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NAD(P)H (QUINONE ACCEPTOR) OXIDOREDUCTASE (DT-DIAPHORASE)-MEDIATED TWO-ELECTRON REDUCTION OF ANTHRAQUINONE-BASED ANTITUMOUR AGENTS AND GENERATION OF HYDROXYL RADICALS

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Abstract—The anthraquinone-based antitumour agents mitoxantrone, daunorubicin and ametantrone were found to be substrates for NAD(P)H (quinone acceptor) oxidoreductase (DT-diaphorase)[QAO] isolated from rat liver. This was indicated by the stimulation of QAO-dependent NADPH oxidation by these agents. This effect followed Michaelis–Menten kinetics and was dependent on the concentration of QAO, inhibited by the specific QAO inhibitor dicumarol (15 μM) and enhanced by the QAO activators bovine serum albumin (0.01%) and Triton X-100 (0.03%). As indicated by the V_{max}/K_m ratio, mitoxantrone (26.53) was considerably more active than ametantrone (11.25) or daunorubicin (7.35). Metabolism of these anthraquinones was associated with the formation of superoxide anions, hydrogen peroxide and hydroxyl radicals as indicated by electron spin resonance spin trapping studies with 5,5-dimethyl-1-pyrroline-*N*-oxide. This is likely to be due to the slow auto-oxidation of the respective dihydroquinones in the presence of molecular oxygen. QAO needs to be considered as a possible route of bioreductive activation of these agents.

The role of NAD(P)H (quinone acceptor) oxidoreductase (QAO‡) in quinone metabolism has been described as a cellular protective mechanism [1] against the toxicity of quinones such as menadione [2, 3]. This mechanism proceeds by two-electron reduction of quinone to the hydroquinone which can be conjugated with polar endogenous molecules and is consequently readily excretable [4, 5]. This pathway has been suggested to compete with flavin reductase-mediated one-electron reduction of quinones and the concomitant reactive oxygen formation which is believed to be responsible for quinone toxicity [6]. Supportive of this deactivation role QAO of Walker tumour cells has been shown to be involved in the detoxification of the bioreductive antitumour agent SR4233, a benzotriazine di-*N*-oxide [7]. Furthermore, dicumarol, a specific inhibitor of QAO has been found to increase the cytotoxic potential of benzoquinone mustard and benzoquinone dimustard in L5178Y/HBM10 mouse lymphoblasts [8]. An increased level of QAO appeared to contribute to the resistance of these cells to these two agents. QAO has also been shown to protect against the cytotoxicity of mitomycin C and porfiromycin under hypoxic conditions [9, 10]. However, QAO appears to be involved in the bioreductive activation of these agents to cytotoxic species under oxic conditions. To support this Marshall *et al.* [11] have shown that oxic cells resistant to mitomycin C have lower QAO levels. However, other evidence suggests that mitomycin C is not a substrate for QAO (reviewed in Ref. 12).

In contrast, some studies have revealed a role for QAO in the activation of bioreductive quinones. AZQ has recently been shown to be a substrate for QAO in HT29 human colon carcinoma cells [13, 14], rat liver [14] and MCF-7 human breast cancer cells [15]. In HT29 cells QAO appears to play a role in DNA strand breakage and cytotoxicity by AZQ [14]. In MCF-7 cell S9 fraction metabolism of AZQ by QAO results in formation of AZQ semiquinone and concomitant reactive oxygen (O_2^-, H_2O_2, ·OH) formation [15]. This was proposed to be as a result of auto-oxidation of AZQ hydroquinone by two one-electron steps. Consistent with QAO activating AZQ, dicumarol was found to protect MCF-7 cells against AZQ cytotoxicity [16]. A form of QAO has recently been identified in Walker 256 rat carcinoma cells which activates 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) to a cytotoxic DNA interstrand crosslinking agent [17].

Quinone-containing antitumour agents which are currently of interest include the clinically effective daunorubicin and mitoxantrone, and ametantrone which is currently undergoing clinical trials (Fig. 1). Reductase-mediated redox cycling has been implicated in the mechanism of antitumour action and cardiotoxicity of daunorubicin (reviewed in Ref.

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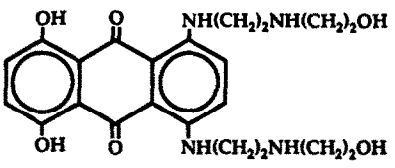
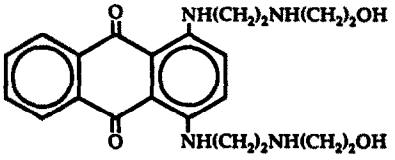
‡ Abbreviations: QAO, NAD(P)H (quinone acceptor) oxidoreductase; AZQ, diaziquone; CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; ESR, electron spin resonance; DCPIP, 2,6-dichlorophenolindophenol; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; BSA, bovine serum albumin; SOD, superoxide dismutase.

MITOXANTRONE



AMETANTRONE



DAUNORUBICIN

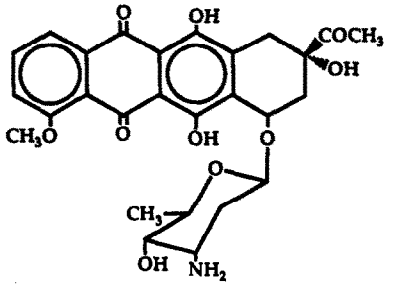


Fig. 1. Structures of mitoxantrone, ametantrone and daunorubicin.

