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INITIAL CHARACTERIZATION OF THE MAJOR MOUSE CYTOCHROME P450 ENZYMES INVOLVED IN THE REDUCTIVE METABOLISM OF THE HYPOXIC CYTOTOXIN 3-AMINO-1,2,4-BENZOTRIAZINE-1,4-DI-*N*-OXIDE (TIRAPAZAMINE, SR 4233, WIN 59075)

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Abstract—The benzotriazine di-*N*-oxide SR 4233 (tirapazamine, WIN 59075) is currently in phase I clinical trials as the lead compound in a series of novel and highly selective antitumour hypoxic cytotoxins. Reductive bioactivation is thought to proceed via a one-electron reduced, oxidizing nitroxide radical and also forms the inactive single *N*-oxide SR 4317 via radical disproportionation or a second one-electron reduction. In mouse liver microsomes reductive metabolism is catalysed predominantly by cytochrome P450 (70%) and cytochrome P450 reductase (30%). The aim of the present study was to examine which cytochrome P450 isozymes may be involved. Reduction of SR 4233 to SR 4317 was monitored by HPLC analysis. Metabolism by microsomes from both control and dexamethasone-induced BALB/c male mice was 70% inhibited by carbon monoxide. The cytochrome P450 inhibitor SKF 525A, following aerobic preincubation, also inhibited SR 4233 reduction by 58%. Reduction was induced 2–3-fold by dexamethasone and was not accountable by increases in cytochrome P450 reductase or DT-diaphorase. The induction data and the greater degree of inhibition of SR 4233 reduction by metyrapone compared to α-naphthoflavone suggested a possible involvement of Cyp2b, Cyp2c and Cyp3a cytochrome P450 subfamilies. Both Cyp3a (7.4-fold) and Cyp2b (1.8-fold) type enzymes were shown by western immunoblot analysis to be induced by dexamethasone, the latter correlating more closely with increased SR 4233 reductase activity and also with the 2-fold induction of benzphetamine *N*-demethylase, a Cyp2b-type enzyme. No inhibition of SR 4233 reduction was seen with erythromycin or cyclosporin A which act as substrates/inhibitors for Cyp3a-type enzymes, but inhibition was seen with *p*-nitrophenol and tolbutamide which are substrates for Cyp2e1- and Cyp2c-type enzymes, respectively (11% and 25% inhibition in induced microsomes). SR 4233 itself inhibited benzphetamine *N*-demethylase, which is catalysed by Cyp2b-type enzymes but not erythromycin *N*-demethylase which is catalysed by Cyp3a-type isoforms. Immunoinhibition studies with epitope specific monoclonal antibodies were consistent with the major involvement of phenobarbitone- and steroid-inducible products of the Cyp2b and Cyp2c subfamilies. These forms contributed at least 53% and 26%, respectively, of the cytochrome P450-associated SR 4233 reductase activity in the induced microsomes. The findings support our earlier conclusion that cytochrome P450 is the major SR 4233 reductase in mouse liver and provides leads as to the possible involvement of specific isoforms in human tumours and normal tissues.

SR 4233‡ (3-amino-1,2,4-benzotriazine-1,4-di-*N*-oxide; tirapazamine; WIN 59075) is the lead compound in a series of highly selective hypoxic cell cytotoxins [1, 2] and is currently in Phase I clinical trials at Stanford, Harvard and Glasgow Universities. The impressive hypoxic selectivity of this novel anticancer drug is thought to be the consequence of one-electron reductive bioactivation within the target hypoxic tumour cells. Reduction of SR 4233 may be catalysed by various reductase enzymes, most notably cytochrome P450 reductase (EC 1.6.2.4) and cytochrome P450 itself [3–6]. The rates of hepatic microsomal reduction of SR 4233 are extremely rapid [3–5, 7] with a V_{max} around 900 nmol SR 4233 reduced/min/mg protein but a rather high K_m of 1.4 mM. Recent experiments have supported the hypothesis that the reactive metabolic intermediate is an oxidizing nitroxide radical [7] which exerts its cytotoxic effects through DNA single and double strand breaks, probably as a result of hydrogen abstraction, but not by direct binding to DNA [8–10]. In normoxic tissues, the drug free radical may auto-oxidize and generate reactive oxygen species, which can usually be detoxified adequately by various

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‡ Abbreviations: SR 4233, 3-amino-1,2,4-benzotriazine-1,4-di-*N*-oxide (tirapazamine, WIN 59075); SR 4317, 3-amino-1,2,4-benzotriazine-1-oxide; SR 4330, 3-amino-1,2,4-benzotriazine; DT-diaphorase, NADP(H): (quinone acceptor) oxidoreductase (EC 1.6.99.2); cytochrome P450 reductase, NADPH: cytochrome P450 reductase (EC 1.6.2.4); SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; DMSO, dimethyl sulphoxide; SKF 525A, proadifen hydrochloride; mAbs, monoclonal antibodies.

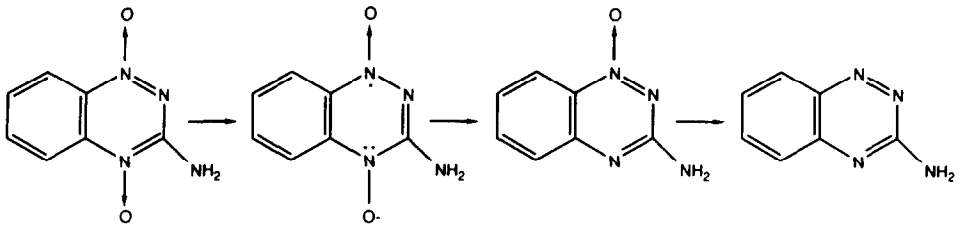

Fig. 1. Reductive metabolism of SR 4233. One-electron reduction forms the nitroxide free radical which is the putative DNA-damaging species [7, 10]. Further one-electron reduction or disproportionation can form the two-electron reduced product SR 4317. This is the major detectable reduction product when SR 4233 is reduced under hypoxic conditions by intact cells, cell or tissue homogenates and most purified reductases, and is an inactive metabolite [1–10]. SR 4233 may undergo a "direct" two-electron reduction to SR 4317 and more predominantly a "direct" four-electron reduction to another inactive metabolite SR 4330 when the reductive metabolism is catalysed by purified DT-diaphorase or cell homogenates rich in this enzyme [5, 13]. The current view is that reactions favouring the formation of the one-electron reduced nitroxide radical will be bioactivating in nature, whereas those bypassing this radical will be bioprotective [68].

cellular defence mechanisms [11]. The differential reactivity of the nitroxide radical under aerobic and hypoxic conditions is thought to be responsible for the highly selective hypoxic cytotoxicity of SR 4233.

