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THE ROLE OF CYTOCHROME P450 AND CYTOCHROME P450 REDUCTASE IN THE REDUCTIVE BIOACTIVATION OF THE NOVEL BENZOTRIAZINE DI-*N*-OXIDE HYPOXIC CYTOTOXIN 3-AMINO-1,2,4-BENZOTRIAZINE-1,4-DIOXIDE (SR 4233, WIN 59075) BY MOUSE LIVER

M. I. WALTON,*† C. R. WOLF‡ and P. WORKMAN*§‖

* MRC Clinical Oncology Unit, MRC Centre, Hills Road, Cambridge CB2 2QH;
‡ ICRF Laboratory of Molecular Pharmacology and Drug Metabolism, Edinburgh EH8 9XD; and
§ CRC Department of Medical Oncology, University of Glasgow, CRC Beatson Laboratories, Alexander Stone Building, Garscube Estate, Bearsden, Glasgow G61 1BD, U.K.

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Abstract—SR 4233 or WIN 59075 (3-amino-1,2,4-benzotriazine-1,4-dioxide) is a novel and highly selective hypoxic cell cytotoxin requiring reductive bioactivation for its impressive antitumour effects. Expression of appropriate reductases will contribute to therapeutic selectivity. Here we provide more detailed information on the role of cytochrome P450 and cytochrome P450 reductase in SR 4233 reduction by mouse liver microsomes. Reduction of SR 4233 to the mono-*N*-oxide SR 4317 (3-amino-1,2,4-benzotriazine-1-oxide) is NADPH, enzyme and hypoxia dependent. An inhibitory antibody to cytochrome P450 reductase decreased the microsomal SR 4233 reduction rate by around 20%. Moreover, studies with purified rat cytochrome P450 reductase showed unequivocally that this enzyme was able to catalyse SR 4233 reduction at a rate of 20–30% of that for microsomes with equivalent P450 reductase activity. Exposure to the specific cytochrome P450 inhibitor carbon monoxide (CO) inhibited microsomal reduction by around 70% and CO plus reductase antibody blocked essentially all activity. Additional confirmation of cytochrome P450 involvement was provided by the use of other P450 ligands: β-diethylaminoethyl diphenylpropylacetate hydrochloride gave a slight stimulation while aminopyrine, *n*-octylamine and 2,4-dichloro-6-phenylphenoxyethylamine were inhibitory. Induction of SR 4233 reduction was seen with phenobarbitone, pregnenalone-16-α-carbonitrile and β-napthoflavone, suggesting that cytochrome P450 subfamilies IIB, IIC and IIIA may be involved. Since cytochrome P450 and P450 reductase catalyse roughly 70 and 30%, of mouse liver microsomal SR 4233 reduction respectively, we propose that expression of these and other reductases in normal and tumour tissue is likely to be a major factor governing the toxicity and antitumour activity of the drug.

SR 4233 (3-amino-1,2,4-benzotriazine-1,4-dioxide, WIN 59075, NSC 130181,¶ Fig. 1) is a novel benzotriazine di-*N*-oxide which shows unusually high selective cytotoxicity towards hypoxic mammalian cells *in vitro* [1, 2]. As such it represents the lead compound in a new class of agents [3] which may be clinically useful in the treatment of solid tumours containing radioresistant and chemoresistant hypoxic cells [4, 5].

The mechanism of SR 4233 hypoxic cell selectivity is thought to result from reductive bioactivation to a cytotoxic species, putatively a one-electron oxidizing radical intermediate [6–8]. Direct evidence of a nitroxide-free radical has been obtained [9]. Radical formation would be rapidly reversed by molecular oxygen (O_2) in aerobic cells.

We have proposed that in addition to the O_2 concentration, the nature and level of expression of reducing enzymes in normal and malignant cells will influence markedly their response to bioreductive agents [10, 11]. Several enzymes have been implicated in the reduction of *N*-oxides [12] and of SR 4233 in particular [9, 11, 13–15]. Previous studies have shown that purified buttermilk xanthine oxidase can bioactivate SR 4233 under nitrogen (N_2) to a species which causes extensive single strand breaks in plasmid DNA, with minimal DNA drug binding [8, 14]. The involvement of an oxidizing radical [8, 9] in the mechanism of DNA damage is unusual compared with most other bioreductive drugs such as nitro and quinone compounds [16–18] which adds to the novelty of the benzotriazine di-*N*-oxide series.