18). However, mitoxantrone, based on daunorubicin, is considered to have structural features which appear to reduce its potential to undergo one-electron reductive activation [19]. Indeed, mitoxantrone has been shown to have reduced propensity to redox cycle in human liver tissue [20], in the presence of purified rat liver NADPH-cytochrome P450 reductase [21] and in MCF-7 human breast cancer cell S9 fraction [22]. Given the conflicting reports of the involvement of QAO in the bioreductive activation of certain quinone antitumour agents and the deactivation of others we have explored the role of this enzyme in the reduction of mitoxantrone, daunorubicin and ametantrone.

MATERIALS AND METHODS

Materials. Cytochrome P450 reductase was a gift from C. R. Wolf, ICRF, University of Edinburgh, U.K. Rat liver QAO for ESR studies was isolated by Dr P. C. Preusch and provided by D. Ross, University of Colarado, Denver, CO, U.S.A. Rat liver QAO for other studies was isolated by following the method of Ernster [23] and was purified to a specific activity of 125 U/mg protein as measured by NADPH-dependent reduction of DCPIP. Mitoxantrone dihydrochloride was supplied by Lederle Laboratories (Gosport, U.K.). The spin trap DMPO was obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). BSA was obtained from Miles Laboratories (Elkhart, IN, U.S.A.). Dicumarol [3,3'-methylenebis(4-hydroxycoumarin)], DCPIP, NADH, NADPH, SOD, catalase, EDTA, Triton X-100 and Tween-20 were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Daunorubicin was obtained from Rhone Poulenc (Dagenham, U.K.).

Determination of drug metabolism by rat liver QAO. The effect of anthraquinones on QAO-dependent NADPH oxidation was used as an indicator of drug metabolism. NADPH oxidation was determined spectrophotometrically at 340 nm, 37° in a system of phosphate buffer (50 mM, pH 7.5), rat liver QAO (1.6 μg/mL), NADPH (100 μM) and anthraquinone.

Determination of superoxide anion formation. Superoxide anion formation was determined by following the SOD-inhibitable rate of reduction of acetylated cytochrome *c*(70 μM) [24] in the presence of anthraquinone, QAO 2.0 μg/mL) and NADPH (500 μM). Acetylated cytochrome *c* reduction was followed spectrophotometrically at 550 nm.

Electron spin resonance spectroscopy (ESR). ESR spectra were obtained at room temperature with a Varian E109 Century Series X-band (9.3 GHz) spectrometer (Varian Instruments, Palo Alto, CA, U.S.A.). Spin trapping studies were carried out in the following system: Tris buffer (25 mM, pH 7.4), rat liver QAO (0.58 μg/mL), NADH (500 μM), drug (200 μM), BSA (0.23 mg/mL), Tween-20 (0.01%) and DMPO (100 mM). In the above system NADH was substituted for NADPH. These two cofactors have been found to be interchangeable as electron donors for QAO activity [23]. BSA and Tween-20 were used as activators of QAO [23]. Determinations were carried out in the presence or absence of dicumarol (50 μM). Anaerobic studies were carried out after bubbling the sample with N_2 (10 min) in a septum sealed vial. The sample was then transferred to the ESR flat cell under anaerobic conditions. ESR parameters were microwave frequency 9.51 GHz, microwave power 10 mW, scan range 80 G, modulation amplitude 1.25 G, time constant 0.25 sec, scan time 4 min and gain 6.3×10^4.

RESULTS

Anthraquinone metabolism by purified rat liver QAO

Studies were carried out to determine whether the quinone-based antitumour agents daunorubicin, mitoxantrone and the mitoxantrone analogue ametantrone were reduced by QAO in the presence of NADPH. Figure 2 shows that these agents (100 μM) stimulated QAO-dependent NADPH oxidation, the degree of stimulation being dependent on the QAO concentration whilst no oxidation of NADPH was observed in the absence of enzyme. Mitoxantrone-mediated stimulation of QAO-dependent NADPH oxidation was 4-fold greater than that produced by daunorubicin or ametantrone.

Figure 3 shows the effect of known activators of QAO [23] on the rate of NADPH oxidation in the presence of anthraquinones. The presence of BSA or Triton X-100 greatly enhanced anthraquinone-stimulated, QAO-dependent NADPH oxidation. In contrast, dicumarol (15 μM), a specific inhibitor of QAO [22] was found to totally abolish anthraquinone-

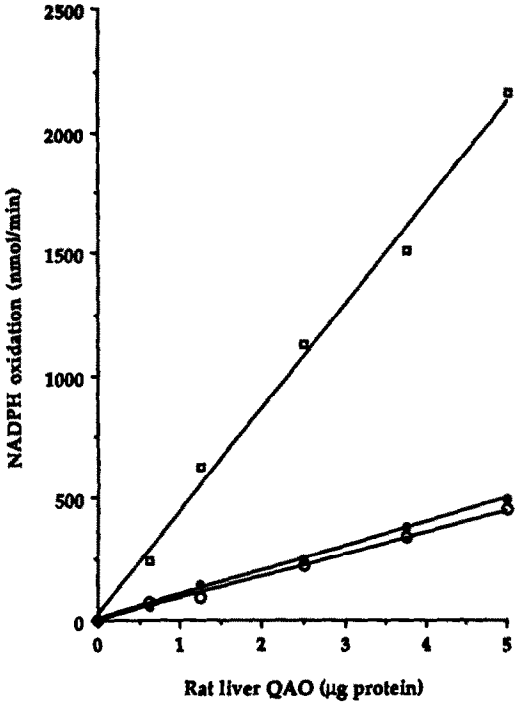


Fig. 2. Effect of rat liver QAO concentration on mitoxantrone- (□), daunorubicin- (○) and ametantrone- (●) stimulated NADPH oxidation. Drug concentration was 100 μM. See Materials and Methods for full experimental details.

stimulated NADPH oxidation in this system (Fig. 3). These results indicated that all three compounds studied were being metabolized by QAO. Since QAO is an obligate two-electron reducing enzyme it can be presumed that the anthraquinones are being metabolized to their corresponding dihydroquinones.

