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ENZYMOLOGY OF THE REDUCTION OF THE POTENT BENZOTRIAZINE-DI-*N*-OXIDE HYPOXIC CELL CYTOTOXIN SR 4233 (WIN 59075) BY NAD(P)H: (QUINONE ACCEPTOR) OXIDOREDUCTASE (EC 1.6.99.2) PURIFIED FROM WALKER 256 RAT TUMOUR CELLS

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Abstract—3-Amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233; WIN 59075) is a highly selective hypoxic cell cytotoxin soon to enter phase I clinical trial. The compound is thought to exert its action through a toxic one-electron reduced free radical intermediate. Preliminary data have suggested that SR 4233 may be metabolized by DT-diaphorase [NAD(P)H: (quinone acceptor) oxidoreductase (EC 1.6.99.2)] to both two- and four-electron reduced products and that this route of biotransformation may represent a bioprotection pathway. In this study, a highly purified enzyme preparation was employed in order to investigate further the metabolism of SR 4233 by DT-diaphorase and to examine the mechanism of reduction in more detail. Spectrophotometric analysis showed that SR 4233 underwent reduction by DT-diaphorase with an apparent K_m of 1.23 ± 0.27 mM and V_{max} of 8.55 ± 1.67 nmol/min/μg protein. This reaction was inhibited completely by dicoumarol (100 μM) and partially by an antiserum raised against the purified enzyme. Characterization of the products of SR 4233 reduction by reverse-phase HPLC confirmed that both two- (SR 4317) and four- (SR 4330) electron reduction products were generated, the latter being the predominant metabolite, particularly in prolonged incubations. Further experiments showed that the four-electron reduction product, but not the two-electron reduction product, was also a substrate for DT-diaphorase with an apparent K_m of 1.14 mM and a V_{max} of 57.12 nmol/min/μg protein. The results presented confirm that SR 4233 is indeed a substrate for DT-diaphorase and that a mixture of two-, four- and six-electron reduced products may be formed. The possible toxicological and pharmacodynamic significance of this metabolism is discussed.

Hypoxic fractions of solid tumours are widely believed to contribute to the limitations of radiation and chemotherapy in the treatment of neoplastic disease [1–3]. However, several features of these hypoxic cells may be exploited in the rational design of new anti-cancer agents [4–6]. SR 4233† (WIN 59075), 3-amino-1,2,4-benzotriazine-1,4-dioxide, is the lead compound in a series of highly selective hypoxic cytotoxins, the benzotriazine di-*N*-oxides, which appear to exhibit improved efficacy and increased selectivity compared with their predecessors, the mitomycins and nitroimidazoles [7].

SR 4233 has been shown to be highly selective towards hypoxic mammalian cells *in vitro* [7] and appears to be an effective anti-tumour agent *in vivo* when combined with radiation [8] or compounds which enhance tumour hypoxia [9]. Mechanistic toxicity studies have shown that SR 4233 can be reduced preferentially under anaerobic conditions to both two-electron and four-electron reduction products [10, 11], neither of which is toxic to cells under aerobic or hypoxic conditions [10, 12]. Consequently, it has been postulated that the one-electron reduced species, by definition a free radical intermediate, is the most likely damaging metabolite [10, 13, 14]. In the absence of oxygen this is unable to redox cycle but probably produces cell death via hydrogen abstraction from DNA and other macromolecules [10, 12, 13]. Although a variety of reductase enzymes may catalyse this bioreductive activation of SR 4233, recent evidence suggests that cytochrome P450 and NADPH: cytochrome P450 reductase are the major reductases involved in SR 4233 bioactivation in hepatic microsomal fractions [11, 15] and cells [16, 17] *in vitro*.

Brown and colleagues [17] have proposed that the selective action of SR 4233 towards hypoxic cells will be dependent on the rate of drug activation by cellular reductases and the ability of the target cells to repair damaged DNA. Tumour cells have been shown to express a number of reductases, including NADPH: cytochrome reductase [16] and DT-diaphorase [NAD(P)H: (quinone acceptor) oxidoreductase (EC 1.6.99.2)] [18, 19]. These different enzymes may play conflicting roles in the biotransformation of SR 4233. Whilst the presence of significant amounts of P450 reductase would be advantageous in the selective hypoxic cytotoxic action of the drug, the elevated levels of DT-diaphorase observed in some tumours [18, 19] would be

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† Abbreviations: SR 4233, 3-amino-1,2,4-benzotriazine-1,4-dioxide (WIN 59075); SR 4317, 3-amino-1,2,4-benzotriazine-1-oxide; SR 4330, 3-amino-1,2,4-benzotriazine; DT-diaphorase, NAD(P)H: (quinone acceptor) oxidoreductase.

likely to catalyse the obligatory two-electron reduction of SR 4233 to the inactive two- and, possibly, four-electron reduced products (SR 4317 and SR 4330, respectively). Therefore, it is likely that the selective toxicity of SR 4233 against hypoxic tumour cells will depend not only on the degree of hypoxia and the cells' repertoire of defense mechanisms but also on the relative expression of activating and detoxifying reductases, as proposed in our "enzyme-directed approach" to bioreductive drug development [6]. Although preliminary findings from this laboratory have suggested that SR 4233 may indeed be a substrate for DT-diaphorase [11], definitive characterization of the metabolism of SR 4233 by this enzyme has yet to be conducted.