In view of the peculiar mechanism of SR 4233 cytotoxicity and its therapeutic potential, we have undertaken a detailed characterization of the enzymology of its reductive metabolism. Our earlier work indicated that SR 4233 was reduced to its single *N*-oxide SR 4317 (Fig. 1) by several mouse liver enzymes, particularly in the microsomal fraction where both cytochrome P450 and its reductase were implicated [10, 13]. Other studies with rat liver

† Present address: The Institute of Cancer Research, Royal Cancer Hospital, Drug Development Section, 15 Cotswold Road, Belmont, Sutton, Surrey SM2 5NG, U.K.
‖ Corresponding author: Prof. P. Workman at the Glasgow address. Tel. (041) 330 4886; FAX (041) 330 4063.

¶ Abbreviations: SR 4233 (WIN 59075, NSC 130181), 3-amino-1,2,4-benzotriazine-1, 4-dioxide; SR 4317, 3-amino-1,2,4-benzotriazine-1-oxide; SR4330, 3-amino-1,2,4-benzotriazine; PB, phenobarbitone; PCN, pregnenalone 16-α-carbonitrile; βNF, β-napthoflavone; DPEA, 2,4-dichloro-6-phenylphenoxyethylamine; SKF 525A, β-diethylaminoethyl diphenylpropylacetate hydrochloride.

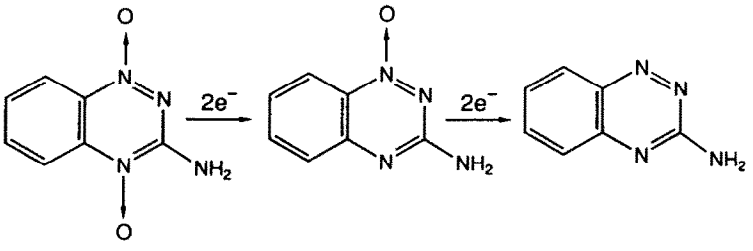

Fig. 1. Reduction pathway for SR 4233.

microsomes supported a role for cytochrome P450 reductase in the formation of SR 4317 and the nitroxide radical [9, 15], but queried the involvement of cytochrome P450. Here, we provide evidence that cytochrome P450 is a major microsomal SR 4233 reductase and, in addition, that there is unambiguously a direct catalytic role for P450 reductase. We also show that cytochrome P450s inducible by phenobarbitone (PB) and pregnenalone 16-α-carbonitrile (PCN) are more efficient than those inducible by β-naphthoflavone (βNF). We consider that these studies are important because of the scheduled clinical development of SR 4233 and in view of the *in vitro* hepatotoxicity which has been observed [6], together with the possible involvement of the same enzymes in the antitumour effect of the drug.

MATERIALS AND METHODS

Chemicals. SR 4233, SR 4317 (3-amino-1,2,4-benzotriazine-1,4-dioxide) and SR 4330 (3-amino-1,2,4-benzotriazine) (Fig. 1) were kindly supplied by Drs M. Tracy and W. W. Lee of SRI International (Menlo Park, CA, U.S.A.). The 2-nitroimidazole HPLC internal standard 2-nitroimidazol-1-yl-3-ethoxypropan-2-ol was supplied by Dr C. E. Smithen of Roche Products (Welwyn Garden City, U.K.). The *N*-oxide HPLC internal standard 4-nitroquinoline *N*-oxide, NADPH, NADH, *n*-octylamine, βNF, cytochrome *c* and bovine albumin were purchased from the Sigma Chemical Co. (Poole, U.K.). PB was obtained from Evans Medical Ltd (Greenford, U.K.). PCN was a gift from G. D. Searle and Co. (Stokie, IL, U.S.A.), 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) was from Lilly Research Laboratories (Indiannapolis, IN, U.S.A.) and β-diethylaminoethyl diphenylpropylacetate hydrochloride (SKF 525A) was from Smith Kline and French Research Ltd. (Welwyn, U.K.). Arachis oil was purchased from Pearce Laboratories (Leeds, U.K.). Zero grade N_2 (<5 ppm O_2) was purchased from the British Oxygen Company (London, U.K.) and research grade carbon monoxide (CO, <10 ppm O_2) from Argo International (Barking, U.K.).

Mouse liver microsome preparation. Adult male C3H/He mice were obtained from our own breeding colony or purchased from OLAC (Bicester, U.K.) and used at 6–12 weeks old. For cytochrome P450 induction animals were administered either PB (80 mg/kg), PCN (200 mg/kg) or β-NF (60 mg/kg) i.p. once daily for 4 days and killed 24 hr after the last injection. Controls received either saline (PB, PCN) or arachis oil vehicle (βNF).