Further studies were carried out to determine the kinetic parameters for metabolism of these compounds by QAO. Figure 4 shows that for mitoxantrone, daunorubicin and ametantrone the rate of QAO-mediated NADPH oxidation was dependent upon the respective drug concentration. Interpretation of this kinetic data using Hanes–Woolf plots (Fig. 4a–c inserts) shows that stimulation of QAO-dependent NADPH oxidation by all three anthraquinones followed Michaelis–Menten kinetics. Table 1 summarises the Michaelis–Menten kinetic constants observed for each compound. As indicated by the V_{max}/K_m ratio the order of anthraquinone activity was mitoxantrone (26.53) > ametantrone (11.25) > daunorubicin (7.35). To investigate the structural requirements for anthraquinone reduction by QAO the effect of anthraquinone and 1,4-dihydroxyanthraquinone on QAO-dependent NADPH oxidation was determined. Table 1 shows the Michaelis–Menten kinetic constants determined for these compounds and demonstrates that anthraquinone *per se* was not a substrate but 1,4-dihydroxyanthraquinone was as active as daunorubicin and ametantrone. This indicates that the minimum requirement for QAO substrate specificity

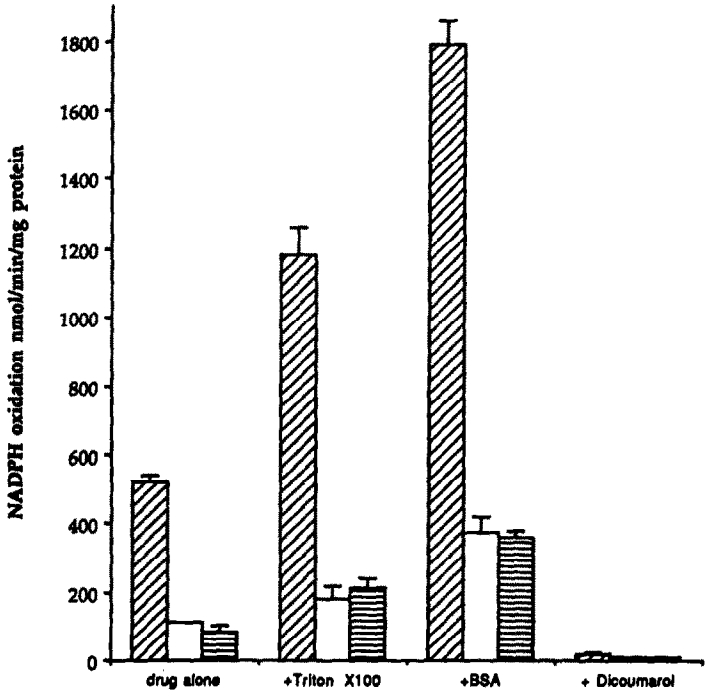


Fig. 3. Effect of QAO activators and inhibitors on anthraquinone- (100 μM) stimulated NADPH oxidation by mitoxantrone (▨), ametantrone (▤) and daunorubicin (□). Concentrations were Triton X-100 (0.03%), BSA (0.01%) and dicumarol (15 μM). See Materials and Methods for full experimental details.

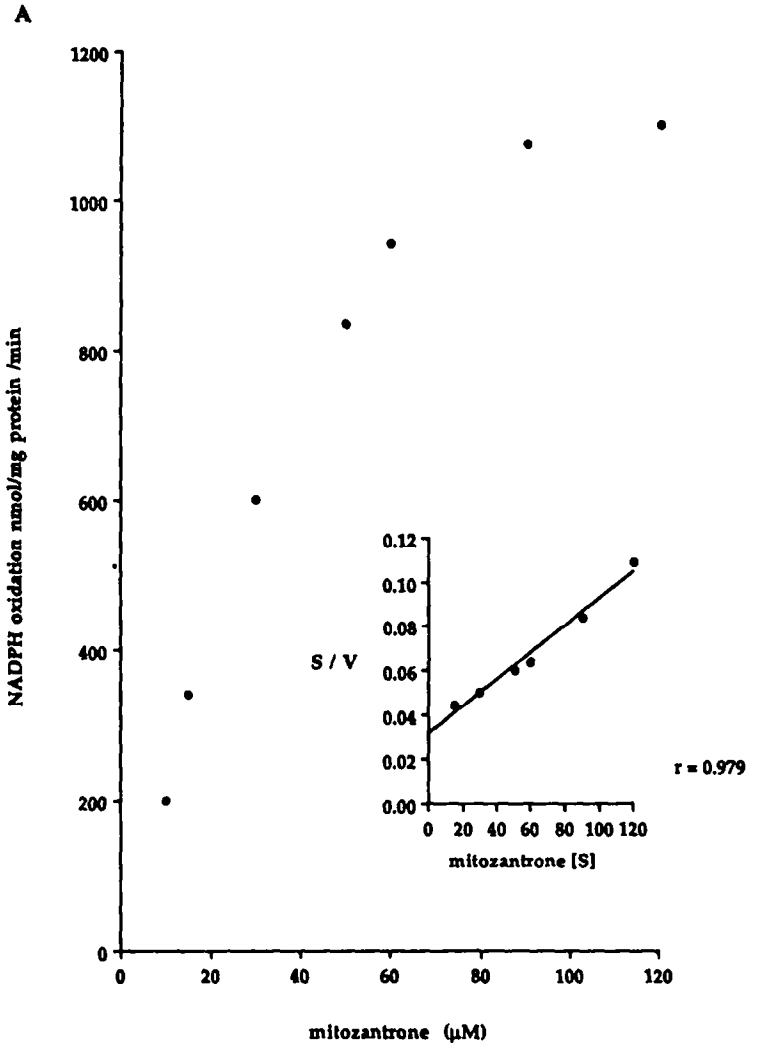


Fig. 4.

is towards anthraquinones with substituted amino and/or hydroxy groups. Despite the K_m values of the clinically relevant anthraquinone-based agents used in this study lying between 50 and 75 μM, this is likely to approximate to the intracellular drug concentration of these agents since they are known to bind to DNA with high affinity [18, 19].

