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THE ENZYMOLOGY OF DOXORUBICIN QUINONE REDUCTION IN TUMOUR TISSUE

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Abstract—We have reported previously that enzymes present in the Sp 107 rat mammary carcinoma catalyse doxorubicin quinone reduction (QR) to 7-deoxyaglycone metabolites *in vivo* [Willmott and Cummings, *Biochem Pharmacol* **36**: 521–526, 1987]. In order to provide insights into the role of QR in the antitumour mechanism of action of doxorubicin, we have attempted in this work to identify the enzyme(s) responsible. NAD(P)H: (quinone acceptor) oxidoreductase (DT-diaphorase) was the major quinone reductase in the tumour accounting for approximately 70% of all the activity measured in microsomes and cytosols (microsomal activity, 28.4 ± 4.6 nmol/min/mg; cytosolic activity, 94.3 ± 11.9 nmol/min/mg). Its presence was confirmed by western blot analysis. Low levels of NADH cytochrome b_5 reductase (15.6 ± 6.3 nmol/min/mg) and NADPH cytochrome P450 reductase (14.5 ± 4.0 nmol/min/mg) were detectable in microsomes. The presence of the latter was confirmed by western blot analysis. Pretreatment of tumours with doxorubicin (48 hr) at a therapeutic dose decreased the level of activity of all the reductases studied by at least 2-fold ($P < 0.01$, Student's *t*-test). Doxorubicin was shown not to be a substrate for purified rat Walker 256 tumour DT-diaphorase with either NADH or NADPH as co-factor and utilizing up to 20,000 units of enzyme/incubation but was confirmed to be a substrate for purified rat liver cytochrome P450 reductase. 7-Deoxyaglycone metabolite formation by purified cytochrome P450 reductase had an absolute requirement for NADPH as co-factor, was inhibited by molecular oxygen and dicoumarol (IC_{50} approx. 50 μM), and modulated by specific reductase antiserum. Reductive deglycoslation of doxorubicin to 7-deoxyaglycones was localized to the microsomal fraction of the Sp 107 tumour, with negligible activity being found in cytosols (NADH, NADPH and hypoxanthine as co-factors) and mitochondria (NADH and NADPH). The tumour microsomal enzyme had an absolute co-factor requirement for NADPH, was inhibited by oxygen and dicoumarol, and modulated by cytochrome P450 reductase antiserum. These data indicate strongly that NADPH cytochrome P450 reductase is the principal enzyme responsible for catalysing doxorubicin QR in the Sp 107 tumour.

Quinone reduction (QR¶) and the subsequent generation of drug free radicals and reactive oxygen species (ROS) have been implicated in the cytotoxic mechanism of action of doxorubicin since as early as 1975 [1]. For even longer, reductive metabolism has been connected with drug glycosidic cleavage and production of a 7-deoxyaglycone metabolite [2], which is now generally considered to be a pathway of drug inactivation [3]. Despite almost 20 years of ensuing intensive investigation an involvement for free radicals in the *in vivo* antitumour activity of doxorubicin remains controversial. Those who argue for and against a role can both cite a large body of experimental results in support of their case. These data have been marshalled elegantly in recent reviews [4, 5].

It is now clear that whether QR is an activating process or a pathway of drug inactivation will depend on both a series of physicochemical and biochemical factors, and the local physiological conditions that prevail in the target tissue, the tumour [6]. As a consequence, cell lines utilized as *in vitro* models to study the role of free radicals in the mechanism of action of doxorubicin have been criticised as not being representative of the *in vivo* situation [4]. Reductive deglycosylation of doxorubicin *in vitro* requires anaerobic incubations [7] and presumably has the same dependency *in vivo*. Due to their short biological half-lives, the free radical species generated in aerobic incubations (particularly the hydroxyl radical) would have to be produced in a site-specific manner to ultimately damage DNA and result in cell death [8]. However, there is little evidence to suggest that this actually happens *in vivo*. In addition, cells are equipped with an array of "scavenger" molecules and enzymes to protect against free radical-induced toxicity, so that the net effect of a ROS insult will depend not only on the rate of doxorubicin bioreduction but also on the levels of detoxification pathways. Two electron reduction could theoretically circumvent oxidative stress to the cell and inactivate the drug, analogous to the metabolism of many other quinone-containing

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¶ Abbreviations: QR, quinone reduction; ROS, reactive oxygen species; BSA, bovine serum albumin; i.t., intratumoural.