Studies in our laboratory using CO and an inhibitory antibody to cytochrome P450 reductase as individual diagnostic probes, have shown that this enzyme contributes around 30% of the microsomal SR 4233 reductase activity while cytochrome P450 contributes the remaining 70% [12]. Moreover, the combination CO and antibody eliminates essentially all microsomal SR 4223 reductase activity. Further work with selective inducers of cytochrome P450 enzymes suggested that SR 4223 may be reduced by members of the Cyp2b, Cyp2c and Cyp3a subfamilies* [12]. However, the precise quantitative involvement of each of these subfamilies was not determined in these studies.

The clinical effectiveness of SR 4233 may be governed by a number of physiological and biochemical differences between hypoxic tumour and normal tissues. In addition to the differential oxygen tensions, the relative expression of potential detoxifying and activating cellular reductases may profoundly influence the inter-individual toxic and/or therapeutic responses to the drug. Most reductases would be expected to catalyse one-electron activation to the nitroxide free radical, although this also leads in turn to the formation of the two-electron reduction product SR 4317 (3-amino-1,2,4-benzotriazine-1-oxide) either by radical disproportionation or a second one-electron reduction (Fig. 1). Enzymes catalysing one-electron reductive activation include cytochrome P450, cytochrome P450 reductase, xanthine oxidase and aldehyde oxidase [3–7]. By contrast, DT-diaphorase is an obligate two-electron reducing enzyme and has been shown to metabolize SR 4233 directly to both two- and four-electron reduced products [SR 4317 and SR 4330 (3-amino-1,2,4-benzotriazine), respectively]: it may thus operate as a cellular defence mechanism [5, 13]. The comparative levels of this enzyme in tumour and normal tissues have been investigated extensively [14–16]. The characterization of the particular cytochrome P450 forms responsible for the metabolism of SR 4233 and the quantitation of their expression in potential target tumours are areas which now require further attention, prior to the establishment of clinically applicable "tumour enzyme profiling". We believe that such studies are an essential component of the rational development of the drug and could be used to guide both patient selection and further drug development.

A useful means of assessing the relative contribution of different forms of cytochrome P450 to the metabolism of a given substrate is through the combined use of diagnostic isozyme-selective chemical inhibitors and epitope-specific inhibitory antibodies. The present study has attempted to combine this approach with hepatic enzyme induction by dexamethasone in an attempt to provide an initial characterization of the cytochrome P450 subfamilies which may be predominantly responsible for the metabolism of SR 4233. Dexamethasone was selected because it acts as a prototype inducer of members of the P450 Cyp2b, Cyp2c and Cyp3a subfamilies in mice [17]. Although we recognize that there are inherent complexities in the use of diagnostic inducers, inhibitors and antibodies, particularly with respect to species variation [18], the results obtained constitute a valuable basis for future studies, as well as providing further confirmation of the involvement of cytochrome P450 in SR 4233 metabolism [5, 12].

* Note that throughout the manuscript we have adopted the cytochrome P450 nomenclature recommended by Nebert *et al.* (Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fuji-Kuriyama Y, Gonzalez F, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR and Waxman DJ, The P450 superfamily: update on new sequences, gene mapping and recommended nomenclature. *DNA Cell Biol* **10**: 1–14, 1991).

MATERIALS AND METHODS

Chemicals and reagents. 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.). SKF 525A (proadifen hydrochloride) was provided by SmithKline and French Research Ltd (Welwyn, U.K.). The *N*-oxide HPLC internal standard 4-nitroquinoline *N*-oxide and all other chemicals (of the highest grade commercially available) were purchased from the Sigma Chemical Co. (Poole, U.K.). Zero grade nitrogen (<5 vpm oxygen) was obtained from the British Oxygen Co. (London, U.K.) and research grade CO from BDH Ltd (Poole, U.K.). Electrophoresis reagents were obtained from Bio-Rad Laboratories Ltd (Watford, U.K.). Horseradish peroxidase-conjugated protein A, nitrocellulose membranes (Hybond C Super) and chemiluminescence reagents were purchased from Amersham International plc (Aylesbury, U.K.). Rabbit polyclonal antibodies against rat cytochrome P450 CYP2B1 and CYP3A were obtained from Oxygene (Dallas, TX, U.S.A.). Anti-cytochrome P450 monoclonal antibodies (MAbs) produced by the hybridoma technique at the U.S. National Cancer Institute Laboratory of Molecular Carcinogenesis (Bethesda, MD, U.S.A.) and tested and characterized as described previously [19–22] were generously donated by Dr H. V. Gelboin. All solvents used were HPLC grade (Fisons plc, Loughborough, U.K.).

Isolation of hepatic microsomes. Male BALB/c mice (18–21 g) were administered dexamethasone (100 mg/kg) by i.p. injection in corn oil once daily for 4 days. A control group of animals received the equivalent dose volume of vehicle (corn oil; 10 mL/kg) once a day for 4 days. Following the final injections the mice were fasted for 24 hr. The animals were then killed by cervical dislocation, their livers removed and weighed and washed microsomes prepared by standard differential ultracentrifugation procedures as reported previously [23]. The microsomes were stored as intact pellets or concentrated suspensions (20 mg/mL protein) at −70°. Total cytochrome P450 content was measured by the method of Omura and Sato [24] and microsomal protein was determined using the dye-binding method of Bradford [25]. NADPH: cytochrome P450 reductase activity was measured by the reduction of cytochrome *c*, monitored at 550 nm using an extinction coefficient of 21 mM/cm according to the method of Yasukochi and Masters [26]. Microsomal DT-diaphorase levels were quantified as reported previously [13] with NADH (2 mM) as cofactor, menadione (20 μM) as the intermediate electron acceptor and cytochrome *c* (77 μM) as the terminal electron acceptor, using a modified version of the assay described by Ernster [27]. The specific activity of the microsomal enzyme was taken as that fraction of the menadione-coupled cytochrome *c* reductase activity which was inhibited by dicoumarol (10 μM) [13].

SDS–PAGE and immunoblot analysis. SDS–PAGE was conducted according to Laemmli [28] on 10% resolving mini-gels (5.0 cm long, 1.5 mm thick) using a Mini-Protean II Dual Slab electrophoresis unit (Bio-Rad). Microsomal samples were diluted in sample buffer, boiled for 5 min and loaded at 5–20 μg protein per 5 mm lane. OXYgene standards were processed in a similar fashion and used according to the manufacturer's instructions. The samples were electrophoresed at 50 mA for 60 min and transferred electrophoretically to nitrocellulose (Hybond C Super, Amersham) with 0.8 mA/cm^2 for 45 min using a Semi-dry Electroblot Apparatus (Electrophoresis Systems, Biotechnology Instruments Ltd, Beds, U.K.) and a buffer consisting of 48 mM Tris, 39 mM glycine, 0.0375% SDS and 20% methanol.