Oxygen free radical formation mediated by anthraquinones during metabolism by QAO

It has been shown previously that formation of dihydroquinones from certain quinones can result in the formation of reactive oxygen species due to autoxidation of the dihydroquinone in the presence of molecular oxygen (e.g. Refs 23 and 25). Oxygen free radical formation during metabolism of the anthraquinones by QAO was therefore studied using an assay for superoxide anions based on the SOD-sensitive reduction of acetylated cytochrome *c*. In this system superoxide anion formation appeared to be very low for daunorubicin, mitoxantrone and ametantrone (Table 2). However, when cytochrome P450 reductase was used instead of QAO, mitoxantrone and daunorubicin stimulated superoxide anion formation but ametantrone had no effect (Table 2).

Spin trapping studies were carried out to further investigate reactive oxygen formation by these anthraquinones in the presence of QAO. Figure 5 shows the ESR spectra observed in an incubate of mitoxantrone, NADH, purified rat liver QAO and DMPO. In the complete system a 1:2:2:1 spectrum

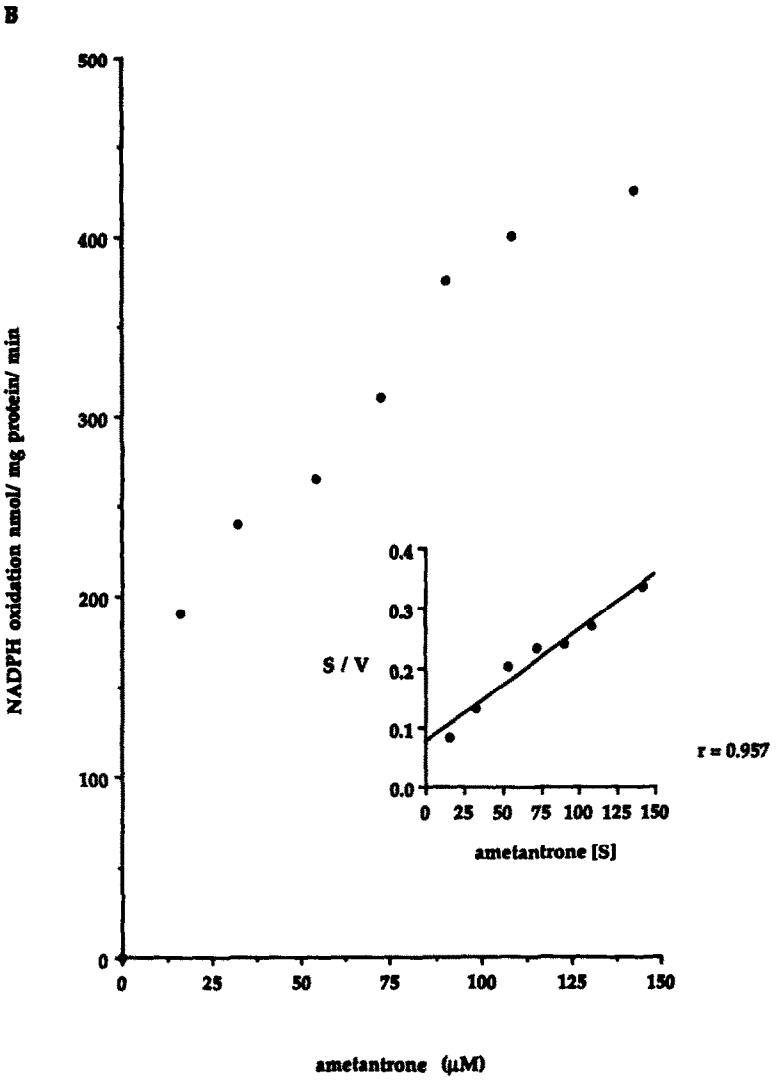


Fig. 4.

with hyperfine splittings of $A^N = A^H = 14.9$ G, characteristic of the DMPO–hydroxyl radical (DMPO–OH) spin adduct was observed [26], indicating formation of hydroxyl radicals in this system. The appearance of this spectrum was inhibited by dicumarol (50 μM) indicating its formation was dependent upon QAO. Figure 6 shows the kinetics of DMPO–OH formation by mitoxantrone in the presence of QAO. The intensity of the spectrum increased with time up to 30 min. To further investigate the origin of DMPO–OH adducts the effect of catalase and SOD on DMPO–OH formation in an incubate of mitoxantrone, NADH and QAO was determined. Catalase (666 U/mL) totally abolished formation of DMPO–OH adducts (Fig. 6). Figure 7 shows that SOD (1332 U/mL) enhanced DMPO–OH formation. However, SOD (1332 U/mL) combined with catalase (666 U/mL) totally abolished the DMPO–OH adduct spectrum.

In an incubate of ametantrone or daunorubicin, NADH, QAO and DMPO, DMPO–OH adducts were also detected. The spectra observed were dependent upon the presence of QAO and drug (Fig. 8). The intensity of the spectra observed increased with time up to 30 min of incubation (Fig. 8).