Liver microsomes were prepared using standard techniques from pretreated and untreated mice which had been fasted overnight [19, 20]. Microsomes were washed once in 20 mM Tris–HCl containing 1.15% KCl (pH 7.4) and resuspended at 10 mg/ml protein in 100 mM sodium phosphate buffer (pH 7.4). Microsomes were stored at −70° for up to 6 weeks without loss of activity.

Purified enzyme and antibody preparations. Cytochrome P450 reductase was purified from the liver microsomes of male Wistar rats pretreated with PB (80 mg/kg i.p. × 4 days) [21, 22]. A polyclonal goat anti-rat inhibitory antibody to this enzyme was prepared as described previously [23, 24].

Enzyme assays. Mouse liver microsomal and purified rat liver enzyme cytochrome P450 reductase were assayed by the reduction of cytochrome *c* [25]. The quantity of the polyclonal antibody to cytochrome P450 reductase was optimized to give at least 85% inhibition of mouse microsomal cytochrome *c* reduction. Equivalent amounts of preimmune serum or bovine albumin were used as controls. Cytochrome P450 concentrations were obtained from CO binding spectra [26]. Protein concentrations were determined using bovine albumin as standard [27].

SR 4233 metabolism. Reductive metabolism was carried out under N_2 or CO at 37° in specially modified 25-ml Erhlenmeyer flasks as described previously [13, 28]. Aerobic incubations were performed in the same flasks open to the air with vigorous shaking. Each incubation contained enzyme (0.1 mg/mL microsomal protein or purified rat liver cytochrome P450 reductase at activity equal or 5 times the activity present in 0.1 mg/mL control microsomes) and 0.9 mM NADPH in a final volume of 1.5 or 3 mL of 100 mM sodium phosphate buffer (pH 7.4). Thus, where the purified P450 reductase was used the reaction were identical to those employed with microsomes. Inhibitory antibody or the relevant control was included where appropriate. The inhibitor SKF 525A (5 mM) was added in 50 μL of 100 mM sodium phosphate buffer, pH 7.4. Aminopyrine (5 mM) and DPEA (2 mM) were

added in dimethyl sulphoxide (50 μL) and *n*-octylamine was added in 50 μL of alkalinized reaction buffer. Flasks were preincubated under N_2 or CO as appropriate, with inhibitor if required. A period of 5–7 min was allowed to ensure consumption of any residual O_2. No increase in rate was seen with longer preincubations.

Reactions were started by the addition of SR 4233 (0.5–10 mM) in dimethyl sulphoxide (50 μL). Aliquots of the reaction mixture were removed at 3–7 consecutive time points through air-tight septa and added to 2 vol. of methanol containing internal standard 2-nitroimidazol-1-yl-ethoxypropan-2-ol at 60 mg/L or 4-nitroquinoline *N*-oxide at 25 mg/L). Samples were centrifuged at 4° (15,000 rpm × 5 min) and the supernatant injected into the HPLC system for analysis. Initial reaction velocities were calculated from linear progress curves constructed from at least four time points.

HPLC analysis. Concentrations of SR 4233 and its reduced metabolites, SR 4317 and SR 4330 (two and four electron reduction products, respectively, Fig. 1), were analysed by isocratic, reverse-phase HPLC [29]. Chromatography was carried out using modular HPLC equipment and columns from Waters Assoc. (Milford, MA, U.S.A.). Separations were performed on μBondapak Rad-Pak phenyl columns (8 mm × 10 cm; 10 μm beads) fitted in Z-module compression units and protected by cyanopropyl (CN) Guard-Pak precolumns. Drugs were eluted from the column isocratically at a constant flow rate of 2–3 mL/min using 33% methanol:water as the mobile phase. Routine detection was by UV absorbance at 254 nm. Drugs and metabolites were identified by co-chromatography with authentic material and spectral characteristics. Quantification was by reference to linear calibration curves covering an appropriate concentration range.

RESULTS

The sensitive and specific HPLC assay permitted simultaneous analysis of the loss of SR 4233 and the formation of the single *N*-oxide SR 4317, together with the benzotriazine SR 4330, as catalysed by microsomes or purified enzymes. We confirmed our original observations [13] that liver microsomes from C3H mice rapidly reduced SR 4233 to SR 4317 under N_2 in the presence of reduced NADPH. Under optimal conditions with 2 mM substrate the rates of SR 4233 loss and of SR 4317 formation for the uninduced microsomal preparations used in the present studies were 240 ± 19 and 121 ± 15 nmol/min/mg, respectively (mean ± SE, N = 3). As noted previously [13], the reaction rates in air, omitting NADPH or using boiled microsomes were less than 5% of those using the complete system.