xenobiotics such as menadione [9]. However, two electron reduction has also been proposed to activate doxorubicin to a quinone methide aglycone intermediate formed after the fully reduced hydroquinone rearranges chemically [10]. The quinone methide aglycone is sufficiently long lived to diffuse through the cell and alkylate DNA and opens up the prospect of another mechanism of action derived from bioreduction, namely drug–DNA covalent adducts [11]. The aerobic phase and the anaerobic phase of doxorubicin bioreduction are not necessarily mutually exclusive and can function in a sequential manner [12]. One electron reduction first produces ROS by redox cycling without consumption of doxorubicin; as oxygen is exhausted bioreduction continues and results ultimately in 7-deoxyglycone metabolite formation.

In a sister paper [13], we have investigated the occurrence of the three major consequences of doxorubicin quinone reduction *in vivo* in tumour tissue: ROS generation as lipid peroxidation, drug–DNA covalent adducts by ^{32}P-post-labelling and 7-deoxyaglycone metabolite formation by HPLC. Here, the final aim was to establish whether or not there was a role for bioreduction in the drug's mechanism of action. The model used was a subcutaneously growing rat mammary carcinoma termed Sp 107 chosen in order to reflect more accurately physiological conditions which may exist in human tumours. Doxorubicin was administered by intratumoural (i.t.) injection either as free drug or incorporated in albumin microspheres which appeared to enhance active drug QR by a factor of up to 155-fold. The study arrived at two main conclusions: (a) that the major consequence of doxorubicin QR in tumour tissue *in vivo* is 7-deoxyaglycone formation without concomitant lipid peroxidation or increased formation of drug–DNA adducts, and (b) QR is not involved in antitumour activity. The validity of these conclusions, however, depends on the enzymology of doxorubicin QR in the Sp 107 both qualitatively and quantitatively. If, for instance, DT-diaphorase is catalysing the reaction, then ROS generation may well be circumvented. Alternatively, the level of quinone reductase activity may be too low to result in a significant production of free radical species. Therefore, in this paper we have attempted to define the enzymology of doxorubicin QR in the Sp107 tumour. Three approaches have been adopted to identify the enzymes involved and characterize their properties: (1) measure levels of activity and expression of the major quinone reductases in the tumour; (2) incubate doxorubicin with purified forms of these enzymes and examine their propensity to metabolize the drug, their co-factor requirements, and the effects of oxygen and specific inhibitors; and (3) fractionate the Sp 107 tumour into subcellular components, incubate these with doxorubicin, and characterize their co-factor requirements, the effect of oxygen and the response to specific inhibitors.

MATERIALS AND METHODS

Animal model and drug treatments. The animal model used throughout was the WAB/NOT strain of rat and the syngeneic, undifferentiated mammary carcinoma Sp 107, a tumour that originally arose spontaneously in a female rat [14]. Tumours were transplanted s.c. into the flanks of animals and were allowed to grow up to 2.5–5 g prior to drug treatment and collection. Drug was administered i.t. either as free doxorubicin in solution (158 or 160 μg) or doxorubicin incorporated in albumin microspheres (160 or 185 μg). The method of preparation of microspheres was as described previously [15]. Tumours were collected 48 hr after drug treatment, at the point of maximum formation of 7-deoxyaglycone metabolites *in vivo* [16], and immediately frozen on solid CO_2. When tumours and livers were collected from controls they received no treatment.

Quinone reductase activity and expression in the Sp 107 rat mammary carcinoma. Cytosolic and microsomal fractions were prepared by differential ultracentrifugation [17]. NAD(P)H: (quinone acceptor) oxidoreductase (EC 1.6.99.2, DT-diaphorase) activity was determined spectrophotometrically by following the reduction of cytochrome *c* using a modification of the method of Ernster [18] as reported in detail elsewhere [19]. Essentially, the reaction mixture contained cytosolic (65–100 μg) or microsomal (17–33 μg) protein, cytochrome *c* (77 μM), bovine serum albumin (BSA) (0.14%, w/v), NADH or NADPH (2 mM) as co-factor, and menadione (20 μM) as the intermediate electron acceptor. Reactions were conducted at 37° in a total volume of 1 mL Tris–HCl buffer (pH 7.4) in the presence and absence of 10 μM dicoumarol. NADPH-cytochrome P450 reductase (EC 1.6.2.3) activity in tumour microsomes was measured using an assay based on the reduction of cytochrome *c* as described by Yasukochi and Masters [20]. Cytochrome b_5 reductase (EC 1.6.2.2) activity was calculated as the dicoumarol-insensitive microsomal cytochrome *c* reductase activity measured with NADH as co-factor. All enzyme activities are expressed as nmol cytochrome *c* reduced/min/mg protein.