Spin trapping studies suggested reactive oxygen species were being formed during metabolism of mitoxantrone, ametantrone and daunorubicin by

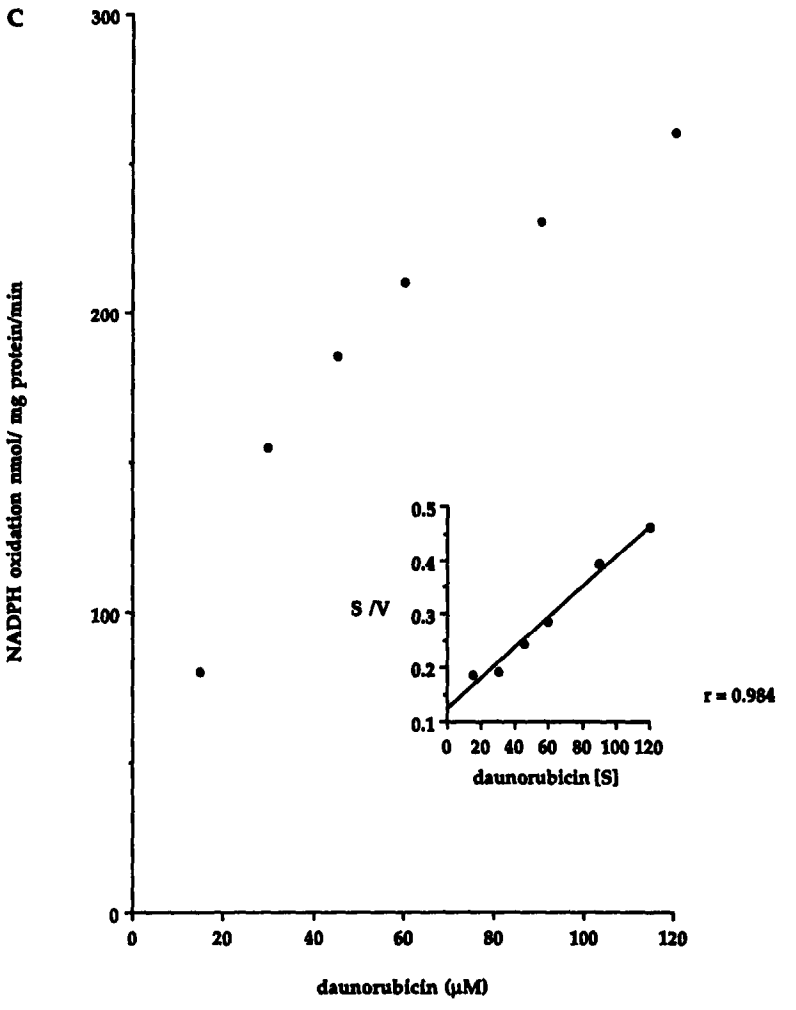


Fig. 4. Effect of (A) mitoxantrone, (B) ametantrone and (C) daunorubicin on QAO-dependent NADPH oxidation. Insert shows kinetic analysis using Hanes–Woolf plot. See Materials and Methods for full experimental details.

Table 1. Michaelis–Menten kinetic constants determined for the stimulation of QAO-dependent NADPH oxidation by anthraquinones

Drug	K_m (μM)	V_{max}	V_{max}/K_m
Daunorubicin	51.7	380.1	7.35
Mitoxantrone	71.7	1902.3	26.53
Ametantrone	53.5	602.2	11.25
Anthraquinone	ND	ND	—
1,4-Dihydroxyanthraquinone	43.0	349.9	8.14

V_{max} = nmol NADPH oxidized/mg protein/min corrected for basal rate oxidation of NADPH in absence of drugs.
Kinetic constants determined using Hanes–Woolf plot illustrated in Fig. 3.
ND, not detected.

QAO. The kinetics and intensity of DMPO–OH adduct observed for each anthraquinone was similar (compare Fig. 6 and Fig. 8).

DISCUSSION

This study shows that the quinone-based anti-tumour agents mitoxantrone, ametantrone and daunorubicin (Fig. 1) are substrates for purified rat liver QAO. This is indicated by the stimulation of QAO-depdendent NADPH oxidation by these agents (see Figs 2, 3 and 4). The consumption of NADPH by QAO was dependent on the concentration of enzyme present (Fig. 2), was inhibited by the specific QAO inhibitor dicumarol and enhanced by the QAO activators BSA and

Table 2. Superoxide anion formation mediated by anthraquinone in the presence of NADPH and rat liver QAO or cytochrome P450 reductase

Drug	Superoxide anion formation QAO	Cytochrome P450 reductase
None	1.7 ± 0.2	40 ± 5
Daunorubicin		
50 μM	2.3 ± 0.4	1010 ± 85
100 μM	3.3 ± 0.1	1460 ± 55
Mitoxantrone		
50 μM	1.8 ± 0.3	400 ± 55
100 μM	1.9 ± 0.4	810 ± 35
Ametantrone		
50 μM	1.1 ± 0	20 ± 0
100 μM	1.5 ± 0.2	25 ± 5

Superoxide anion formation nmol acetylated cytochrome *c* reduced/min/mmol enzyme.
Results are mean ± SD of three determinations.