The present communication describes attempts to define the detailed kinetics of the reduction of SR 4233 by purified DT-diaphorase from Walker 256 rat carcinoma cells and speculates on the relevance of the findings to the mode of action, selective tumour-directed toxicity and clinical development of this attractive bioreductive agent.

MATERIALS AND METHODS

Chemicals. SR 4233, SR 4317 and SR 4330 were kindly provided by Drs M. Tracy and W. W. Lee of SRI International (Menlo Park, CA, U.S.A.). The 2-nitroimidazole HPLC internal standard Ro 07-0913 (2-nitroimadazol-1-yl-3-ethoxypropan-2-ol) was supplied by Dr C. E. Smithen of Roche Products (Welwyn Garden City, U.K.). NADH, cyochrome *c*, menadione, dicoumarol and bovine albumin were purchased from the Sigma Chemical Co. (Poole, U.K.). Highly purified DT-diaphorase from Walker 256 rat carcinoma cells and a purified rabbit polyclonal antiserum raised against the purified enzyme were prepared as reported previously [20] and kindly donated by Dr R. Knox (Institute of Cancer Research, Sutton, U.K.). SDS–PAGE and immunoblotting showed that the antiserum specifically recognized a protein with a M_r of 33 kDa in the purified enzyme preparation as described previously [21].

DT-diaphorase activity. The DT-diaphorase activity of the purified enzyme was determined spectrophotometrically by following the reduction of cytochrome *c* at 550 nm, using a modified version of the procedure described by Ernster [22]. The reactive mixture contained purified enzyme (7 ng), cytochrome *c* (77 μM), bovine albumin (0.14%, w/v), NADH (2 mM) as cofactor and menadione (20 μM) as the intermediate electron acceptor. Reactions were conducted at 37° in a total volume of 1 mL 50 mM Tris–HCl buffer (pH 7.4) in the presence and absence of the specific inhibitor dicoumarol (100 μM). Preliminary experiments showed that this concentration of dicoumarol inhibited DT-diaphorase activity > 95%; subsequently, all activities were expressed as the fraction of activity measured which was dicoumarol-inhibitable. In the inhibition studies using the antiserum against DT-diaphorase, the reaction was initiated by addition of NADH after the purified enzyme had been incubated with the antiserum for 5 min at 37°. Rates of reduction were calculated from the linear portion of the reaction progress curves, using 5–30 sampling points over a period of 90 sec. Cytochrome *c* (ε 21.1 × 10^3 M/cm) reduction was monitored in a Lambda 2 spectrophotometer (Perkin–Elmer, Beaconsfield, U.K.). One unit (U) of enzyme activity is defined as the amount of enzyme which catalysed the reduction of 1 μmole of cytochrome *c* per minute. Protein concentrations were determined by the dye-binding method of Bradford [23] using bovine albumin as standard.

SR 4233 metabolism. The reduction of SR 4233 by DT-diaphorase was assayed both spectrophotometrically and by measuring the reduction of the parent drug to its reduction products by reverse-phase HPLC. For spectrophotometric analysis, SR 4233 replaced the benchmark quinone menadione in the assay described above. Incubations were conducted under conditions found to be linear with respect to both time and enzyme concentration. A typical reaction mixture contained SR 4233 (0.3–10 mM), purified enzyme (146 ng) and NADH (200 μM). NADH and/or enzyme were omitted from appropriate control incubations. The pH dependence of the reduction of SR 4233 catalysed by the purified DT-diaphorase preparation was investigated in 50 mM Tris–HCl buffers (pH 5.8, 7.4 and 8.0).

In order to identify the products of SR 4233 reduction by DT-diaphorase, incubations were conducted at 37° in test-tubes open to air with vigorous shaking. Standard incubations contained purified DT-diaphorase (4.45 μg), NADH (2 mM) and bovine albumin (0.14%, w/v). Incubations were conducted in 50 mM Tris–HCl buffer (total volume 1 mL) in the absence and presence of dicoumarol (100 μM). Aliquots (100 μL) of the reaction mixtures were removed at consecutive time points and added to two volumes of ice-cold methanol containing internal standard (Ro 07-0913; 60 mg/L). Samples were then centrifuged at 4° (1700 *g* for 5 min) and the supernatants injected into the HPLC system for analysis. Control incubations were conducted in the absence of cofactor or enzyme.

HPLC analysis of SR 4233 metabolism. HPLC analysis of the reduction of SR 4233 by DT-diaphorase to its two- and four-electron reduction products (SR 4317 and SR 4330, respectively) was determined using isocratic reverse-phase HPLC as described previously [24]. Chromatography was carried out using modular HPLC equipment and columns from Waters Associates (Milford, MA, U.S.A.). Separation was achieved on μBondapak Rad-Pak phenyl columns (8 mm × 10 cm; 10 μm beads) fitted in a RCM 8 × 10 compression unit. The mobile phase consisted of 25% methanol in water and was delivered at a flow rate of 2.5 mL/min using a Waters 660 E System Controller. Absorbance of the column effluent was monitored using a Waters 990 Photodiode Array Detector. Routine detection was at 254 nm and drug and metabolites were identified by chromatographic and spectral analysis. Quantitation was by peak area ratio with reference to standards spiked into appropriate blank sample material.