Verification of protein transfer and the location of molecular mass markers was determined by staining the nitrocellulose membrane with Ponceau S according to the manufacturer's instructions (Sigma, Poole, U.K.). The blot was then immersed in blocking buffer [10 mM Tris, 154 mM NaCl, 0.1% (w/v) thimerosal and 0.2% (w/v) Tween 20] containing 3% (w/v) skimmed milk powder and gently rocked for 2 hr at room temperature to block any non-specific protein binding sites. The blocking buffer was then decanted from the nitrocellulose sheets and replaced with a 1/100 dilution of rabbit polyclonal anti-cytochrome P450 antibodies. Following an overnight incubation at 4°, the antibody solutions were removed and the blots washed three times in blocking buffer.

Enhanced chemiluminescence detection was used to visualize bound antibody as reported previously [29]. Horseradish peroxidase-conjugated protein A was diluted 1/5000 in blocking buffer containing 3% skimmed milk powder and incubated with the blots for 15 min at room temperature. The blots were washed as described above and then incubated for 1 min at room temperature with equal volumes of the enhanced chemiluminescence reagents and exposed to Kodak X-OMAT AR Scientific Imaging Film (IBI Ltd, Cambridge, U.K.) for 15 sec to 2 min. Band intensities were quantified using a Joyce-Loebl Sandig Densitometer (Gateshead, U.K.).

Erythromycin and benzphetamine N-*demethylase activities.* These isozyme-specific cytochrome P450 marker activities were determined at 37° in an incubation mixture containing 1 mM substrate, 0.7 mg microsomal protein and 1.25 mM semicarbazide hydrochloride in a final volume of 2 mL 100 mM Tris–HCl buffer, pH 7.4. The reaction was initiated by the addition of 1 mM NADPH, conducted for 15 min and the rate of formaldehyde formation measured according to the method of Nash [30]. For experiments to determine the effects of SR 4233 (5 mM) on cytochrome P450 marker activities, semicarbazide was omitted from the incubation mixture and the rate of formaldehyde formation was measured, as described by Sawicki *et al.* [31].

SR 4233 metabolism. Reductive metabolism of SR 4233 was conducted under N_2 or CO at 37° in specially adapted 25 mL Ehrlenmeyer flasks as detailed previously [5, 13]. Incubation mixtures were pre-gassed for 5–7 min at 37° with humidified zero grade N_2 which had been deoxygenated further by passage through a 15% (w/v) alkaline solution of pyrogallol at a flow rate of 50 mL/min. Aerobic metabolism was conducted in flasks or test-tubes

open to air. CO inhibition was examined by bubbling the reaction mixtures with the gas for 1 min at 0–4° prior to the addition of substrate; the flasks were then immediately stoppered and the reaction initiated. Studies with the same CO method using purified xanthine oxidase as the enzyme source have ruled out the possibility that inhibition might be due to residual oxygen rather than CO [12]. All reactions were carried out under conditions shown to be linear with respect to microsomal protein and time and at which cofactors were non-limiting [5, 12]. Standard incubations contained 0.1 mg microsomal protein and 1 mM NADPH in a total volume of 50 mM Tris–HCl buffer, pH 7.4. Reactions were started by the addition of substrate in 10 μL dimethyl sulphoxide (DMSO) (SR 4233; 1.5 mM final concentration) through air-tight seals. Aliquots (100 μL) of the reaction mixture were removed at 3–5 consecutive time points and added to two volumes of methanol containing internal standard (4-nitroquinoline *N*-oxide, 25 mg/L). Samples were then centrifuged at 4° (1700 *g* for 5 min) and the supernatants analysed by HPLC.

HPLC analysis of SR 4233 metabolism. SR 4233 and its stable, reduced metabolites were analysed by isocratic reverse-phase HPLC as described previously [5, 13, 32]. Chromatography was carried out using modular HPLC equipment incorporating a WISP 712 autosampler, a model 600WE system controller with quaternary pump, and a model 990 photodiode array detector, all from Waters Associates (Milford, MA, U.S.A.). Separation was achieved on μBondapak Rad-Pak phenyl columns (8 mm × 10 cm; 10 μm beads) in a RCM 8 × 10 compression unit (Waters). The mobile phase consisted of 33% methanol in water and was delivered at a flow rate of 2 mL/min. Routine detection was at 254 nm and drug and metabolites were identified by chromatographic and spectral analysis with reference to authentic standards. Quantitation was by peak area ratio compared to standards spiked into appropriate blank sample material.

Chemical and immunoinhibition of SR 4233 metabolism. Incubations and analyses were performed as described above with 1.5 mM SR 4233, except that various diagnostic cytochrome P450 substrates/inhibitors were added at concentrations of 5–2500 μM. Where appropriate, control experiments were conducted with the same amount of buffer or vehicle (DMSO or methanol, 0.5% v/v) required for solubilization of the test compounds. Following the establishment of preliminary dose–response curves, the cytochrome P450 inhibitors were used at concentrations which have been chosen routinely to monitor prototype cytochrome P450 isozyme-dependent activities.

Immunoinhibition experiments were conducted in a similar way, using four mAbs which have been shown to be specific towards different cytochrome P450s. These were: clones 1-7-1, 4-29-5, 1-91-3 and 1-68-11 (Table 3), as described by Park and colleagues [19–22]. A control mAb (Hy-Hel) against chicken lysozyme was used to determine any non-specific reaction. Pooled microsomes were incubated with the reference protein (Hy-Hel) or mAb at a ratio of 1:1 on ice for 15 min prior to the initiation of the metabolism of SR 4233 by adding NADPH and substrate. A limited supply of these inhibitory antibodies precluded the establishment of full concentration–effect curves and hence these conditions which have been shown to be optimal for several substrates [33 and references therein] were employed throughout.

Statistical analyses. Mean values between control and test groups were compared by Student's *t*-test. The 0.05 level of probability was chosen as the criterion of significance.

RESULTS

Treatment of male BALB/c mice with dexamethasone (100 mg/kg i.p. for 4 days) increased the total hepatic cytochrome P450 levels from 0.65 ± 0.03 to 1.83 ± 0.20 nmol/mg microsomal protein (mean ± SE, N = 4; $P < 0.01$). This increase in spectrally determined cytochrome P450 was accompanied by a shift in the Soret maximum by 1–2 nm towards the blue as reported previously [34]. There were no significant effects on either the total body weight, mass of excised livers or the yield of microsomal protein in dexamethasone-treated animals compared with vehicle (corn oil)-treated controls (Table 1).