The level of expression of cytochrome P450 reductase in tumour microsomes was measured by western blot analysis after SDS–PAGE using 7.5% polyacrylamide gels [21]. Western blots were developed with a polyclonal goat antibody to rat P450 reductase and a peroxidase-conjugated rabbit anti-goat IgG Vectastain colour detection kit (Vector Laboratories, Bretton, Peterborough, U.K.). The level of expression of DT-diaphorase was measured by western blot analysis after SDS–PAGE using either 12 or 15% acrylamide gels. Western blots were developed with a primary purified polyclonal rabbit antibody raised against purified rat Walker 256 tumour DT-diaphorase [22]. Autoradiograms were exposed at −70° for 1–7 days.

Measurement of doxorubicin anaerobic QR to a 7-deoxyaglycone metabolite by tumour sub-cellular fractions and purified quinone reductases. Cytochrome P450 reductase purified from rat liver microsomes by affinity chromatography was a gift from Dr Roland Wolf, Imperial Cancer Research Fund, Laboratory of Molecular Pharmacology and

Drug Metabolism, University of Edinburgh, U.K. One unit of its activity is defined as the amount of enzyme which converts 1 nmol of cytochrome *c* to its reduced form per minute at 37°. DT-diaphorase purified from Walker 256 tumour cells of the rat was a gift from Dr Richard J. Knox, Molecular Pharmacology Unit, Section of Drug Development, Institute of Cancer Research, Sutton, U.K. One unit of its activity is also defined as the amount of enzyme which converts 1 nmol of cytochrome *c* to its reduced form per minute at 37°. Rat liver microsomes were prepared by a standard differential centrifugation method in 0.25 M sucrose. Sub-cellular fractions for incubation with doxorubicin were prepared from the Sp 107 rat mammary carcinoma as follows. Whole tumours (6–10 g) were first washed in saline and then transferred to 0.25 M sucrose, 5 mM Tris buffer, 0.5 mM EDTA, pH 7.4 (STE) in which a homogenate was produced (33% w/v). Portions of this homogenate were either used directly in drug incubations or processed further to isolate mitochondrial, microsomal and cytosolic fractions. The mitochondrial fraction was produced after a low speed spin (600 *g* for 10 min at 4°) to remove cellular debris and a high speed spin at 32,000 *g* for 10 min at 4°. The pellet was resuspended in STE and subjected to a further low and high speed spin. Afterwards, the final pellet was resuspended in 15 mL of STE. Microsomes were isolated from the post-mitochondrial supernatant by centrifugation at 155,000 *g* for 1 hr at 4°. In this case, the pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.4 and centrifuged again at 155,000 *g*. The final pellet was resuspended in a small volume (5–7 mL) of 10 mM Tris–HCl buffer, pH 7.4 containing 20% glycerol and 0.1 M EDTA. The cytosolic fraction was produced from the post microsomal STE supernatant by centrifugation at 155,000 *g* for 4 hr at 4°. At the end of this run the supernatant (20–25 mL) was retained for drug incubations. The protein content of all fractions was determined by the Biuret method [23] using BSA as a standard and typical yields were: mitochondrial fractions, 5 mg protein/mL (final concentration); microsomes, 4–7 mg/mL; and cytosols, 11 mg/mL.

Drug incubations were performed at 37° in a final volume of 5 mL placed in 50 mL tapered test-tubes stoppered with rubber bungs. In order to maintain an anaerobic environment, a constant flow of helium gas was delivered to these tubes and allowed to vent off through an inlet and outlet tube placed in the rubber bung. Additions were made by simply unstoppering test-tubes. Each incubation mixture contained 10 μg/mL doxorubicin, 1 mL of tumour homogenate or sub-cellular fraction (or control rat liver microsomes), and 1.1 mM co-factor (NADPH, NADH or hypoxanthine) in 0.1 M sodium phosphate buffer, pH 7.4. Mixtures were helium sparged for 20 min prior to addition of drug which started the reaction. In incubations with purified enzymes, 1000 U of cytochrome P450 reductase and 1000–20,000 U of DT-diaphorase both in a small volume (0.15 mL) of sterile phosphate-buffered saline were used. In the case of enzyme inhibitors, both dicoumarol (1–200 μM, made up in a 50:50 mixture of 0.1 M sodium phosphate buffer:0.1 M sodium