Kinetic analyses. Michaelis–Menten enzyme kinetics were defined under optimal conditions for each substrate using the criteria described by Henderson

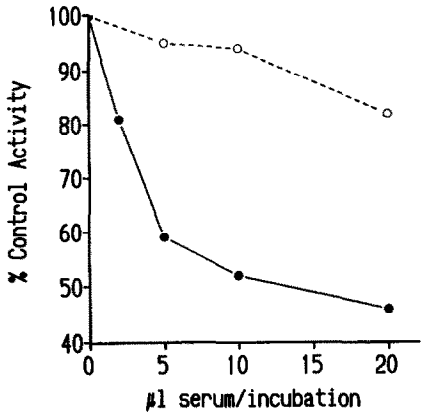

Fig. 1. Inhibition of activity of purified DT-diaphorase preparation towards the benchmark quinone menadione by a rabbit antiserum raised against the enzyme (●) and pre-immune rabbit serum (○). Incubation conditions were as described in Materials and Methods. Serum was pre-incubated with the enzyme for 5 min at 37° prior to initiation of the reaction. The respective concentrations of protein in the pre-immune serum and the purified antiserum were 70 and 66 mg/mL. Uninhibited activity was 3461 nmol/min/μg protein. Results are means of two experiments (SD < 15%).

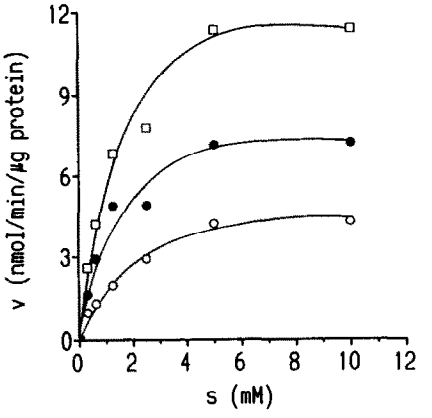

Fig 2. Plots of v versus s for the reduction of SR 4233 mediated by NADH (200 μM) (○), NADH plus purified DT-diaphorase (□) and the corrected enzyme-dependent rate of reductive metabolism (●), which was completely dicoumarol-inhibitable. Rates of reduction were measured spectrophotometrically by the reduction of the terminal electron acceptor cytochrome c, as detected by the change in absorbance at 550 nm. Control incubations contained SR 4233 (0.3–10 mM), cytochrome c (77 μM), bovine serum albumin (0.14%) in 1 mL of Tris–HCl buffer, pH 7.4. Units of s and v are mM and nmol/min/μg protein, respectively. Results shown are from a typical experiment. Similar results were obtained in repeat experiments.

[25]. Apparent K_m and V_{max} values were determined by computer graphics analysis (GraphPAD InPlot, GraphPAD Software, San Diego, CA, U.S.A.) of linear transform (Hanes–Woolf) plots using at least six substrate concentrations.

RESULTS

Using the prototype quinone menadione, the activity of the highly purified preparation was found to be 3417 ± 237 nmol/min/μg or 3.42 ± 0.24 U/μg (mean ± SD, N = 4). Dicoumarol (100 μM) almost completely inhibited this activity, the equivalent rate in the presence of dicoumarol being 98 ± 51 nmol/min/μg (mean ± SD, N = 3). In addition, the polyclonal rabbit antiserum raised against the purified enzyme was found selectively to inhibit the reduction of menadione by DT-diaphorase (Fig. 1). However, in agreement with previous studies [26], the degree of inhibition afforded by the antiserum was far less than that achieved with dicoumarol (only 50–60% maximal inhibition being achieved with 10 μL serum per incubation). In contrast, pre-immune rabbit serum exhibited minimal inhibition of DT-diaphorase activity.

SR 4233 was found to be a substrate for DT-diaphorase purified from the Walker rat mammary tumour cell line. Spectral analysis indicated that neither SR 4233 nor its reduced metabolites exhibited appreciable absorbance at 550 nm and hence were unlikely to interfere with the monitoring of cytochrome c reduction. With 2 mM NADH as cofactor, significant non-enzymatic reduction of SR 4233 was observed, as evidenced by the finding that under these conditions dicoumarol only inhibited the rate of reduction of cytochrome c by 30–40%. Subsequently, the concentration of NADH was decreased to 200 μM in order to minimize this non-enzymatic reduction. At this concentration, NADH itself produced negligible direct reduction of cytochrome c. However, as shown in Fig. 2, even at 200 μM measurable non-enzymatic reduction of SR 4233 still occurred (approximately 33% of the total activity measured). Control incubations were therefore adjusted in order to account for this by including cofactor as well as drug in the reference cuvette and the activities were then calculated from the enzyme-dependent rate which was inhibited by dicoumarol. No reduction of SR 4233 was observed with enzyme alone (i.e. in the absence of cofactor). Under the conditions employed, the enzymatic rate of reduction of SR 4233 was linear with time up to 180 sec and also with enzyme concentration (in the range 15–1000 ng/mL) and was completely inhibited by 100 μM dicoumarol.