Under the conditions chosen, the reduction of SR

Table 1. General characteristics and specific reductase activities in hepatic microsomes from male BALB/c mice treated with corn oil (controls) or dexamethasone (induced)

	Control	Induced
Body mass (g)	22.17 ± 0.33	21.18 ± 0.46
Liver mass (g)	0.95 ± 0.04	1.40 ± 0.20
Microsomal protein (mg/g liver)	16.34 ± 2.47	16.99 ± 1.61
Total cytochrome P450 content (nmol/mg)‖	0.65 ± 0.03	1.83 ± 0.20†
DT-diaphorase (nmol‡/min/mg)‖	139.1 ± 18.8	215.5 ± 28.2*
NADPH: P450 reductase (nmol‡/min/mg)‖	182.1 ± 11.4	223.0 ± 17.4
SR 4233 reductase (nmol§/min/mg)‖	181.7 ± 8.9	371.7 ± 31.1

Values are mean ± SE, N = 4–16 animals.
* Significantly different from control values, $P < 0.05$.
† Significantly different from control values, $P < 0.01$.
‡ Cytochrome *c* reduced.
§ SR 4317 formed.
‖ Microsomal protein.

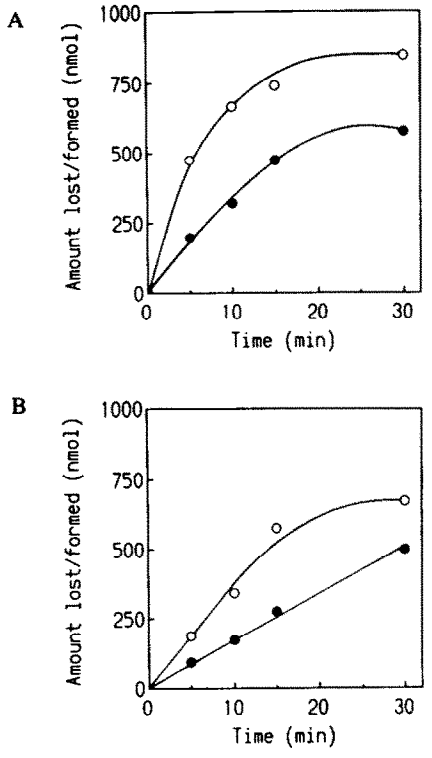

Fig. 2. Typical reaction progress curves for the reduction of SR 4233 (1.5 mM) by (A) control and (B) dexamethasone-induced mouse hepatic microsomes. Full experimental details are given in Materials and Methods. The curves show the rate of substrate loss (open symbols) and the rate of formation of the two-electron reduction product SR 4317 (closed symbols) and are from one experiment representative of four independent repeats (SE <10%).

4233 to SR 4317 (the major detectable stable metabolite) by control mouse hepatic microsomes was linear up to 30 min (Fig. 2A). Parent drug loss was linear up to 15 min. The reaction stoichiometry of parent loss: metabolite (SR 4317) formation was similar to that reported previously for C3H mice [3, 5] at 2.5 ± 0.2 (mean ± SE, N = 3). Table 1 shows the hepatic enzyme induction by dexamethasone resulted in a 2-fold increase in the rate of SR 4317 formation (371.7 ± 31.1 vs 181.7 ± 8.9 nmol/min/mg; mean ± SE, N = 4, $P < 0.01$). However, metabolite formation was linear only up to 15–20 min and parent loss only to 5–10 min with dexamethasone-induced mouse liver microsomes (Fig. 2B). During the initial linear phase of the reduction profiles, the reaction stoichiometry for dexamethasone-induced mouse liver microsomes was similar to controls at 2.6 ± 0.2 (mean ± SE, N = 3). Increased levels of the four-electron reduced metabolite (SR 4330) were also observed in incubations conducted with dexamethasone-induced mouse liver microsomes, although the amounts detected were <4% of the levels of SR 4317. Typical HPLC profiles are shown in Fig. 3. The remaining SR 4233 is believed to be lost on bioreduction at the level of the one-electron radical [12].

The reduction of SR 4233 by microsomes from control or induced animals was entirely dependent on the presence of NADPH and active microsomes, and was almost completely inhibited by oxygen. The cofactor requirements for the reductive metabolism of SR 4233 have been reported in detail elsewhere [3, 5]. The reaction was inhibited by CO in both control (% inhibition = 70 ± 19; mean ± SE, N = 3) and induced (% inhibition = 74 ± 11) microsomes, indicating the major role of cytochrome P450 in this biotransformation with both types of microsomes. This was also confirmed by experiments with the established cytochrome P450 inhibitor SKF 525A using control microsomes. At an inhibitor concentration of 2.5 mM, previously activated for 8 min with the microsomes under aerobic conditions, the inhibition by SKF 525A was 58 ± 5% (N = 3). Subsequent experiments were aimed at identifying the subfamilies of cytochrome P450 which were predominantly responsible for catalysing SR 4233 reduction.

Despite the fact that the CO and SKF 525A data were consistent with a major role for cytochrome P450 reduction, it was important to consider the possible involvement of other microsomal enzymes, especially for induced microsomes. Dexamethasone produced a modest (23%) and in fact statistically non-significant increase in the activity of hepatic microsomal NADPH: cytochrome P450 reductase (Table 1). Although previous data have indicated that cytochrome P450 reductase may reduce SR 4233 directly in the absence of cytochrome P450 [3, 4], this increase was not sufficient to account for the enhanced rate of SR 4317 formation seen in induced microsomes. However, dexamethasone also produced a significant elevation in the levels of microsomal DT-diaphorase [NAD(P)H: (quinone acceptor) oxidoreductase] (139.1 ± 18.8 to 215.5 ± 28.2 nmol/min/mg). This enzyme too is able to reduce SR 4233 [13]. However, from the marked inhibition of the microsomal reduction by oxygen and the resultant metabolite profile (i.e. the predominant formation of the two-electron reduced metabolite SR 4317 rather than the four-electron reduced product SR 4330, see Ref. 13) it was clear that DT-diaphorase was not significantly involved in the anaerobic microsomal reduction of SR 4233. In further support of this view, dicoumarol (10 μM), a potent inhibitor of NAD(P)H: (quinone acceptor) oxidoreductase [27], did not inhibit significantly the reductive metabolism of SR 4233 by hepatic microsomes from either control or dexamethasone-treated animals (data not shown). Subsequent studies therefore concentrated on the chemical and immunoinhibition of specific cytochrome P450 isozymes.