Involvement of cytochrome P450 reductase

An inhibitory antibody to cytochrome P450 reductase was added to liver microsomes in an amount which gave >85% inhibition of the cytochrome *c* reductase activity present. Figure 2 shows that this did inhibit both SR 4233 loss and SR 4317 formation. In the particular experiment shown the antibody inhibited SR 4233 loss by 8% and SR 4317 formation by 21%. Note that there is greater inaccuracy in assessing the relatively small decrease in the large initial substrate peak than is the case for the production of a new metabolite by HPLC. The degree of inhibition calculated from three independent experiments was 22.7 ± 10.0% for SR

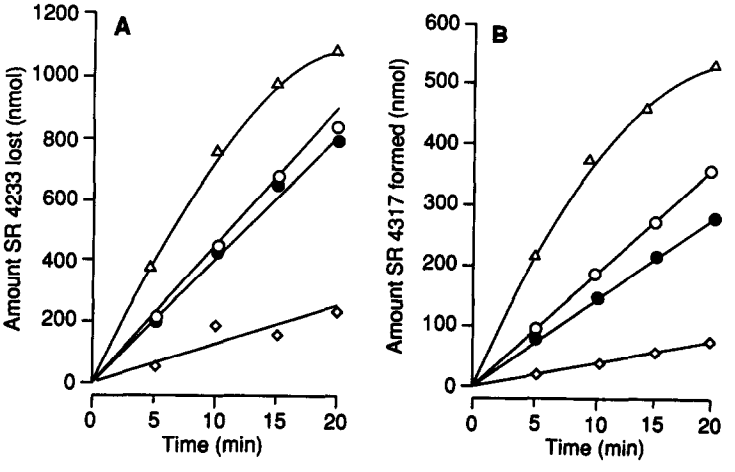

Fig. 2. Characterization of the role of microsomal cytochrome P450 reductase in SR 4233 reduction to SR 4317. (A) SR 4233 loss and (B) SR 4317 formation. Symbols: (○) mouse liver microsomal control, (●) microsomes plus anti-P450 reductase antibody (~85% inhibitory), (◇) purified rat liver microsomal cytochrome P450 reductase at an equivalent activity to that present in the microsomal control, and (△) purified rat liver microsomal P450 reductase at a 5-fold higher concentration than in control microsomes. Incubations were carried out under N_2 and contained 0.1 mg/mL microsomal protein, 2 mM SR 4233 and 0.9 mM NADPH in a final volume of 3 mL 100 mM sodium phosphate buffer, pH 7.4. Results are shown for one of three independent experiments.

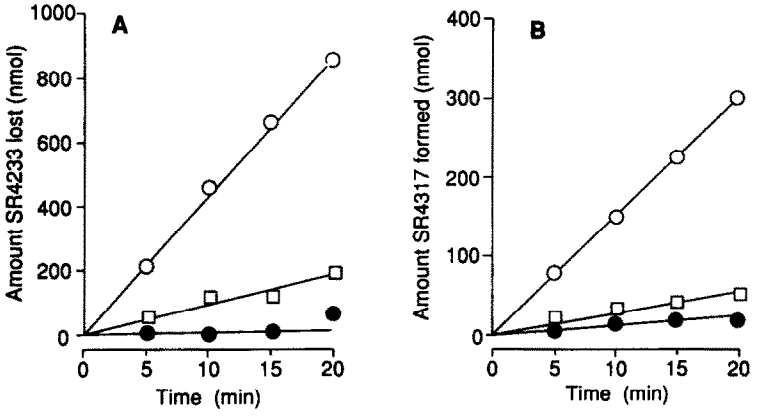

Fig. 3. Characterization of the role of cytochrome P450 in mouse liver microsomal SR 4233 reduction to SR 4317. (A) SR 4233 loss and (B) SR 4317 formation. Symbols: (○) control microsomes, (□) microsomes plus CO and (●) microsomes plus CO plus anti-P-450 reductase antibody. Reaction conditions were as for Fig. 2. Results are shown for one of three repeat experiments.

4233 loss and 18.6 ± 6.3% for SR 4317 formation (mean ± SE).