Plots of v against s were rectangular hyperbolae and s/v versus s plots were linear (Fig. 3) indicating that the DT-diaphorase-mediated reduction of SR 4233 conformed to Michaelis–Menten kinetics. Values of apparent K_m and V_{max} were 1.23 ± 0.27 mM and 8.55 ± 1.67 nmol/min/μg, respectively (mean ± SD, N = 3). Thus, the V_{max} was some 400-fold lower than that previously found in this laboratory with menadione and the K_m some 400-fold higher [27]. Equivalent values were obtained when NADH was replaced by NADPH. The polyclonal antibody to DT-diaphorase (10 μL/incubation) was inhibitory with regard to the metabolism of SR 4233 by DT-

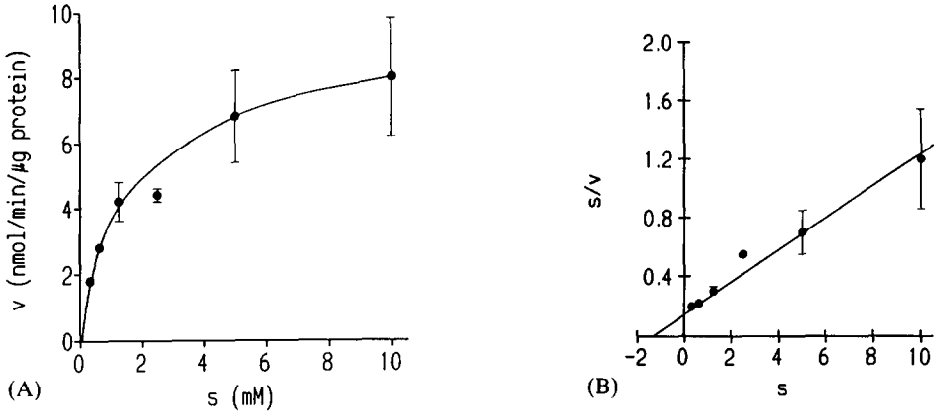

Fig. 3. (A) Plot of v versus s for the reduction of SR 4233 by purified DT-diaphorase as measured spectrophotometrically by the reduction of the terminal electron acceptor cytochrome c and (B) the corresponding s/v versus s (linear transform) plot. Incubation conditions were as detailed in Materials and Methods. Units of s and v are mM and nmol/min/μg protein, respectively. Data are means ± SD (not shown where smaller than symbol) for three independent experiments.

Table 1. Effect of pH on the reduction of SR 4233 (2.5 mM) by purified DT-diaphorase as determined by the reduction of cytochrome c at 550 nm

pH	Rate of reduction (nmol/min/μg)
5.8	4.22 ± 0.65
7.4	4.22 ± 0.10
8.0	5.84 ± 1.29

Incubation conditions were as outlined in Materials and Methods. Values are means ± SD of three independent experiments.

diaphorase (23% inhibition with 2.5 mM SR 4233). Table 1 shows that the reduction of SR 4233 by purified DT-diaphorase did not show any marked pH-dependence as there were no significant differences between the rates of reduction measured at the three pH levels chosen.

HPLC analysis of the products of SR 4233 reduction by DT-diaphorase produced intriguing results. A small but detectable amount of the two-electron reduction product SR 4317 (<2% of the total activity measured in the presence of enzyme and cofactor) was observed in incubations which contained cofactor alone (data not shown). This direct reduction of SR 4233 would account for the non-enzymatic rate of reduction measured spectrophotometrically in the cytochrome c assay and probably proceeds via two sequential one-electron reduction stages. The data shown in Fig. 4 and those obtained at all other substrate concentrations were therefore corrected for this effect. No SR 4317 was detected in incubations conducted with enzyme alone (i.e. in the absence of cofactor). As noted previously by us in crude Walker cell extracts [11] both SR 4317 and SR 4330 were identified as the only detectable stable metabolites. Under the conditions employed, SR 4233 loss did not exceed 5% and hence the rate of loss of parent compound could not be quantified accurately in these experiments. Figure 4 shows typical reaction progress curves for the formation of SR 4317 and SR 4330 from SR 4233 (2.5 mM) with time and the respective inhibition of the production of the two metabolites by dicoumarol. Similar profiles were observed at all the substrate concentrations examined. The four-electron reduction product, SR 4330, was found to be the major metabolite. Moreover, the production of this metabolite was inhibited 82–100% by 100 μM dicoumarol and exhibited a biphasic profile over the time range studied. By contrast, although SR 4317 was produced with equal facility to SR 4330 at early time points, the formation of the two-electron reduced species declined progressively beyond 15 min. In addition, the dicoumarol-inhibitable portion of this activity was only 53–63%. Kinetic analysis of the initial linear phase of SR 4233 reduction to SR 4317 and SR 4330 as measured by HPLC showed that only the formation of SR 4330 obeyed Michaelis–Menten kinetics. Values of apparent K_m from two independent experiments were 7.2 and 5.1 mM, while the corresponding V_{max} values were 0.49 and 0.34 nmol/min/μg. Plots of s/v versus s for the production of SR 4317 and the combined formation of the two reduction products were non-linear (data not shown). Note that the K_m value for SR 4330 formation was about 4-fold higher than that noted for SR 4233 reduction using the cytochrome c assay while the V_{max} was about 20-fold lower.

In order to investigate further the apparent discrepancy between the rate of SR 4233 reduction by DT-diaphorase measured spectrophotometrically and the rate of production of SR 4317 and SR 4330 measured by HPLC, the metabolism of SR 4317 and SR 4330 by the flavoenzyme was assessed using the