Experiments with metyrapone and α-naphthoflavone in control microsomes showed that the reduction of SR 4233 to SR 4317 was preferentially inhibited by metyrapone, suggesting that this reaction was predominantly mediated by phenobarbitone-inducible cytochrome P450 enzymes. Metyrapone (1 mM) inhibited the formation of SR 4317 by ≥70%, indicating that essentially all of the CO-sensitive microsomal SR 4233 reductase activity was blocked. In contrast, the maximal inhibition produced by α-naphthoflavone (1 mM) was around

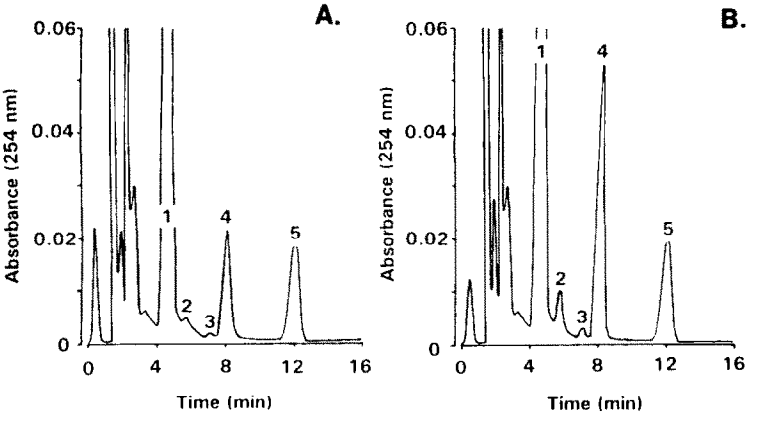

Fig. 3. Representative HPLC chromatograms of SR 4233 and its metabolites from incubations containing 1.5 mM substrate, 1 mM NADPH and 0.1 mg microsomal protein from control (trace A) or induced (trace B) animals. HPLC conditions are given in the text. Incubations were conducted under nitrogen for 10 min and stopped and processed as reported in Materials and Methods. Peaks correspond to SR 4233 (1), an unidentified metabolite (2), SR 4330 (3), SR 4317 (4) and the internal standard (4-nitroquinoline *N*-oxide) (5).

30% of the microsomal activity or 43% of the cytochrome P450-associated activity. These data are consistent with the view that cytochrome P450 isoforms of the mouse Cyp2b, Cyp3c and Cyp3a, but not the Cyp1a families, might be the main enzymes involved.

Further experiments with a range of isozyme-selective chemical inhibitors of cytochrome P450 are summarized in Table 2. Considering first the control microsomes, the macrolide antibiotic erythromycin, a substrate and competitive inhibitor for members of the rat CYP3A subfamily [35], did not inhibit the reduction of SR 4233. Indeed, this compound actually stimulated the metabolism of SR 4233 to SR 4317 up to 167%. This effect was dose-dependent up to an inhibitor concentration of 5 mM (data not shown). Tolbutamide, a substrate for the rat CYP2C enzymes and probably other phenobarbitone-inducible members of the cytochrome P450 superfamily [36, 37], exerted no significant effect on the metabolism of SR 4233. *p*-Nitrophenol, a substrate and selective inhibitor of the rat CYP2E1 [38], was also without effect.

Studies with epitope-specific, inhibitory mAbs in control microsomes showed that at an antibody: microsomal protein ratio of 1 : 1 none of the antibodies investigated appeared to inhibit significantly the reductive metabolism of SR 4233 (Table 3). Indeed, as noted for some of the putative chemical inhibitors, the monoclonal antibodies appeared to stimulate the reaction, a phenomenon also observed with different cytochrome P450 substrates [32, 39]. Hence, members for the Cyp1a and Cyp2e subfamilies did not appear to be responsible for SR 4233 metabolism and the members of the Cyp2b and Cyp2c subfamilies which appeared to catalyse this reaction were not

Table 2. Effect of various chemical inhibitors on the microsomal reduction of SR 4233 to SR 4317

Inhibitor	Concentration (μM)	% Uninhibited activity	
		Control	Induced
α-Naphthoflavone	50	84 ± 9	81 ± 5*
Erythromycin	500	167 ± 7†	139 ± 19*
p-Nitrophenol	100	105 ± 6	89 ± 8
Tolbutamide	500	92 ± 6	75 ± 6†
Metyrapone	500	59 ± 9†	68 ± 2†

Microsomes (0.1 mg) from control or dexamethasone-treated (induced) animals were pre-incubated with NADPH (1 mM) under nitrogen in the presence of the putative inhibitor at the concentration indicated. Reactions were initiated by the addition of the substrate (SR 4233; 1.5 mM) through an air-tight seal and incubated for 15 min.

Values represent % control (uninhibited) activities obtained with the appropriate solvent and are mean ± SE of 3–4 independent experiments. None of the solvents exerted an effect on SR 4233 metabolism at the concentration used (<1%, v/v). Uninhibited activities for control and induced microsomes were 181.7 ± 8.9 nmol/min/mg and 371.7 ± 31.1 nmol/min/mg, respectively.

* Significantly different from respective control values, $P < 0.05$.

† Significantly different from respective control values, $P < 0.001$.

Table 3. Effect of four epitope-specific mAbs against cytochrome P450s on the reduction of SR 4233 catalysed by hepatic microsomes from control or dexamethasone-treated male BALB/c mice

mAb	P450	% Uninhibited activity	
		Control	Induced
1-7-1	CYP1A1/2	159, 166 (162)	93, 139 (116)
4-29-5	CYP2B1/2	174, 157 (166)	60, 67 (63)
1-91-3	CYP2E1	178, 138 (158)	96, 106 (101)
1-68-11	CYP2C11/6	125, 138 (131)	84, 80 (82)

Values represent % remaining activity expressed as (activity with mAb/activity with Hy-Hel control) ×100% and are from individual experiments performed in duplicate. Uninhibited activities refer to the rates of reduction observe with Hy-Hel; at the same concentration used for the mAbs and were 172.8 and 193.0 nmol/min/mg for control microsomes and 321.8 and 317.6 nmol/min/mg for induced microsomes. The rat cytochrome P450s recognized by the mAbs are indicated.

inhibited significantly by antibodies 4-29-5 (or 2-66-3) and 1-68-11, suggesting that these antibodies were not cross-reactive towards the particular constitutive mouse enzymes. However, given the inhibitory effect of metyrapone and the lack of inhibition by anti-CYP2B1/2 antibodies, a role for constitutive Cyp2a enzymes cannot be entirely excluded. This possibility was not examined further but may be addressed in future studies.

Turning to the induced microsomes, western immunoblot analysis showed that the elevation in total cytochrome P450 content of the livers of dexamethasone-treated mice was largely attributable to an increase in the specific hepatic content of Cyp3a-type enzymes by 7.4-fold (Fig. 4A). This increase in the levels of immunoquantifiable protein was paralleled by a similar enhancement in the activity of erythromycin *N*-demethylase, a specific substrate probe for members of the CYP3A subfamily [35] (Fig. 5). However, the CYP3A substrate and competitive inhibitor erythromycin [35] did not inhibit the reduction of SR 4233 catalysed by dexamethasone-induced mouse microsomes (Table 2), indicating that the steroid-inducible cytochrome P450 enzyme(s) efficient in the reduction of SR 4233 was probably not a Cyp3a enzyme. Similar results were obtained in experiments where erythromycin was pre-incubated with NADPH-fortified microsomes in air. In addition, cyclosporin A, another substrate and competitive inhibitor for CYP3A [40], was also without effect at a concentration of 100 μM (data not shown). Both α-naphthoflavone and *p*-nitrophenol exhibited modest and variable effects, suggesting at most a minor role for Cyp1a1/2 and Cyp2e1. Metyrapone inhibited the reduction of SR 4233 to SR 4317 by induced microsomes to a similar extent to that observed for control microsomes (30–40%). Interestingly, the CYP2C substrate tolbutamide appeared to inhibit the metabolism of SR 4233 in induced microsomes only.