Figure 2 shows that purified rat P450 reductase was able to reduce SR 4233 to SR 4317. In the experiment shown, when added at an equivalent activity to that present in whole mouse liver microsomes, the observed rate with the purified enzyme was 30% of that with microsomes for SR 4233 loss and 22% for SR 4317 formation. The proportional rates calculated from three independent experiments were 34.0 ± 7.5% and 18.5 ± 5.7% for SR 4233 loss and SR 4317 formation, respectively (mean ± SE). Thus purified P450 reductase is able to reduce SR 4233 to SR 4317 in the absence of cytochrome P450. When five times the amount of purified P450 reductase was added the reaction proceeded at a proportionally faster rate, in excess of that for control microsomes (Fig. 2).

The reaction stoichiometry remained generally unchanged under these different conditions. Calculated from reaction rates, the values obtained were 2.4 ± 0.5 mol SR 4233 consumed per mol of SR 4317 formed for control microsomes compared with 2.4 ± 0.2 in the presence of inhibitory antibody and 1.9 ± 0.4 for purified cytochrome P450 reductase (mean ± SE, N = 3). This implies that drug loss at the one-electron level is similar for both the complete microsomal enzyme system and purified P450 reductase.

Involvement of cytochrome P450

Figure 3 confirms that inhibition of cytochrome P450 by CO reduced markedly the rate of conversion of SR 4233 to SR 4317 by mouse liver microsomes. In the particular experiment shown the extent of inhibition was 79% for SR 4233 loss and 83% for SR 4317 formation. Furthermore, when the anti-reductase antibody was combined with CO the microsomal reaction was inhibited by 80–95% (Fig. 3). The stoichiometry of the reaction was unaltered under CO at 2.5 ± 0.7 moles of SR 4233 lost for each mole of SR 4317 formed (mean ± SE, N = 3).

The effects of a range of other cytochrome P450 ligands [30, 31] are summarized in Table 1. In the presence of SKF 525A, which causes a type I spectral change, reduction of SR 4233 was stimulated slightly but reproducibly compared with controls, e.g. by 7–8% for SR 4317 formation. By contrast, both SR 4233 loss and SR 4317 formation rates were inhibited markedly by the type II ligands *n*-octylamine and DPEA (Table 1). Aminopyrine was also inhibitory (Table 1). These data further support a role for cytochrome P450 in SR 4233 reduction, as do the enzyme induction experiments summarized below.

Table 2 gives the activities of liver microsomes from mice pretreated with the cytochrome P450-inducing agents PB, PCN and βNF. With the protocols used, PB and PCN treatment had little effect on P450 reductase activity compared with control C3H/He mouse liver, whereas total P450 concentrations were increased by approximately 50%. βNF pretreatment produced a 35% lower P450 reductase activity but a 2-fold higher P450 concentration relative to controls. Table 2 shows that SR 4317 formation rates were increased by 38–54%, 16–31% and 22–40% for PB-, PCN- and βNF-induced microsomes, respectively. As these three induction protocols gave no increase in P450 reductase activity over controls, the enhanced SR 4317 formation rates must be due solely to elevated P450 expression. After normalization for the different amounts of total P450 induced by each protocol, it can be seen that the isozymes induced by PB and PCN have comparable efficiencies to the constitutive forms whereas those induced by βNF may be less efficient (Table 2).

DISCUSSION

SR 4233 is preferentially cytotoxic to a range of mammalian cells under hypoxic conditions [1, 2, 6, 7], probably as a result of reductive bioactivation to a reactive one-electron reduced, oxidizing nitroxide

Table 1. Effects of cytochrome P450 inhibitors on the reduction of SR 4233 to SR 4317 by mouse liver microsomes *in vitro*

Inhibitor		% rate of reduction in control	
Type	Agent (concentration)	SR 4233 loss	SR 4317 formation
	Control	100	100
I	SKF 525A (5 mM)	105, 103	108, 107
II	Aminopyrine (5 mM)	58, 64	72, 72
	n-Octylamine (5 mM)	71, 73	85, 84
	DPEA (2 mM)	18, 46	50, 42

Separate values were determined in independent experiments. Reactions were carried out under N_2 in the presence of 0.1 mg/mL microsomal protein, 0.9 mM NADPH, 0.5 mM SR 4233 and inhibitor (2 or 5 mM as appropriate) in a final volume of 3 mL sodium phosphate buffer, pH 7.4.

Control reaction rates were 163 ± 15 and 102 ± 13 nmol/min/mg protein (mean ± SE, N = 3) for SR 4233 loss and SR 4317 formation, respectively.

radical [6–9]. We have shown previously that a variety of reductases can metabolize SR 4233 to SR 4317 [11, 13], particularly cytochrome P450 and cytochrome P450 reductase in the microsomal fraction of mouse liver [11, 13]. Other work with rat liver microsomes supported a role for cytochrome P450 reductase, but not for cytochrome P450 [9, 15]. We therefore sought further evidence to clarify the role of these enzymes in SR 4233 reduction.