Table 1. Quinone reductase activity (menadione as substrate) in sub-cellular fractions isolated from the Sp 107 rat mammary carcinoma

	Cytosolic		Microsomal				
			DT-diaphorase		Cytochrome P450 reductase	Cytochrome b_5 reductase
Pretreatment	DT-diaphorase	"Other"	NADH	NADPH	NADPH	NADH
None	94.25 ± 11.9*	27.1 ± 4.7	25.3 ± 3.2	28.4 ± 4.6	14.5 ± 4.1	15.6 ± 6.3
Doxorubicin	42.4 ± 4.0†	8.1 ± 0.8†	ND	ND	ND	ND
Doxorubicin-loaded microspheres	47.2 ± 5.9†	8.4 ± 1.0†	15.5 ± 4.2†	15.0 ± 1.9†	ND	ND

Cytosols and microsomes were isolated as described in Materials and Methods from control rats and animals pretreated for 48 hr with either i.t. free doxorubicin (150 μg) or i.t. doxorubicin loaded in albumin microspheres (180 μg). Enzyme activities were measured as described in Materials and Methods. Each value represents the mean ± SD from six separate tumours for the no pretreatment group and three separate tumours for the free doxorubicin and doxorubicin-loaded microsphere groups.

* All enzyme activities are in nmol/min/mg protein.

† $P < 0.01$, Student's *t*-test compared to no pretreatment.

ND, not detectable; activity had fallen to below the limit of detection of the assay method.

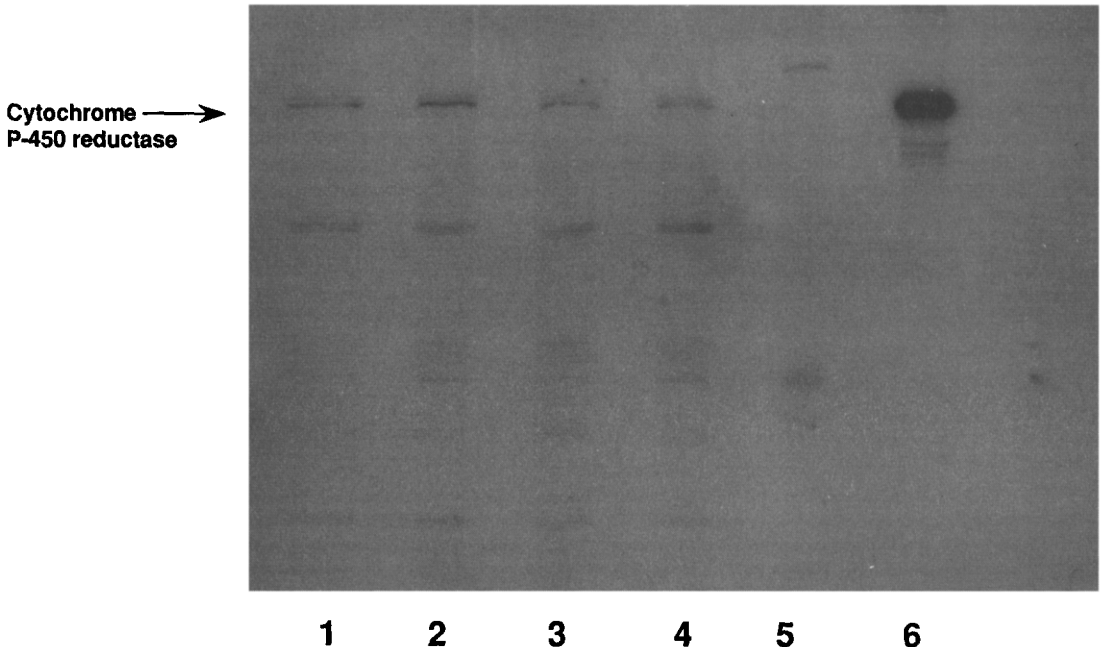

Fig. 1. Western blot analysis of the cytochrome P450 reductase content of the Sp 107 rat mammary carcinoma. Tumour microsomes were isolated as described in Materials and Methods from control rats or animals pretreated for 48 hr with either i.t. free doxorubicin (150 μg) or i.t. doxorubicin loaded in albumin microspheres (180 μg). In each case 25 μg was loaded on to a 7.5% SDS–PAGE gel for electrophoresis and the western blots were developed with a polyclonal goat antibody to rat liver cytochrome P450 reductase as described in the text, after a 2 week exposure. Lane 1, control tumour; lane 2, microsphere-pretreated tumour; lane 3, free drug-pretreated tumour; lane 4, free drug-pretreated tumour; lane 5, blank; and lane 6, 5 pmol purified rat liver cytochrome P450 reductase.

hydroxide) and cytochrome P450 reductase antiserum (10–200 μL) were added to anaerobic incubations for 30 min at 37° prior to starting the reaction with doxorubicin.