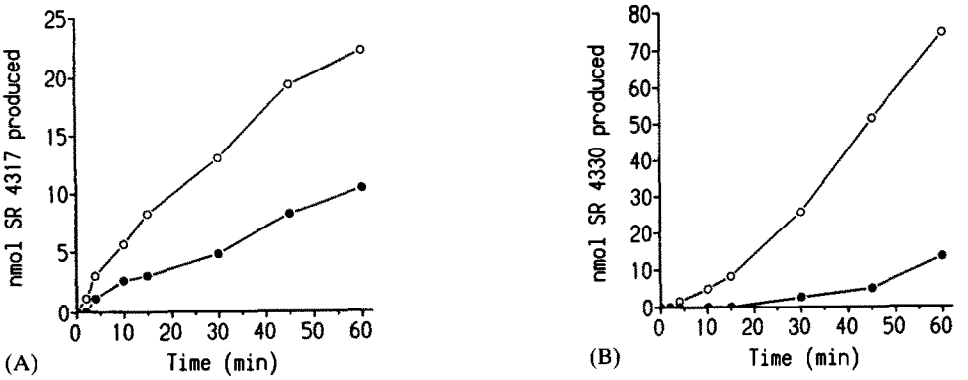

Fig. 4. Reaction progress curves for the reduction of SR 4233 to the two-electron reduced product SR 4317 (A) and the four-electron reduced product SR 4330 (B) as measured by reverse-phase HPLC. Incubations were conducted with SR 4233 (2.5 mM), purified DT-diaphorase (4.45 μg), bovine serum albumin (0.14%) and NADH (2 mM) in the presence (●) and absence (○) of dicoumarol (100 μM). Results shown are for a typical experiment. Similar profiles were obtained in repeat experiments and at other substrate concentrations (0.3–10 mM).

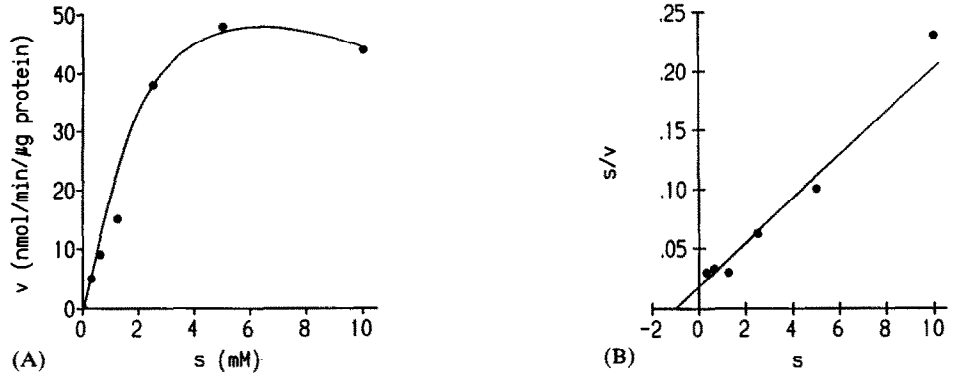

Fig. 5. (A) Plot of v versus s for the reduction of SR 4330 by purified DT-diaphorase as measured spectrophotometrically by the reduction of the terminal electron acceptor cytochrome c and (B) the corresponding s/v versus s (linear transform) plot. Incubation conditions were as detailed in Materials and Methods. Units of s and v are mM and nmol/min/μg protein, respectively. Results are shown for a typical experiment. Similar results were obtained in a repeat experiment (see Table 2).

cytochrome c reduction method. These experiments gave the surprising result that SR 4330 was a substrate for DT-diaphorase but SR 4317 was not. Figure 5 shows that the reduction of SR 4330 conformed to Michaelis–Menten kinetics: plots of v against s were rectangular hyperbolae and s/v versus s plots were linear. Interestingly, SR 4330 appeared to be a better substrate for the purified DT-diaphorase than the parent benzotriazine di-*N*-oxide with K_m and V_{max} values of 1.14 mM and 57.12 nmol/min/μg (means of two independent experiments; Table 2). Although the enzyme exhibited a similar apparent affinity for SR 4330, the V_{max} value was some 7-fold higher. Inhibition of this reaction by dicoumarol was >95% and the polyclonal antibody also produced inhibition of the reduction of SR 4330 by DT-diaphorase (15% at 1.0 mM SR 4330). Table 2 summarizes the data obtained with the cytochrome c reduction assay for the DT-diaphorase-mediated reduction of SR 4233 and SR 4330. Taking intrinsic clearance, CL_{int} (V_{max}/K_m), as a measure of the relative molecular efficiency of substrate reduction [28], the four-electron product SR 4330 is apparently a better substrate for DT-diaphorase than the parent compound SR 4233.

DISCUSSION

Central to the application of an enzyme-directed approach to the design of new bioreductive anticancer drugs is the identification of the major reductases catalysing their reductive bioactivation and detoxication and the factors which govern the expression and regulation of the relevant enzymes

Table 2. Summary of Michaelis–Menten kinetic parameters for the reduction of SR 4233, SR 4317 and SR 4330 by purified DT-diaphorase

Substrate	K_m (mM)	V_{max} (nmol/min/μg)	CL_{int} (V_{max}/K_m)
SR 4233	1.23 ± 0.27	8.55 ± 1.67	6.98 ± 0.29
SR 4317	ND	ND	ND
SR 4330	1.07, 1.22	50.13, 66.72	46.85, 54.68

Values are means ± SD of three independent experiments for SR 4233 and data obtained from two experiments for SR 4330. Activity of the purified enzyme preparation towards the benchmark quinone menadione was 3417 ± 237 nmol/min/μg protein (mean ± SD of three independent experiments; inhibited > 95% by dicoumarol).
Units of intrinsic clearance, CL_{int} are μL/min/μg protein.
ND, not detectable.

in both normal and tumour tissue [6]. With regard to the novel benzotriazine di-*N*-oxide SR 4233, much emphasis has been focused on the elucidation of the reductases involved in the bioactivation of this prodrug [10–12, 15, 16]. Experiments have shown unequivocally that both cytochrome P450 and NADPH: cytochrome P450 reductase play a significant role in the reductive metabolism of this compound to the cytotoxic one-electron reduction product [11, 15–17]. The experiments undertaken in the present study were aimed at examining the possible involvement of DT-diaphorase [NAD(P)H: (quinone acceptor) oxidoreductase (EC 1.6.99.2)] in the metabolic reduction and detoxication of SR 4233.