Immunoinhibition experiments with dexamethasone-induced mouse microsomes showed that the 4-29-5 (CYP2B-specific) and 1-68-11 mAbs (CYP2C11-specific) both inhibited the formation of SR 4317 (Table 3). Results similar to those with 4-29-5 were also obtained with another anti-CYP2B1/2 antibody, 2-66-3 (data not shown). Inhibition with 4-29-5 (37%) was greater than for 1-68-11 (18%), suggesting a major role for Cyp2b enzymes. Note that since the CO inhibition experiments showed that 70% of the microsomal rate was attributable to cytochrome P450, the antibody inhibition data indicated that the isoforms recognized by the anti-CYP2C11 antibody contributed at least 26% of the total cytochrome P450-related SR 4233 reductase activity, while those recognized by the anti-CYP2B antibody contributed at least 53% of this activity. Western immunoblot analysis confirmed that treatment with dexamethasone increased the levels of CYP2B-related proteins 1.8-fold (Fig. 4B). This was accompanied by a similar increase in the activity of benzphetamine *N*-demethylase, a reaction diagnostic for members of the CYP2B subfamily [41] (Fig. 5). With respect to the involvement of a dexamethasone-inducible mouse Cyp2c enzyme in the reactive metabolism of SR 4233, mAb 1-68-11 has been shown to cross-react with rat P450 CYP2C6, an inducible member of the subfamily in this species [42].

The major involvement of Cyp2b but not Cyp3a isoforms was further tested by determining the effect of SR 4233 on microsomal N-demethylation of benzphetamine and erythromycin, by dexamethasone-induced microsomes under aerobic conditions. These have been used routinely as diagnostic reactions for CYP2B and CYP3A, respectively, in the rat. Despite its relatively high K_m values for microsomal reduction (around 1 mM [5]), SR 4233 at 5 mM was found to inhibit benzphetamine N-demethylation (% activity remaining = 76.3 ± 1.4%, n = 3). By contrast, the same concentration of SR 4233 did not inhibit erythromycin N-demethylation, but in fact stimulated this reaction (126.6 and 128.7% of control activity in two repeat experiments). This provides further support for the interaction of SR 4233 with Cyp2b but not Cyp3a isoforms.

DISCUSSION

Previous studies have shown that the developmental antitumour agent SR 4233 exerts highly selective toxicity towards hypoxic cells as a result of metabolic activation to an oxidizing drug free radical [10]. Reductive bioactivation by both buttermilk xanthine oxidase [10] and rat cytochrome P450 reductase (unpublished data) results in the generation of a species which induces strand breaks in co-incubated plasmid DNA. While a variety of cellular reductases are able to catalyse the reductive metabolism of SR 4233 [3–7, 10], cytochrome P450 and cytochrome P450 reductase have been reported to be the major enzymes responsible for this reaction in both rodent hepatic microsomes and certain tumour cells *in vitro* [3–7, 12]. Although two different laboratories have shown unequivocally that cytochrome P450 reductase can reduce SR 4233 [4, 12] there remains a degree of debate as to the precise role of cytochrome P450.