At various time points (normally 0, 15, 30, 45 and 60 min) an aliquot (100 μL) was withdrawn from an incubation and 20 μL was immediately subjected to HPLC to determine the concentration of doxorubicin and its metabolites: doxorubicin 7-deoxyaglycone and, when present, doxorubicinol 7-deoxyaglycone [24]. Reaction rates were calculated from linear or near-linear portions of reaction curves and are expressed as nmol/30 min/mg of protein or 1000 U of enzyme.

RESULTS

Quinone reductase activity and expression in the Sp 107 rat mammary carcinoma

The level of quinone reductase activity (menadione as substrate) determined in the microsomal and cytosolic fractions of the Sp 107 rat mammary carcinoma are shown in Table 1. For the cytosolic fractions enzymes grouped into the "other" category would include xanthine oxidase (EC 1.2.3.2) and aldo-keto reductase. Pretreatment of tumours with either free doxorubicin or doxorubicin incorporated in microspheres by i.t. injection 48 hr prior to collection resulted in at least a 2-fold reduction ($P < 0.01$, Student's t-test) in the activity of all the enzymes investigated consistent with previously published studies [25, 26]. DT-diaphorase accounted for almost 70% of all the quinone reductase activity measured in the two fractions. Western blot analysis confirmed the presence of both DT-diaphorase (data not shown) in the cytosolic fraction and cytochrome P450 reductase in the microsomal fraction (Fig. 1). The level of expression of cytochrome P450 reductase appeared to be unaltered by pretreatment with either free doxorubicin or doxorubicin incorporated in microspheres in contrast to its activity which decreased significantly to below the assay detection limit (Table 1).

Characteristics of doxorubicin bioreductive glycosidic cleavage to a 7-deoxyaglycone metabolite by liver microsomes and purified quinone reductases

The time course of doxorubicin biotransformation to 7-deoxyaglycone metabolites by anaerobic liver microsomes is illustrated in Fig. 2. These data confirm previous results that their formation is rapid (maximum conversion to doxorubicin 7-deoxyaglycone in under 15 min) and that they are produced in a linear sequential pathway where doxorubicin is first converted to doxorubicin 7-deoxyaglycone which is then converted to doxorubicinol 7-deoxyaglycone [27, 28]. Metabolite formation was dependent on the presence of a reduced co-factor (NADPH) and active microsomes, and was completely inhibited by molecular oxygen.

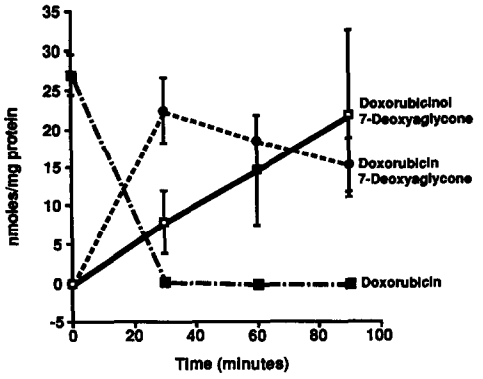

Fig. 2. Biotransformation of doxorubicin to 7-deoxyaglycone metabolites by anaerobic NADPH fortified rat liver microsomes. Incubations were performed as described in Materials and Methods. The starting concentration of doxorubicin was 10 μg/mL (approx. 20 μM). Concentrations of doxorubicin (—·—·—·—), doxorubicin 7-deoxyaglycone (----) and doxorubicinol 7-deoxyaglycone (———) were determined by HPLC at the various time points indicated and are expressed as nmol/mg of protein. Each value represents the mean ± SD from three separate experiments.