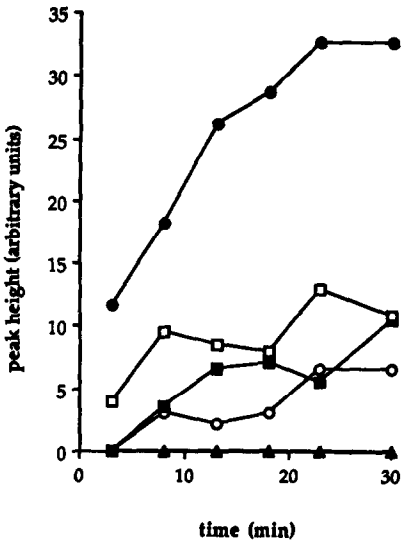


Fig. 6. Kinetics of DMPO–hydroxyl radical adduct formation in a system containing mitoxantrone (200 μM), rat liver QAO (0.58 μg/mL), NADH (500 μM), BSA (0.23 mg/mL), Tween-20 (0.01%) and DMPO (100 mM). Curves represent complete system (●), + dicumarol 50 μM (■), + catalase 666 U/mL (▲), NADH + QAO (□) and NADH + mitoxantrone (○). Signal intensity was calculated from peak to peak height of the second low field line of the DMPO–OH spectrum. See Materials and Methods for details of ESR.

Triton X-100 (Fig. 3). Further evidence for the involvement of QAO in the reduction of these anthraquinones was the stimulation of QAO-dependent NADPH oxidation by these agents following Michaelis–Menten kinetics.

Mitoxantrone produced the greatest stimulation of NADPH oxidation and appeared to be a much better substrate for QAO compared to daunorubicin and ametantrone as indicated by the higher V_{max}/

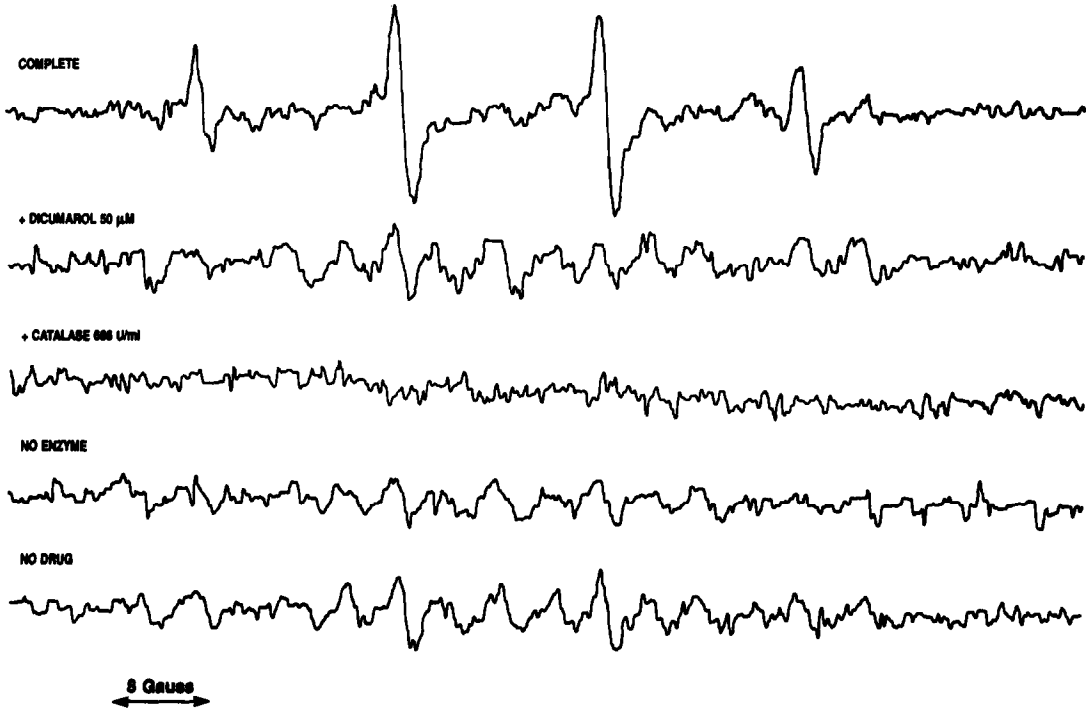


Fig. 5. ESR spectra observed in a system of mitoxantrone (200 μM), rat liver QAO (0.58 μg/mL), NADH (500 μM), BSA (0.23 mg/mL), Tween-20 (0.01%) and DMPO (100 mM). See Materials and Methods for details of ESR. Dicumarol concentration was 50 μM. Catalase concentration was 666 U/mL.

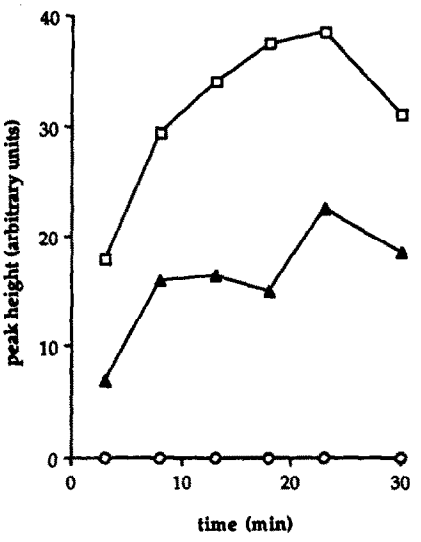


Fig. 7. Effect of SOD on the kinetics of DMPO–hydroxyl radical adduct formation in a system of mitoxantrone (200 μM), rat liver QAO (0.58 μg/mL), NADH (500 μM), BSA (0.23 mg/mL), Tween-20 (0.01%) and DMPO (100 mM). Curves represent complete system (▲), + SOD 1332 U/mL (□), + SOD 1332 U/mL + catalase 666 U/mL (○). See Materials and Methods for details of ESR.