DT-diaphorase is a flavoenzyme which has been shown to catalyse the reduction of a wide range of chemically diverse, potentially toxic compounds, including quinones, nitro compounds, azo dyes and C-nitroso derivatives [22, 26, 29]. An important and unusual feature of DT-diaphorase is that it catalyses the obligatory two-electron reduction of its substrates [30]. In addition, the enzyme has been shown to be over-expressed in a variety of tumour cell lines [18, 21] and solid tumours (particularly of the breast and colon) [19, 31]. The identification of these properties has stimulated current interest in the development of new bioreductive drugs aimed at exploiting differences in tumour physiology and enzymology [5, 6]. Preliminary data from our laboratory suggested that DT-diaphorase may metabolize SR 4233 predominantly to the four-electron reduction product SR 4330 [11]. In the present study, a highly purified enzyme preparation (≥95% pure) was employed in order to investigate further the metabolism of SR 4233 by DT-diaphorase and to examine the mechanism of reduction in more detail. SR 4233 was found to be reduced by the purified DT-diaphorase, confirming the initial observations [11], and kinetic analysis showed that the reduction of SR 4233 by DT-diaphorase conformed to classical Michaelis–Menten kinetics (Fig. 3). However, the apparent K_m and V_{max} values (1.23 mM and 8.55 nmol/min/μg protein, respectively) obtained with the sensitive spectrophotometric assay based on the reduction of cytochrome *c* showed that SR 4233, like mitomycin C [32] and CB 1954 [21], was a much poorer substrate than the prototype quinone menadione. In contrast to mitomycin C [32] but in agreement with other DT-diaphorase substrates [22, 33], the DT-diaphorase-mediated metabolism of SR 4233 did not exhibit any significant pH dependence. Table 1 shows that the metabolic reduction of SR 4233 by DT-diaphorase was remarkably constant at the three pH levels previously shown to demonstrate the marked pH dependence of mitomycin C reduction [32].

Although the highly sensitive spectrophotometric assay demonstrated that SR 4233 was indeed a substrate for DT-diaphorase, it did not allow characterization of the products of this reductive metabolism. HPLC analysis of the products of the metabolic reduction of SR 4233 by DT-diaphorase confirmed that the four-electron reduction product SR 4330 was the major stable metabolite formed after prolonged incubation, although similar levels of SR 4317 (the two-electron reduction product) were detected up to 10–15 min (Fig. 4). These findings are in close agreement with previous data obtained with a crude Walker cell sonicate [11] and suggest that the substrate remains bound at the active site of the enzyme during catalytic reduction, without releasing all of the two-electron reduced intermediate, giving rise to elevated levels of the terminal four-electron reduced product. Interestingly, although the formation of SR 4330 was almost completely inhibited by dicoumarol, the reduction of SR 4233 to SR 4317 was only inhibited by about 50%. Subsequent attempts to define the kinetic parameters for the production of these two stable metabolites proved largely unsuccessful as only the initial linear portion of SR 4330 formation yielded linear transform (s/v versus s) plots. The apparent K_m and V_{max} for the formation of SR 4330 were markedly different from the values obtained with the spectrophotometric assay for the overall reduction of SR 4233.

Although, depending on the chemistry of the substrate, DT-diaphorase may either bioactivate or detoxify xenobiotics [34, 35], the reduction of SR 4233 to SR 4330 and SR 4317 by DT-diaphorase may represent an important route of detoxification, since neither the two- or four-electron reduced products are significantly cytotoxic under normoxic or hypoxic conditions [10, 12]. Furthermore, the K_m

determined in the spectrophotometric assay was similar to that previously reported for the bioactivation of SR 4233 by cytochrome P450 in rodent hepatic fractions *in vitro* [11, 15], which indicates that DT-diaphorase may function as a competing detoxifying pathway. However, competition between different routes of biotransformation will depend on both the K_m and V_{max} of the different enzymes and, possibly, their subcellular distribution. Hence, given the relative inefficiency of the reduction of SR 4233 by DT-diaphorase, it remains to be determined whether any pharmacological and/or toxicological relevance can be assigned to the metabolism of SR 4233 by this enzyme. Indeed, further studies are in progress which will establish whether SR 4233, like other relatively poor DT-diaphorase substrates, including mitomycin C [32, 36] and CB 1954 [21], may actually be regarded as an inhibitor of this flavoenzyme.

Experiments conducted in order to explain the apparent quantitative discrepancy between the data obtained in the spectrophotometric and HPLC analyses produced the surprising observation that the four-electron reduction of SR 4233 metabolism, SR 4330, was a better substrate for DT-diaphorase than SR 4233 itself (Fig. 5 and Table 2). By contrast, as indicated previously [11], SR 4317 did not appear to be a substrate for DT-diaphorase.