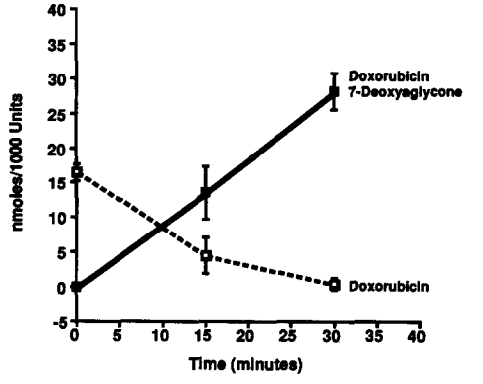

Fig. 3. Biotransformation of doxorubicin (----) to doxorubicin 7-deoxyaglycone (———) by anaerobic purified rat liver cytochrome P450 reductase (1000 U per incubation). Incubations were performed and concentrations determined as in Fig. 2. Results are expressed as nmol/1000 U and represent the means ± SD from five separate experiments.

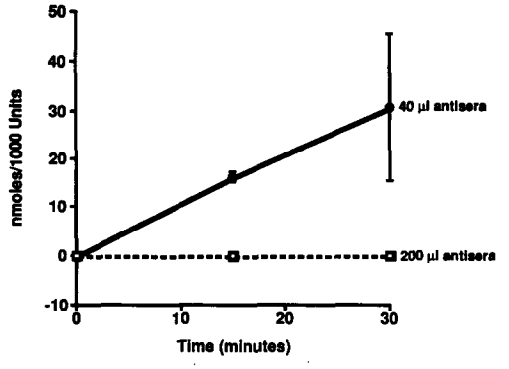

Fig. 4. Inhibition of anaerobic purified rat liver cytochrome P450 reductase (1000 U)-catalysed biotransformation of doxorubicin to doxorubicin 7-deoxyaglycone by goat antiserum to cytochrome P450 reductase. The following dilutions of antiserum were investigated: 5, 20, 40, 100 and 200 μL. For clarity the effect of only two dilutions of antiserum on 7-deoxyaglycone metabolite formation are shown: 40 (———) and 200 (----) μL. Results are expressed as nmol/1000 U and represent the means ± SD of two to three separate experiments.

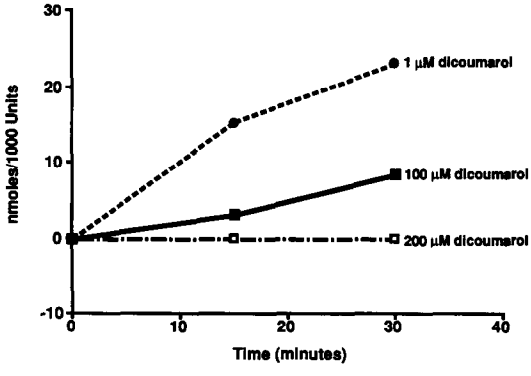

Fig. 5. Inhibition of anaerobic purified rat liver cytochrome P450 reductase (1000 U)-catalysed biotransformation of doxorubicin to doxorubicin 7-deoxyaglycone by dicoumarol. The following concentrations of inhibitor were investigated: 1, 10, 50, 100 and 200 μM. Incubations were performed and concentrations determined as in Fig. 2. For clarity the effect of only three dicoumarol concentrations on 7-deoxyaglycone metabolite formations are shown: 1 (----), 100 (———) and 200 (—·—·—·—) μM. Results are expressed as nmol/1000 U and represent the mean values from two separate experiments.

Biotransformation of doxorubicin by cytochrome P450 reductase is illustrated in Fig. 3. In this case, doxorubicin was converted almost stoichiometrically into doxorubicin 7-deoxyaglycone. The reaction was dependent on NADPH as co-factor as well as active enzyme, was inhibited by molecular oxygen and could be completely blocked by addition of an appropriate dilution of a polyclonal goat antiserum to cytochrome P450 reductase (see Fig. 4). In the antibody inhibition experiments it was also noted that at low dilutions (<40 μL) a slight but significant stimulation of glycosidase activity occurred. Cytochrome P450 reductase-catalysed formation of doxorubicin 7-deoxyaglycone was also inhibited by high concentrations of dicoumarol, a well known inhibitor of DT-diaphorase (see Fig. 5) [9]. In this study, the concentration of dicoumarol that produced 50% inhibition of metabolite formation was in the region of 50 μM. Doxorubicin did not act as a substrate for reductive deglycosylation with purified

Table 2. Characterization of doxorubicin reductive glycosidase activity in the Sp 107 rat mammary carcinoma