The present results confirm that liver microsomes from C32H/He mice rapidly reduce SR 4233 to SR 4317 under N_2 *in vitro*, with minimal four-electron product formation. This was the case also for microsomes, nuclei and primary hepatocyte cultures from Fisher 344 rats [6, 15], Chinese hamster ovary cell cultures and various other cell lines [7, 15], buttermilk xanthine oxidase [8, 13], and also several cytosolic enzymes from C3H/He mouse liver [13]. The reaction stoichiometry is constant, with around two moles SR 4233 consumed per mole of SR 4317 formed.

Both our own studies with male C3H/He mouse liver microsomes ([11, 13], present paper) and those with liver microsomes from male Fischer rats [15] are in agreement that reduction of SR 4233 to SR 4317 is dependent on an anaerobic environment, is inhibited almost completely in boiled preparations, and requires NADPH but not NADH. The marked preference for NADPH is in contrast to the reduction of the tertiary amine *N*-oxides of imipramine, tiaramide and *N*,*N*-dimethylaniline by rat liver microsomes, which showed 28–58% activity in the presence of NADH alone [31, 32]. Reduction rates for these compounds were, however, much lower at between 0.9 and 1.5 nmol/min/mg. Rates of liver microsomal SR 4233 reduction have proved remarkably consistent across the various studies. The values for SR 4317 in control, uninduced C3H/He mouse microsomes were 121 nmol/min/mg protein in the present study and 144 nmol/min/mg in our previous report [13] (both 2 mM SR 4233 substrate) compared with 160 nmol/min/mg for male Fischer rat liver microsomes (1 mM SR 4233) [15]. Moreover, our V_{max} value of 945 nmol/min/mg for SR 4233 loss [13] is closely similar to that of 900 nmol/min/mg for oxygen consumption in rat liver microsomes [9], the K_m being 1.4 mM in both cases. These reduction rates observed are extremely rapid.

In view of the major role of cytochrome P450 reductase in catalysing the one-electron reduction of nitrocompounds and quinones [16–18], its participation in SR 4233 warrants particular attention. We show here that specific inhibitory antibody to cytochrome P450 reductase inhibited the intact microsomal SR 4233 reduction by around 20%. This indicates that the reductase does participate in the mechanism of SR 4233 reduction. The process could involve direct reduction of the drug and/or a contribution via reduction of cytochrome P450. The 20% inhibition of microsomal SR 4233 reduction was seen with an amount of anti-cytochrome P450 reductase antibody which inhibited cytochrome *c* reduction by ≥85%. However, quantitative comparison is difficult because the steric considerations involved in the reduction of the relatively small benzotriazine-di-*N*-oxide molecule as against the large protein are quite likely to be dissimilar. For this reason it was important to carry out experiments with the purified enzyme.

When used at an equivalent activity, purified P450 reductase from male Wistar rat liver was able to catalyse SR 4233 reduction at about 20–35% of the rate for intact mouse liver microsomes. Thus, it is clear that cytochrome P450 reductase does participate in the reduction of SR 4233 in rodent liver microsomes, and that the enzyme may reduce the drug directly. The direct contribution of the reductases to microsomal SR 4233 reduction is 20–30%. Direct reduction of SR 4233 to SR 4317 by rat liver cytochrome P450 reductase was also observed independently [15]. This again contrasts with results for imipramine, tiaramide and *N*,*N*-dimethylaniline tertiary *N*-oxide reduction, which did not appear to be catalysed by rat liver cytochrome P450 reductase [31]. The direct reduction of SR 4233 by P450 reductase may contribute in part to the much greater efficiency of SR 4233 reduction compared with the

Table 2. Effects of cytochrome P450-inducing agents on SR 4233 reduction to SR 4317 by mouse liver microsomes *in vitro*

Treatment	Cytochrome P450 reductase activity		Cytochrome P450 content		Rate of SR 4317 formation‡	Induction ratio SR 4317 formation/cytochrome P450 content
	nmol/min/mg*	Induced/control	nmol/mg†	Induced/control	Induced/control	
Saline	416 ± 13	1.0	1.13	1.0	1.0	1.0
Oil	305 ± 8	1.0	0.68	1.0	1.0	1.0
PB (in saline)	398 ± 15	0.96	1.77	1.53	1.38, 1.54	0.9, 1.0
PCN (in saline)	395 ± 14	0.95	1.68	1.48	1.31, 1.16	0.89, 0.78
βNF (in oil)	198 ± 4	0.65	1.41	2.10	1.22, 1.40	0.59, 0.68