Electrochemical studies have shown that SR 4233 can undergo two biologically attainable reduction steps: irreversible four-electron reduction of the two *N*-oxide functions followed by a quasi-reversible two-elecron reduction of the benzotriazine ring [37]. Such a mechanism would be consistent with the results obtained in the present study. The further quasi-reversible two-electron reduction of the benzotriazine ring of SR 4330 may result in an oxygen-sensitive six-electron reduced metabolite of SR 4233 which rapidly auto-oxidizes. Chemical reduction of SR 4317 by sodium dithionite also results in the formation of this six-electron reduced product, which appears to be colourless, only exists in the presence of the reducing agent and may back-oxidize in air to give SR 4330 (M. Tracy, SRI International, personal communication). In support of this hypothesis, attempts to identify such a product by HPLC analysis of incubations with SR 4330 as the substrate for DT-diaphorase proved inconclusive as no consistent decrease in the levels of SR 4330 could be detected nor could the presence of any stable metabolites (data not shown). The reduction of SR 4233 to a combination of two-, four- and six-electron reduced metabolites may explain the observed discrepancy between the two methods used to characterize the metabolic reduction of SR 4233, since the putative colourless six-electron reduced metabolite, the precise chemistry and stability of which has yet to be confirmed, may not be detected under the HPLC conditions employed here and may even auto-oxidize in air giving rise to the higher rates of cytochrome *c* reduction observed in the (indirect) spectrophotometric assay. Further studies are obviously required to clarify these proposals.

In summary, the selective hypoxic cell cytotoxin benzotriazine di-*N*-oxide SR 4233 has been shown to be a substrate for DT-diaphorase purified from Walker 256 rat tumour cells. It is now important to establish the precise role of DT-diaphorase in the cellular activity and toxicity of this promising lead compound. Although the rate of reduction is modest compared to menadione [21, 27 and this paper] and the bioreductive anticancer indoloquinone EO9 [27], comparably low rates were also seen for mitomycin C (at neutral pH) [32] and CB 1954 [21] and yet the cytotoxicity of the reduction products of these agents appears consistent with an activating function of DT-diaphorase. If anything, DT-diaphorase is likely to play a detoxifying role with SR 4233 and studies are underway to test this hypothesis. We envisage that mechanistic and enzymological studies such as those described here will not only aid the clinical development of SR 4233 by optimizing patient selection, but may also lead to the rational development of even more selective, improved analogues of this novel bioreductive agent. If these data are applicable to the clinical scenario, then patients with tumours expressing high levels of DT-diaphorase may not be the most suitable for treatment with SR 4233. Such patients may be treated more appropriately with drugs which are activated by DT-diaphorase to cytotoxic reduction products. In contrast, it may transpire that patients with low levels of DT-diaphorase and increased levels of activating reductase enzymes within the target tumour may be especially suitable for SR 4233 treatment. The application of this sophisticated approach requires more detailed biochemical analysis of the enzyme profiles of biopsies from the relevant target tumours and surrounding normal tissues.

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REFERENCES

1. Coleman CN, Hypoxia in tumours: a paradigm for the approach to biochemical and physiological heterogeneity. *J Natl Cancer Inst* **80**: 310–317, 1988.
2. Workman P, Optimized treatment modalities for hypoxic tumour cells. In: *Drug Delivery in Cancer Treatment II* (Ed. Domellof L), pp. 79–102. Springer, Heidelberg, 1989.
3. Teicher BA, Lazo JS and Sartorelli AC, Classification of antineoplastic agents by their selective toxicities toward oxygenated and hypoxic tumour cells. *Cancer Res* **41**: 73–81, 1981.
4. Kennedy KA, Hypoxic cells as specific targets for chemotherapy. *Anti-Cancer Drug Des* **2**: 181–194, 1987.
5. Kennedy KA, Teicher BA, Rockwell S and Sartorelli AC, The hypoxic tumour cell: a target for selective cancer chemotherapy. *Biochem Pharmacol* **29**: 1–8, 1979.
6. Workman P and Walton MI, Enzyme-directed bioreductive drug development. In: *Selective Activation of Drugs by Redox Processes* (Eds. Adams GE, Breccia A, Wardman P and Fielden EM), pp. 173–191. Plenum, New York, 1990.
7. Zeman EM, Brown JM, Lemmon MJ, Hirst VK and Lee WW, SR 4233: a new bioreductive agent with high selective toxicity for hypoxic mammalian cells. *Int J Radiat Oncol Biol Phys* **12**: 1239–1242, 1986.
8. Zeman EM, Hirst VK, Lemmon MJ and Brown JM, Enhancement of radiation-induced tumor cell killing