Tumour fraction	Doxorubicin 7-deoxyaglycone formation (nmol/30 min/mg protein)	Co-factor requirement	Effect of oxygen	Effect of 100 μM dicoumarol
Homogenate	0.067 ± 0.004*	NADPH +ve NADH −ve	Complete inhibition	
Microsomes	0.91 ± 0.36	NADPH +ve NADH −ve	Complete inhibition	Complete inhibition
Cytosol	0.00	NADPH −ve NADH −ve Hypoxanthine −ve		
Mitochondria	0.00	NADPH −ve NADH −ve		
Liver microsomes	22.8 ± 4.8	NADPH +ve	Complete inhibition	
Rat liver cytochrome P450 reductase (1000 U)	28.3 ± 2.6	NADPH +ve NADH −ve	Complete inhibition	74% inhibition
Rat tumour DT-diaphorase (20,000 U)	0.00	NADPH −ve NADH −ve		

The Sp 107 tumour was fractionated and all the anaerobic drug incubations were performed as described in Materials and Methods. Reductive glycosidase activity was calculated from linear or near-linear reaction curves.

* Each value represents the mean ± SD from three to six separate experiments.

rat Walker 256 tumour cell DT-diaphorase even when up to 20,000 U of active enzyme (determined by cytochrome *c* reduction) was incorporated in incubations and regardless of whether NADH or NADPH was the co-factor.

In vitro *characterization of doxorubicin bioreductive glycosidic cleavage to a 7-deoxyaglycone metabolite by the Sp 107 rat mammary carcinoma*

The characteristics of doxorubicin biotransformation by subcellular fractions isolated from the Sp 107 tumour are summarized in Table 2 and compared to the characteristics of purified enzymes. Glycosidase activity was associated exclusively with the microsomal fraction, cytosols and mitochondria being without detectable levels of activity. Microsomal metabolism had an absolute requirement for NADPH as co-factor, produced only doxorubicin 7-deoxyaglycone and was inhibited by oxygen and dicoumarol, and its activity was modulated by the antiserum to cytochrome P450 reductase. The low level of activity recorded by tumour microsomes is consistent with the low level of expression of the enzyme measured in the tumour.

DISCUSSION

The aim of this present study has been to identify the enzyme(s) catalysing doxorubicin biotransformation in the Sp 107 rat mammary carcinoma and use this information to provide insights into the role of QR in the drug's *in vivo* mechanism of action. In addition, experiments were conducted to identify an inhibitor of this reaction for evaluation of its effects on the drug's metabolism and antitumour activity *in vivo*. The results presented demonstrate that cytochrome P450 reductase is the principal, if not sole, enzyme responsible for the reductive metabolism of doxorubicin and that dicoumarol is an effective inhibitor of this reaction.

In one of the earliest papers characterizing doxorubicin reductive deglycosylation by rat liver, the majority of activity was localized to the microsomal fraction and had an absolute requirement of NADPH as co-factor [7]. Although, some activity was also associated with mitochondria, nuclei and cytosols were essentially inactive. Shortly after this study, the enzyme catalysing this reaction was purified from phenobarbital-pretreated rat liver microsomes and identified as cytochrome P450 reductase [29]. The purified enzyme has also been shown to convert doxorubicin into a semi-quinone free radical [30], support redox cycling to molecular oxygen [12] and promote drug-mediated DNA damage [31]. In a key paper by Gutierrez *et al.* [32] working with purified enzyme it was demonstrated that drug free radical formation (along with ROS generation) and 7-deoxyaglycone formation were linked but the eventual end product depended on the incubation conditions. In aerobic incubations, one electron reduction produced the semi-quinone drug free radical which then preferentially redox cycled with molecular oxygen. Only a small fraction of drug radicals degraded to the aglycone metabolite. In anaerobic incubations, the semi-quinone drug free radical predominately degraded to the 7-deoxyaglycone (anaerobic deglycosylation). Further evidence that cytochrome P450 reductase is a major enzyme catalysing doxorubicin QR came from an extensive study with purified enzyme preparations [33]. Here, cytochrome P450 reductase exhibited 20–50-fold higher specific activity compared to

several one and two electron reductases including xanthine oxidase and NADH dehydrogenase (EC 1.6.99.3), two important enzymes implicated in doxorubicin QR [34, 35].