* Values represent means ± SE of three determinations.
† Standard errors were <5% throughout.
‡ Rates of SR 4317 formation in two independent experiments were 256 and 262 nmol/min/mg for saline controls and 200 and 214 nmol/mg for oil controls. Incubation conditions were as for Table 1 except that the NADPH concentration was 4 mM and the SR 4233 substrate concentration was 10 mM. Similar results were obtained at other substrate concentrations.

various other *N*-oxides. On the other hand, it is possible that a similar direct reaction for P450 reductase also occurs with the tertiary amine *N*-oxides, but because of the far lower rates involved (see above) this may not have been detectable.

In addition to a direct role for cytochrome P450 reductase, we have confirmed and extended our previous evidence [11, 13] for the participation of cytochrome P450 in the reduction of SR 4233. Cytochrome P450 was also shown to be involved in the reduction of the tertiary *N*-oxides of tiraramide, imipramine and *N*,*N*-dimethylaniline in rat liver microsomes [31–34]. Our observation of 70–80% inhibition of SR 4233 reduction by CO indicated that cytochrome P450 was a major microsomal reductase. The fact that inhibition by CO was not 100%, as for example was the case for the anaerobic microsomal dechlorination of trichlorofluoromethane [35], is again consistent with a direct role for cytochrome P450 reductase in SR 4233 reduction at a level of around 20–30%. Since cytochrome P450 reductase is required to reduce cytochrome P450 it might perhaps be expected that the reductase antibody would have had a greater effect on microsomal SR 4233 reduction than the 20% inhibition observed here. The amount of antibody used for the experiments was shown to be around 85% inhibitory towards cytochrome *c* reduction and it is difficult to exceed this value. It therefore seems likely that the cytochrome P450 reductase is in excess with respect to the cytochrome P450 and thus 85% inhibition of the P450 reductase has minimal effect on reduction by P450. Indeed if 70–80% of the SR 4233 is catalysed by P450 (as indicated by CO inhibition) and 20–30% by the reductase, then all of the antibody inhibition can be attributed to blockade of the direct SR 4233 reduction by the reductase, with little or no effect on the P450 component. When the inhibitory P450 reductase antibody was combined with CO the reaction was almost completely inhibited, confirming that cytochrome P450 and cytochrome P450 reductase together account for essentially all the SR 4233 microsomal reduction.

Alternative effects of CO other than P450 inhibition are unlikely, and we have unpublished data which demonstrates that whereas reduction of SR 4233 by xanthine oxidase is inhibited completely in air it is not affected by CO. However, since CO was reported to be without effect on SR 4233 reduction in rat liver microsomes [9, 15] we sought further confirmation of cytochrome P450 catalysis by evaluating the effects of additional P450-modulating agents also used by others to inhibit *N*-oxide reduction by cytochrome P450 [30, 31]. Thus *n*-octylamine and DPEA which cause type II spectral changes were both inhibitory. These have been considered to act by co-ordination at the haem moiety, thereby retarding reduction of cytochrome P450 [36, 37]. The type I ligand SKF 525A did not inhibit but gave a modest stimulation of SR 4233 reduction. Again, similar results have been seen with other *N*-oxides [30, 31]. This type of effect has been attributed to an acceleration of cytochrome P450 reduction in the presence of ligand [37]. Other possibilities are that electrons are being shunted away from a less efficient to a more efficient isozyme

of P450 or that SR 4233 is associated with an unusual substrate binding site on P450.

Further evidence in support of an involvement of cytochrome P450 in SR 4233 reduction is provided by the enzyme induction experiments. SR 4233 reduction was enhanced in cytochrome P450-induced microsomes and in the absence of P450 reductase induction. PB- and PCN-inducible isoforms appeared to be more efficient than those induced by βNF. Whereas P450 reductase is reduced by 35% by βNF, the direct reductase component is reduced by only 20% and so the decreased P450 reductase would only account for a reduction of 7%. Although some quantitative differences were seen between the various inducing agents, the results reported here for SR 4233 reduction by mouse liver microsomes are generally very similar to those seen with imipramine, tiaramide and *N,N*-dimethylaniline *N*-oxides in the rat [31, 32].