by the hypoxic cell cytotoxin SR 4233. *Radiother Oncol* **12**: 209–218, 1988.
9. Brown JM, Exploitation of bioreductive agents with vasoactive drugs. In: *Proceedings of the Eighth International Congress of Radiation Research* (Eds. Fielden EM, Fowler JF, Hendry JH and Scott D), pp. 719–724. Taylor and Francis, London, 1987.
10. Baker MA, Zeman EM, Hirst VK and Broan JM, Metabolism of SR 4233 by Chinese hamster ovary cells: basis of selective hypoxic cytotoxicity. *Cancer Res* **48**: 5947–5952, 1988.
11. Walton MI and Workman P, Enzymology of the reductive bioactivation of SR 4233. A novel benzotriazine di-*N*-oxide hypoxic cell cytotoxin. *Biochem Pharmacol* **39**: 1735–1742, 1990.
12. Costa AK, Baker MA, Brown JM and Trudell JR, *In vitro* hepatotoxicity of SR 4233 (3-amino-1,2,4-benzotrioazine-1,4-oxide), a hypoxic cytotoxin and potential antitumour agent. *Cancer Res* **49**: 925–929, 1989.
13. Lauderoute K, Wardman P and Rauth AM, Molecular mechanisms for the hypoxia-dependent activation of 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233). *Biochem Pharmacol* **37**: 1487–1495, 1988.
14. Laderoute K and Rauth AM, Identification of two major reduction products of the hypoxic cell cytotoxin 3-amino-1,2,4-benzotriazine-1,4-dioxide. *Biochem Pharmacol* **35**: 3417–3420.
15. Walton MI, Wolf CR and Workman P, Molecular enzymology of the reductive bioactivation of hypoxic cell cytotoxins. *Int J Radiat Oncol Biol Phys* **16**: 983–986, 1989.
16. Cahill A and White INH, Reductive metabolism of 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233) and the induction of unscheduled DNA synthesis in rat and human derived cell lines. *Carcinogenesis* **11**: 1401–1411, 1990.
17. Biedermann KA, Wang J, Graham RP and Brown JM, SR 4233 cytotoxicity and metabolism in DNA repair-competent and repair-deficient cell cultures. *Br J Cancer* **63**: 358–362, 1991.
18. Berger MS, Talcott RE, Rosenblum ML, Silva M, AliOsman F and Smith MT, Use of quinones in brain-tumour therapy: preliminary results of preclinical laboratory investigations. *J Toxicol Environ Health* **16**: 713–719, 1985.
19. Schor NA and Cornelisse CJ, Biochemical and quantitative histochemical study of reduced pyridine nucleoside dehydrogenase by human colonic carcinomas. *Cancer Res* **43**: 4850–4855, 1983.
20. Knox RJ, Friedlos F, Jarman M and Roberts JJ, A new cytotoxic, DNA interstrand crosslinking agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(azirin-1-yl)-2,4-dinitrobenzamide (CB 1954) by a nitroreductase enzyme in Walker carcinoma cells. *Biochem Pharmacol* **37**: 4661–4669, 1988.
21. Boland MP, Knox RJ and Roberts JJ, The differences in kinetics of rat and human DT diaphorase result in a differential sensitivity of derived cell lines to CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). *Biochem Pharmacol* **41**: 867–875, 1991.
22. Ernster L, DT-diaphorase. *Methods Enzymol* **10**: 309–317, 1967.
23. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
24. Walton MI and Workman P, High-performance liquid chromatographic assay for the benzotriazine di-*N*-oxide (SR 4233) and its reduced metabolites in biological materials. *J Chromatogr* **430**: 4429–4437, 1988.
25. Henderson PJF, Statistical analysis of enzyme kinetic data. In: *Techniques in Protein and Enzyme Biochemistry* (Eds. Kornberg HL, Metcalfe JC, Northcote DH, Pogson CI and Tipton KF), B1-ii, pp. 1–43. Elsevier Biomedical Press, Amsterdam, 1978.
26. Koga N, Hokama-Kuroki Y and Yoshimura H, Rat liver DT-diaphorase as a nitoroso-reductase. *Chem Pharm Bull* **38**: 1096–1097, 1990.
27. Walton MI, Smith PJ and Workman P, The role of NAD(P)H: quinone reductase (EC 1.6.99.2, DT-diaphorase) in the reductive bioactivation of the novel indoloquinone antitumour agent EO9. *Cancer Commun* **3**: 199–206, 1991.
28. Rane A, Wilkinson GR and Shand DG, Prediction of hepatic extraction ratio from *in vitro* measurement of intrinsic clearance. *J Pharmacol Exp Ther* **200**: 420–424, 1977.
29. Ernster L, DT-diaphorase: a historical review. *Chemica Scripta* **27A**: 1–13, 1987.
30. Iyanagi T and Yamazaki I, One electron transfer reactions in biological systems. V. Difference in the mechanism of quinone reduction by the NADH dehydrogenase and the NAD(P)H dehydrogenase (DT-diaphorase). *Biochim Biophys Acta* **216**: 282–294, 1970.
31. Schlager JJ and Powis G, Cytosolic NAD(P)H: (quinone acceptor) oxidoreductase in human normal and tumour tissue: effects of cigarette smoking and alcohol. *Int J Cancer* **45**: 403–409, 1990.
32. Siegel D, Gibson NW, Preusch PC and Ross D, Metabolism of mitomycin C by DT-diaphorase: role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* **50**: 7483–7489, 1990.
33. Siegel D, Gibson NW, Preusch P and Ross D, Metabolism of diaziquone by NAD(P)H: (quinone acceptor) oxidoreductase (DT-diaphorase): role in diaziquone-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* **50**: 7293–7300, 1990.
34. Smith MT, Eastmond DA and DiMonte D, The activation and detoxication of quinones by DT-diaphorase. *Chemica Scripta* **27A**: 105–107, 1987.
35. Ross D, Siegel D, Gibson NW, Pacheco D, Thomas DJ, Reasor M and Weirda D, Activation and deactivation of quninones catalysed by DT-diaphorase: evidence for bioreductive activation of diaziquone (AZQ) in human tumour cells and detoxification of benzene metabolites in bone marrow stroma. *Free Rad Res Commun* **8**: 373–381, 1990.
36. Schlager JJ and Powis G, Mitomycin C is not metabolised but is an inhibitor of human kidney NAD(P)H: (quinone acceptor) oxidoreductase. *Cancer Chemother Pharmacol* **22**: 126–130, 1988.
37. Tocher JH, Virk NS and Edwards DI, Electrochemical studies and DNA damaging effects of the benzotriazine-*N*-oxides. *Biochem Pharmacol* **29**: 781–786, 1990.