Although, DT-diaphorase was the major quinone reductase present in Sp 107 there was no indication of an involvement in doxorubicin deglycosylation in either cytosols (NADPH and NADH as co-factors) or microsomes (NADH as co-factor). When a negative result was recorded with doxorubicin, it was confirmed with menadione that active DT-diaphorase was present in these fractions. Previous studies with highly purified rat liver DT-diaphorases have produced conflicting results. There have been reports which have shown that doxorubicin is a substrate for the enzyme when glycosidase activity was measured [35] and is not a substrate for the enzyme when NADH utilization was followed [36]. Also, in a recent study with purified rat liver DT-diaphorase and the closely related anthracycline daunorubicin, the enzyme was demonstrated to catalyse the formation of hydroxyl radicals [37]. In that study it was postulated that the two electron reduced hydroquinone form of the anthracycline underwent very slow auto-oxidation (≪menadione, AZQ and mitomycin c) back to native drug via the semi-quinone free radical producing two molecules of superoxide radical. A possible explanation for the above conflicting results is that different DT-diaphorase preparations were used and these can differ in many respects including substrate specificity [38]. At least three unique isoenzymes have been isolated from rat liver cytosols [39] and the inclusion of activators like BSA in preparations can greatly influence results. For example, although rat Walker 256 tumour DT-diaphorase was able to reduce CB 1954 to a cytotoxic DNA interstrand cross-linking agent the enzyme purified from human Hep G2 cells sustained a much lower rate of catalysis (6.4-fold) [22]. It was concluded that whilst CB 1954 acts as a substrate for the rat enzyme, it can be considered more like an inhibitor of the human enzyme. More significantly, despite the fact that purified rat liver DT-diaphorase has been shown to form ROS with anthracyclines, the enzyme present in the S9 fraction of MCF-7 human breast cancer cells was unable to support this reaction with doxorubicin [40]. We have concentrated on rat tumour subcellular fractions and enzyme purified from rat tumour and our results consistently reveal that doxorubicin is not a substrate for DT-diaphorase.

Rat cytosols have been shown to convert doxorubicin into forms that bind covalently to DNA and it was argued that the enzyme catalysing this reaction was DT-diaphorase [10]. Since dicoumarol had little effect on DNA binding in that particular study we believe that it is unlikely that DT-diaphorase was involved and that the cytosols probably contained other quinone reductases.

The major ramification of NADPH cytochrome P450 reductase as the principal enzyme supporting doxorubicin QR in the Sp 107 tumour is that it catalyses redox cycling under aerobic conditions and 7-deoxyaglycone metabolite formation under anaerobic conditions with equal facility. Xanthine oxidase is much less effective at forming the 7-deoxyaglycone metabolite than it is at catalysing redox cycling [33] and DT-diaphorase has only a limited capacity to generate free radicals [37, 40]. The *in vivo* implication of this result is that ROS production should predominate in oxygenated regions of the Sp 107 tumour whereas 7-deoxyaglyxcone metabolite formation should predominate in hypoxic areas. We have shown previously that after i.t. administration of doxorubicin-loaded microspheres there is a 16–24 hr delay before high levels of 7-deoxyaglycone metabolites appear [16] which is in marked contrast to their rapid formation in minutes in normal tissue (liver and heart) after i.p. drug administration [41]. During this time we believe redox cycling may be occurring in the tumour. Therefore, it is interesting that no evidence was detected of oxidative damage at the end of this period as measured by lipid peroxidation [13]. Possibly because of the low rates of reaction (30–40-fold less than in rat liver microsomes) the endogenous levels of free radical detoxification pathways in the tumour may have been adequate to resist this minor ROS insult. After 24 hr, high levels of 7-deoxyaglycones appeared in the tumour which persisted to 72 hr and were still measurable even after 1 week. This effectively means that from 24 hr onwards large areas of the tumour must have become hypoxic. Despite this we were unable to detect an elevation in levels of doxorubicin–DNA covalent adducts consistent with anaerobic bioreductive alkylation [13]. The induction of hypoxia by the microspheres was probably not due to depletion of oxygen by redox cycling since: (a) only a low rate of reaction was sustained in the tumour and (b) aglycone formation did not occur after i.t. administration of free drug which was presumably also undergoing redox cycling. Other factors must have been involved with the microspheres and these are presently under investigation.

In conclusion, we now report that cytochrome P450 reductase is the major enzyme catalysing doxorubicin QR in the Sp 107 rat mammary carcinoma *in vivo*. When the tumour appeared to be well oxygenated, the level of activity of the enzyme was insufficient to induce oxidative stress through redox cycling. When the tumour appeared to contain metabolically active hypoxic regions, the enzyme converted doxorubicin into its 7-deoxyaglycone metabolite which slowly accumulated in the tumour over several days.

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