In the next phase of the work we plan to elucidate the particular P450 subfamilies which may be predominantly involved in SR 4233 reduction. From the results with enzyme inducers described above, it seems likely that constitutive members of the P450 IIB, IIC and IIIA subfamilies which are phenobarbitone and glucocorticoid inducible [38] will participate. However, members of the IA subfamily are unlikely to be involved.

Until recently, the precise molecular mechanism of SR 4233 reduction to SR 4317 was unknown. However, an analysis of the kinetics of nitroxide radical production suggests that the formation of SR 4317 by rat liver microsomal cytochrome P450 reductase involves an initial one-electron reduction to the radical, followed by disproportionation of two nitroxide radical molecules to generate a molecule each of SR 4317 and SR 4233 [9]. Cytochrome P450 may operate by a similar mechanism. However, the lack of effect of CO and metyrapone on the radical formation [9] suggests that two one-electron reduction steps may occur in rapid succession on the enzyme. If so this might be a detoxication reaction. Small amounts of the four-electron reduction product are also formed in liver microsomes and extracts of cell lines. We have shown that DT-diaphorase catalyses predominantly the four-electron reduction, apparently without liberating the two-electron product which is a very poor substrate [13, 39]. Although DT-diaphorase is predominantly cytosolic it may contribute in a minor way to microsomal SR 4233 reduction, and especially to SR 4317 formation. An NADH-dependent cytochrome P450 reductase was also proposed in the microsomal reduction of indicine *N*-oxide [40].

SR 4233 causes extensive DNA damage, especially under hypoxic conditions [7]. Toxicity under aerobic conditions will result from futile cycling of the nitroxide radical generating superoxide whereas under hypoxia the radical will abstract hydrogen from sugar residues in the DNA of hypoxic cells leading to more extensive strand break formation [7–9]. In view of the reactivity of the nitroxide radical, it has been proposed that enzymatic reduction in the proximity of the DNA may be particularly important [15]. SR 4233 is reduced by rat liver nuclei [15] and we have shown recently that anaerobic metabolism of SR 4233 by purified rat liver cytochrome P450 reductase produces strand breaks in naked DNA (R. Riley and P. Workman, unpublished). However, cytochrome P450 isozymes are also present in rat liver nuclei [41]. Of interest with regard to the present work is the observation that reduction of the quinoxaline-1, 4-di-*N*-oxide antibiotic quindoxin by *Escherichia coli* cells and extracts produced a free radical detected by electron spin resonance, as well as the two-electron reduction product quinoxaline-*N*-oxide which was itself inactive [42]. The bacterial cells were more sensitive to the cytotoxicity of the drug under anaerobic conditions, the reduction was supported by both NADH and NADPH, and resistant strains were shown to express lower reductase activities [42]. We plan to determine the relationship between the expression of cytochrome P450 and its reductase and cytotoxicity in yeast cells transfected with these enzymes [43].

Clearly, there is potential for the active one-electron reduced SR 4233 radical to be formed by both cytochrome P450 and P450 reductase in hepatocytes. It is of interest to note that the stoichiometry of the reaction was similar with the purified reductase to that in microsomes at about two moles of parent drug consumed per mole of product formed. The unaccounted for material is presumed to be lost at the one-electron radical level and this may represent the fraction of drug available for mediating cytotoxicity. It is also possible that other metabolites are produced that are not seen with the existing chromatography and we intend to clarify this issue when radiolabelled material becomes available.

In conclusion, SR 4233 is rapidly reduced by mouse liver microsomes *in vitro*. Our results indicate that cytochrome P450 is a major enzyme in this pathway, but cytochrome P450 reductase also has an important role, not only in donating electrons to P450 but also in catalysing direct reduction. The latter function may be more important in normal tissues and tumours which express lower levels of cytochrome P450. Reduction by cytochrome P450 is likely to predominate in the liver (also to some extent in the lung and kidneys). The observed toxicity in primary rat hepatocyte cultures [6] is probably mediated by cytochrome P450 and P450 reductase-generated radicals. Additional enzymes [13] are likely to participate and even predominate in some tissues, and we are now characterizing these in human tumours. It is hoped that mechanistic and enzymological data may facilitate the clinical development of SR 4233. We have proposed that therapeutic selectivity of such agents is likely to relate to the level of expression and substrate specificities of various reductases [10, 11, 13]. It may be that the enzymological profiles of particular kinds of human tumour are especially suited to the bioactivation of SR 4233. In addition, understanding the enzymology of SR 4233 metabolism may lead to the rational design and evaluation of improved SR 4233 analogues.

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