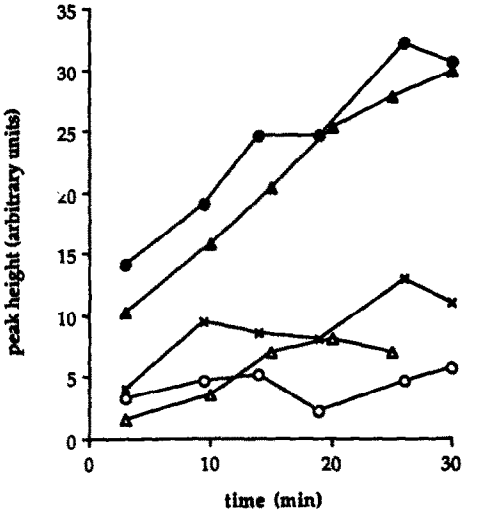


Fig. 8. Kinetics of DMPO–hydroxyl radical adduct formation in a system containing ametantrone (200 μM) (●) or daunorubicin (200 μM) (▲), rat liver QAO (0.58 μg/mL), NADH (500 μM), BSA (0.23 mg/mL), Tween-20 (0.01%) and DMPO (100 mM). Controls were ametantrone + NADH (○), daunorubicin + NADH (△) and QAO + NADH (×). See Materials and Methods for details of ESR. Signal intensity was calculated from peak to peak height of the second low field line of the DMPO–OH spectrum.

K_m ratio obtained (see Table 1). The overall order of activity was mitoxantrone > ametantrone > daunorubicin (see Table 1). Metabolism of these agents by QAO is likely to proceed via direct two-electron reduction to the corresponding dihydroquinone (Eqn 1).

$$AQ \xrightarrow{+2e^- + 2H^+} AQH_2. \quad (1)$$

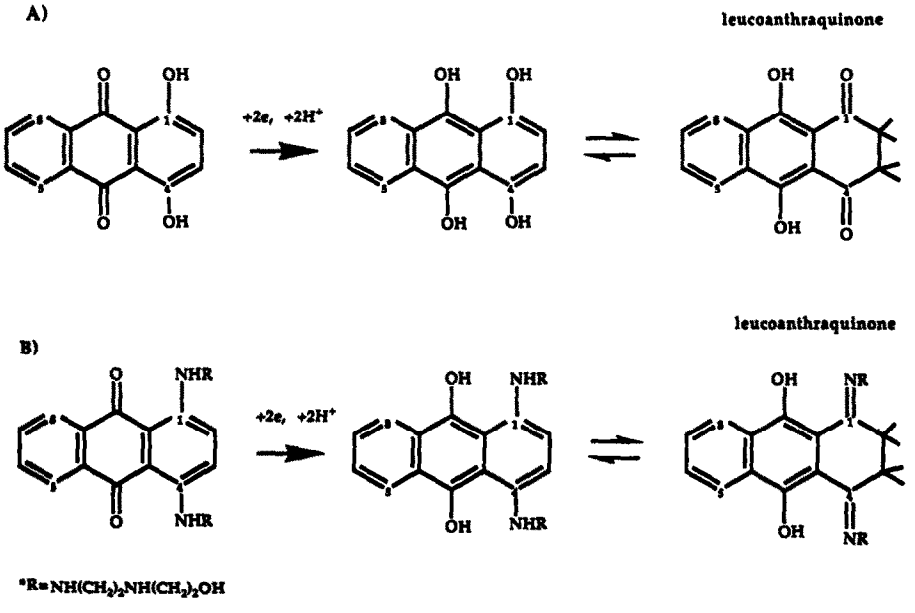


Fig. 9. Scheme for formation of leucoanthraquinones following two-electron reduction of (A) amino-substituted anthraquinones and (B) hydroxy-substituted anthraquinones.

The reduction of anthraquinones by QAO is consistent with the finding of a previous study which showed doxorubicin to be a substrate for QAO in rat liver cytosol [27]. Furthermore, doxorubicin has been shown to induce QAO activity which protected against doxorubicin toxicity in neonatal rat myocytes [28]. A possible interaction between mitoxantrone and QAO has previously been indicated by the inhibition of the acute toxicity of mitoxantrone in human liver hep G2 hepatoma cells by dicumarol [29]. However, in another study daunorubicin was shown not to be a substrate for rat liver QAO [30].

The dihydroquinones of amino- and/or hydroxy-substituted anthraquinones, including mitoxantrone and ametantrone are tautomeric with the respective leucoanthraquinone [31] (Fig. 9) which may be important in their reduction by QAO. Formation of the leucoderivative upon reduction of these anthraquinones by QAO may result in the stabilization of the hydroquinone state. Consequently the equilibrium of reaction 1 will favour hydroquinone formation. In contrast, anthraquinone *per se* does not form a leucoderivative and this may explain why it is not a substrate for QAO (see Table 1). Ametantrone could be reduced via scheme B (Fig. 9), dihydroxyanthraquinone via scheme A and mitoxantrone can be reduced via schemes A and B. This may explain why mitoxantrone is a better substrate than ametantrone or dihydroxy-anthraquinone (Table 1). Daunorubicin may be a special case since two-electron reduction has been shown to result in formation of C_7 deoxydaunorubicin aglycone (reviewed in Ref. 32). However, the daunorubicin hydroquinone has recently been shown to form a leucoderivative without elimination of the sugar moiety [33].

In this study we have shown that metabolism of mitoxantrone, ametantrone and daunorubicin by rat liver QAO was associated with formation of hydroxyl radicals as indicated by ESR spin trapping. This is likely to proceed via auto-oxidation of the dihydroquinone in the presence of molecular oxygen via two one-electron oxidation processes. Firstly one-electron oxidation of the dihydroquinone will result in formation of the semiquinone free radical and superoxide anion (see Eqn 2).