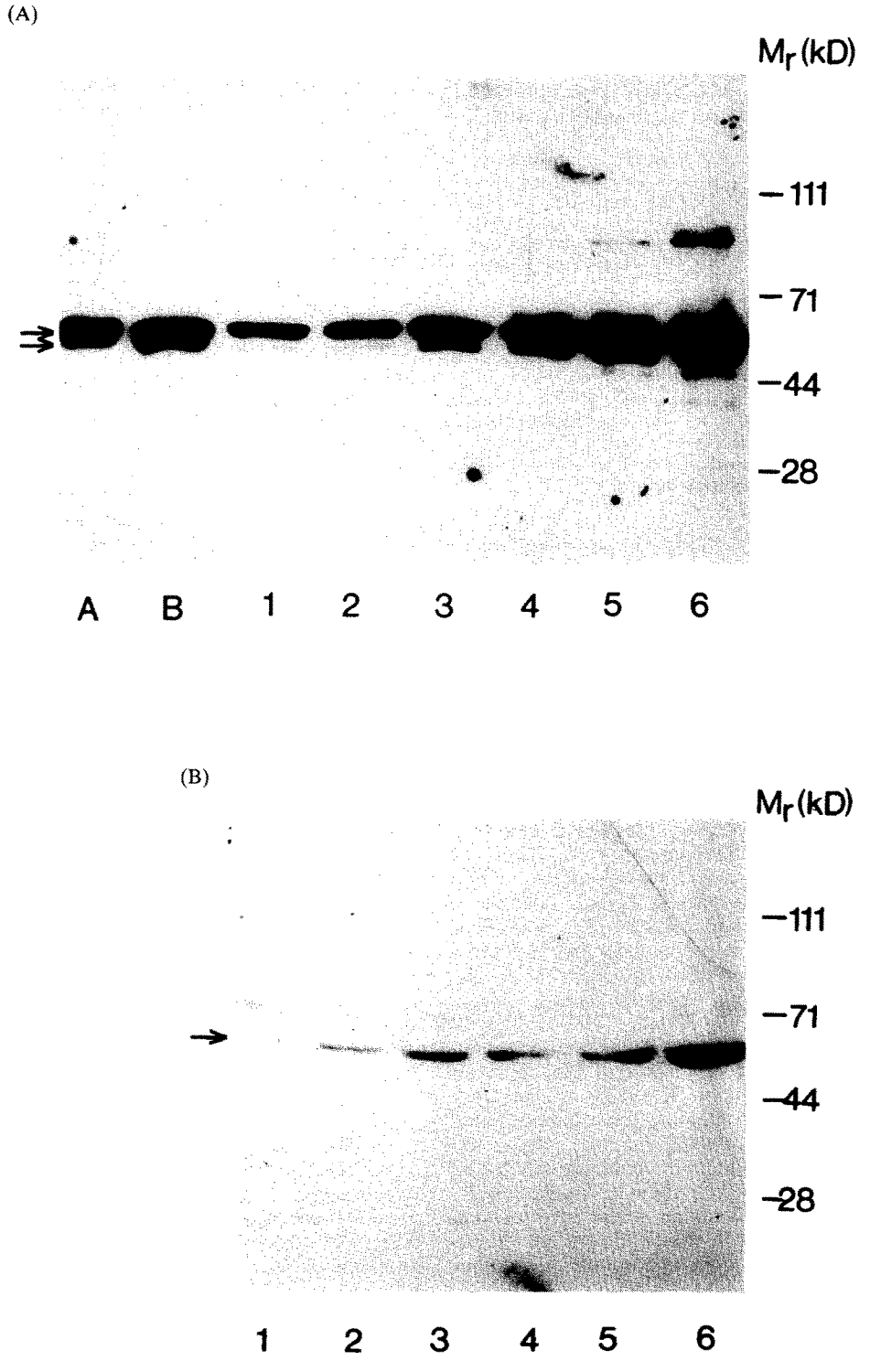

Fig. 4. Western immunoblot analysis of CYP3A- and CYP2B-related proteins in hepatic microsomal fractions from control and dexamethasone-induced animals. (A) Microsomal proteins from control and induced animals were separated on 10% acrylamide gels, transferred to nitrocellulose, blocked and immunoreacted with polyclonal anti-rat CYP3A. Lanes A and B contain 5 and 10 μL of OXYgene standards. Lanes 1, 2 and 3 contain 5, 10 and 20 μg hepatic microsomal protein from control animals and lanes 4, 5 and 6 contain 5, 10 and 20 μg protein from the livers of induced animals. The arrows indicate the positions of the major CYP3A-related proteins detected. The estimated M_r of the major band was 53,000. The relative positions of the molecular mass markers are shown to the right of the blot. (B) As above but immunoreacted with polyclonal anti-rat CYP2B1 antibody. The arrow signifies the position of the major band induced by dexamethasone which had a M_r of 54,000.

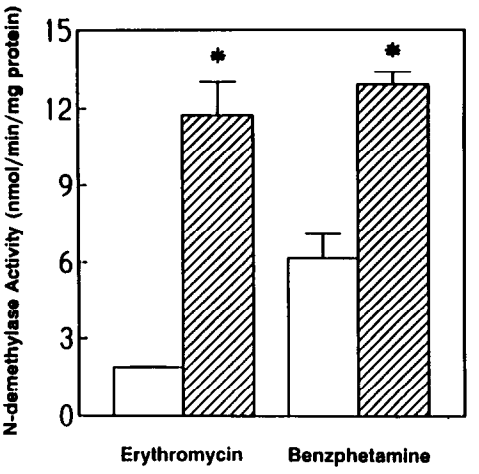

Fig. 5. Effect of hepatic enzyme induction by dexamethasone on marker microsomal monooxygenase activities. Hepatic microsomes from corn oil (open columns) or dexamethasone-treated animals (shaded columns) were assayed for their capacity to catalyse the oxidation of the prototype cytochrome P450 substrates erythromycin and benzphetamine as described in the Materials and Methods. Values are mean ± SE of four independent experiments and are expressed as nmol/min/mg microsomal protein. *Significantly different from values obtained with control microsomes, $P < 0.001$.

Data from our own laboratory have consistently shown that the reduction of SR 4233 by murine hepatic microsomes can be inhibited 70% by the cytochrome P450-specific inhibitor CO [3, 5, 12]. The results of the present study support entirely these earlier findings. Moreover, we show here that the cytochrome P450 inhibitor SKF 525A (2.5 mM), following aerobic preincubation, also inhibited microsomal SR 4233 reduction by 58% and the cytochrome P450-associated component by 83%. Our earlier studies had suggested that this agent was not inhibitory but, in contrast to the present experiments, these did not involve an aerobic preactivation of SKF 525A [12]. Moreover, studies with an inhibitory antibody to cytochrome P450 reductase and incubations conducted with known activities of the purified rat reductase have demonstrated that the residual 20–30% of the total reductase activity can be attributed directly to cytochrome P450 reductase; furthermore, combined use of the reductase antibody and CO produce complete abolition of microsomal SR 4233 reduction [13]. Thus cytochrome P450 and its reductase together account for essentially all the microsomal SR 4233 reductase activity. In contrast to the situation with purified rat DT-diaphorase [13], we found that in both control and dexamethasone-induced mouse liver microsomes the reaction displayed a cofactor preference for NADPH, a low level of production of the four-electron reduction product SR 4233, and was not significantly inhibited by the DT-diaphorase inhibitor dicoumarol [27]. Thus although, as found in other studies [43, 44], the mouse hepatic microsomes we used expressed quite high DT-diaphorase activity it does not appear to play a significant role in SR 4233 reduction by this preparation. A study by Cahill and White [4] compared the reduction of SR 4233 by purified rat liver cytochrome P450 reductase with that of rat liver microsomes and concluded that cytochrome P450 reductase was the major participating enzyme. However, the amount of reductase used in their study was greatly in excess of that measured in rat hepatic microsomes (>6-fold). A more recent study by Lloyd *et al.* [7] also proposed that cytochrome P450 reductase was the major SR 4233-activating enzyme in rat liver microsomes based on the measurement and chemical inhibition of nitroxide radical production. The precise reasons for the apparent discrepancies remain obscure. However, several possibilities may be put forward.

Lloyd *et al.* [7] suggested that the formation of SR 4317 during the microsomal reduction of SR 4233 may proceed by disproportionation of the nitroxide radical produced via one-electron reduction. It is conceivable, however, that cytochrome P450 enzymes may catalyse two rapid sequential one-electron reduction steps, thereby not liberating appreciable levels of the toxic drug radical [7]. If this latter mechanism were correct, metabolism of SR 4233 by cytochrome P450 could be regarded as a detoxication mechanism and this may help to explain the failure of cytochrome P450 inhibitors to block SR 4233 nitroxide radical formation. An alternative explanation might be a species difference in the expression of the cytochrome P450 enzymes which catalyse the reductive metabolism of SR 4233, as the work of Cahill and White [4] and Lloyd *et al.* [7] employed rat liver microsomes, whereas other studies have used tissues derived from mouse liver [3, 5, 12] or human cell lines [6]. It is interesting to note here that such a marked difference has been reported for coumarin hydroxylase (involving CYP2A in humans and Cyp2a in the mouse): this enzyme is expressed at relatively high levels in both mice and humans but virtually no activity is found in the rat [45]. Such an explanation seems less likely but may warrant further investigation.

