implicated in the above process, may actually be involved in the activation of doxorubicin to alkylating species, however, more work is needed to substantiate this conclusion. It is important to note, however, that the relative contribution of different pathways, e.g. topoisomerase II inhibition, covalent binding, redox cycling etc., in the mechanism of action of this compound may well depend on the properties of target cells.

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