Secondly, oxidation of the semiquinone will result in the formation of superoxide anion and regeneration of the parent quinone (Eqn 3).

$$AQH_2 + O_2 \rightarrow AQH\cdot + O_2^{\cdot-} + H^+ \quad (2)$$

$$AQH\cdot + O_2 \rightarrow AQ + O_2^{\cdot-} + H^+. \quad (3)$$

The parent quinone will become available for further enzymic reduction. This process can be described as a type of redox cycle. A role for the compro-portionation/disproportionation equilibrium (Eqn 4) in the formation of semiquinone radical and subsequent reactive oxygen formation does not seem likely since no anthrasemiquinones could be detected following metabolism of the anthraquinones by QAO under anaerobic conditions.

$$AQH_2 + AQ \rightleftharpoons 2AQH\cdot. \quad (4)$$

Formation of superoxide anions leads to production of hydrogen peroxide via spontaneous dismutation (Eqn 5). Formation of hydrogen peroxide can lead to hydroxyl radical formation via the iron catalysed Haber–Weiss reaction (Eqn 5).

Superoxide anion dismutation	$\rightarrow H_2O_2 \rightarrow$	Iron catalysed Haber–Weiss reaction	$\rightarrow$ Hydroxyl radical. (5)

The inhibition of mitoxantrone mediated hydroxyl radical adduct formation in the presence of QAO by catalase (Figs 5 and 6) indicates that DMPO–hydroxyl radical adducts are derived from hydrogen peroxide rather than a breakdown product of the DMPO–superoxide anion adduct (DMPO–OOH) [34]. Spin trapping studies did not detect any superoxide anion formation. This is not surprising since superoxide anions react with DMPO at a much slower rate ($10\ M^{-1}sec^{-1}$) compared to hydroxyl radicals ($2 \times 10^9\ M^{-1}sec^{-1}$) [35]. Furthermore, superoxide anions dismutate spontaneously (Eqn 5) at a much higher rate ($5 \times 10^5\ M^{-1}sec^{-1}$) than they interact with DMPO. A biochemical assay for superoxide anion formation by anthraquinones in the presence of QAO detected negligible superoxide anion formation (Table 2). However, this may reflect the lack of sensitivity of the acetylated cytochrome *c* assay when low levels of superoxide anions are present. In contrast, mitoxantrone and daunorubicin but not ametantrone were observed to mediate superoxide anion formation in the presence of rat liver cytochrome P450 reductase (Table 2), consistent with earlier studies [21]. The results of the present study may indicate that auto-oxidation of the dihydroquinones of mitoxantrone, ametantrone and daunorubicin proceeds at a very slow rate. Formation of the daunorubicin C7 aglycone from the dihydroquinone may prevent dihydroquinone auto-oxidation. The intensity of DMPO–hydroxyl radical spin adduct formation increased with time over 30 min for all three anthraquinones (see Figs 6 and 8). However, the maximum intensity achieved was still 10-fold less than that detected following metabolism of AZQ by rat liver QAO under identical conditions [15]. Consistent with this slow rate of anthraquinone auto-oxidation, it is our experience that leucoderivatives of anthraquinones are very slow to oxidize in solution, requiring many hours in the presence of oxygen (unpublished observations). Furthermore, auto-oxidation of mitoxantrone di-hydroquinone has been found to occur very slowly following its production electrochemically in aqueous medium [36]. However, another explanation for the lack of superoxide anion formation during metabolism of anthraquinones by QAO which cannot be ruled out is direct oxidation of the dihydroquinone by molecular oxygen (Eqn 6).

$$AQH_2 + O_2 \rightleftharpoons AQ + H_2O_2. \quad (6)$$

Indirect evidence was obtained for superoxide anion formation during metabolism of mitoxantrone by QAO since SOD was found to enhance DMPO–hydroxyl radical spin adduct formation in this system (Fig. 7). This is possibly due to SOD driving Eqns 2 and 3 to the right, increasing the rate of dihydroquinone and semiquinone auto-oxidation

concomitant with an increase in the rate of hydrogen peroxide formation from superoxide anions (Eqn 5). This will ultimately lead to an increase in hydroxyl radical formation via iron-catalysed Haber–Weiss reaction (Eqn 5). This effect of SOD also substantiates a role for hydrogen peroxide in the hydroxyl radical formation observed. This was confirmed by the finding that catalase totally abolished DMPO–OH formation both in the presence and absence of SOD (Fig. 7).

Despite the formation of hydroxyl radicals during the metabolism of mitoxantrone, daunorubicin and ametantrone extrapolation of these findings to the designation of a role for QAO in the activation of these anthraquinones in cellular systems is not possible. In the intact cell the presence of detoxification systems which may lead to conjugation of the dihydroxyanthraquinone with glutathione [26] or glucuronic acid [4] may determine the availability of the dihydroquinone for auto-oxidation. Mitoxantrone [37] and doxorubicin [38] have been shown to form such conjugates.

The formation of hydroxyl radicals observed during metabolism of daunorubicin by QAO in the present study may be consistent with the finding that MCF-7 cells resistant to doxorubicin were found to contain reduced QAO activity [39, 40], reduced expression of QAO gene, downregulation of QAO gene transcription [40] and produced significantly less hydroxyl radicals compared to doxorubicin sensitive cells [39].

The results of this study indicate that QAO needs to be considered as an enzyme which is capable of activating mitoxantrone, daunorubicin and ametantrone. The response of some drug resistant and sensitive tumour cells to these agents may be modulated by this enzyme. Levels of QAO in different tumour types and normal tissues vary considerably [41]. Clearly further studies are required to investigate whether modulation of QAO levels/activity in tumour cells can modulate the cytotoxic potential of these and related agents.

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