The present study was conducted in an attempt to obtain an initial assessment of involvement of cytochrome P450 enzymes of the Cyp2b, Cyp2c and Cyp3a subfamilies in the reductive metabolism of SR 4233 in mouse liver. Previous data from hepatic enzyme induction studies conducted in our laboratory had suggested a major role of such isoforms [12]. Calculation of the apparent turnover number for the reduction of SR 4233 to SR 4317 showed that β-naphthoflavone-inducible members of the Cyp1a subfamily were less efficient at catalysing this reaction than constitutive enzymes. By contrast, both phenobarbitone and pregnenolone 16 α-carbonitrile appeared to increase the rates of reduction of SR 4233 through the induction of enzymes which were at least as effective as constitutive forms [12]. The experiments described in this report used hepatic enzyme induction by dexamethasone, a potent inducer of members of the Cyp2c, Cyp2b and Cyp3a subfamilies in mice, and a combination of chemical and immunoinhibition studies to delineate further which cytochrome P450 enzymes are predominantly active in the reductive metabolism of SR 4233.

Our previous studies have shown that type II ligands, such as *n*-octylamine and 2,4-dichloro-6-phenylphenoxyethylamine, are capable of inhibiting the cytochrome P450-mediated reduction of SR 4233 [12]. In the present studies, screening with hepatic microsomes from control animals showed that metyrapone (another type II ligand) was a more potent inhibitor of SR 4233 metabolism to SR 4317 than was α-naphthoflavone. This further suggested that phenobarbitone-inducible Cyp2a, Cyp2b, Cyp2c and Cyp3a isoforms might be predominantly involved in this biotransformation whereas the Cyp1a family was not clearly implicated. It should be noted that metyrapone may also be reduced by carbonyl reductases in both rodent [46] and human [47] hepatic microsomal fractions. These enzymes can operate effectively with either NADH or NADPH as the cofactor and are inhibited by dicoumarol [46, 47]. However, since the microsomal reduction of SR 4233 showed a marked preference for NADPH over NADH and was unaffected by dicoumarol, the participation of such enzymes can be excluded. We may therefore conclude that the metyrapone-sensitive microsomal SR 4233 reductase is a member of the cytochrome P450 superfamily. The subsequent experiments were designed to look more closely at the relative participation of the various possible isoforms.

With control mouse hepatic microsomes, neither erythromycin nor cyclosporin, substrates and competitive inhibitors for members of the rat CYP3A subfamily [35, 40], were inhibitory towards SR 4233 reduction. Indeed, erythromycin appeared to stimulate the metabolic reduction of SR 4233 when co-incubated. However, the mechanism of reduction of CYP3A-type enzymes by erythromycin may involve the production of a mechanistic/suicidal inhibitor via oxidation of the substrate to a reactive nitroso derivative [48]. Experiments were therefore performed in which erythromycin (0.5–2 mM) was pre-incubated with NADPH-fortified microsomes in air (for 5–10 min) prior to incubation with additional NADPH and SR 4233 under nitrogen. The results of these studies also demonstrated that erythromycin was without effect. The highly selective CYP2E1 inhibitor *p*-nitrophenol [38] and the sensitive CYP2C substrate tolbutamide [36, 37] effected no inhibition of SR 4317 formation, suggesting that CYP2E1- and constitutive CYP2C-like enzymes [36, 37] did not contribute significantly to the metabolism of SR 4233.

Inhibitory mAbs raised against the rat CYP1A, CYP2B, CYP2C and CYP2E subfamily members did not inhibit SR 4317 formation catalysed by control mouse microsomes. The selective inhibition by metyrapone suggests the principal involvement of phenobarbitone-inducible and/or steroid metabolizing enzymes, i.e. members of the Cyp2a, Cyp2b, Cyp2c and Cyp3a subfamilies. The most likely candidates would therefore be a Cyp2a isoform or a mouse orthologue of the rat CYP2B3 [49]. The putative constitutive Cyp2b enzyme may not be inhibited significantly by mAb 4-29-5 and may be functionally distinct from the known members of this subfamily. Recent evidence suggests the existence of such isoforms in rat liver which display differential activity towards the marker CYP2B substrates aminopyrine and benzphetamine [50–52]. Interestingly, aminopyrine also inhibits the reduction of SR 4233 catalysed by mouse hepatic microsomes [12]. Collectively, these results support a role for constitutive Cyp2b enzymes in the reductive metabolism of SR 4233.

Hepatic enzyme induction by dexamethasone resulted in an approximate 2-fold increase in the rate of reduction of SR 4233 (Fig. 2) which is consistent with the theory that this reaction is catalysed by members of the Cyp2b, Cyp2c and/or Cyp3a subfamilies [17]. Characterization of hepatic microsomal reductase activities showed that both cytochrome P450 reductase and DT-diaphorase were increased by this induction protocol. However, the cofactor requirement, the metabolite profile, the lack of significant inhibition by dicoumarol and the marked inhibition of SR 4233 metabolism by CO and other reagents provided clear confirmation that cytochrome P450 (which was increased 2–3-fold) was the major SR 4233 reductase in dexamethasone-induced microsomes, as in the uninduced state described earlier. Chemical inhibition experiments supported the theory that phenobarbitone-inducible enzymes were predominantly involved. Metyrapone (500 μM) inhibited the reaction to a similar extent to that seen in control microsomes (30–40%), whereas α-naphthoflavone produced a modest and variable effect. The macrolide erythromycin also exhibited a stimulatory effect similar to that observed in control microsomes indicating that neither constitutive nor inducible Cyp3a enzymes were involved in SR 4233 reduction. Interestingly, tolbutamide was only inhibitory with dexamethasone-induced but not control microsomes. Tolbutamide hydroxylase has been shown to be a member(s) of the CYP2C subfamily in both humans (2C8,9 and 2C10) [36] and rats [37] and is induced by phenobarbitone in the latter species. It is possible, therefore, that a steroid-inducible member of the Cyp2c subfamily is at least partially responsible for the metabolism of SR 4233 in mouse liver microsomes. The differential inhibition of the metabolism of SR 4233 by these structurally diverse cytochrome P450 substrates provides further support for the involvement of cytochrome P450 enzymes.

Immunoinhibition studies with epitope-specific inhibitory mAbs showed that, although none of these produced any inhibition with control microsomes, both the 4-29-5 (CYP2B-specific) and 1-68-11 (CYP2C11-specific) clones were able to inhibit the reductive metabolism of SR 4233 by microsomes from dexamethasone-induced animals. Hence, these studies suggest that the mouse equivalent to CYP2B1 (or a closely related form) and an inducible CYP2C isoform orthologue (against which the 1-68-11 clone is cross-reactive) are the major cytochrome P450 enzymes responsible for the metabolic reduction of SR 4233 in the steroid-induced state. Quantitative comparison indicated that the isoforms recognized by the anti-CYP2C11 antibody contributed a minimum of 26% of the total cytochrome P450-associated SR 4233 reductase activity in induced microsomes, while those recognized by the anti-CYP2B antibody contributed at

least 53%. As anticipated, dexamethasone markedly increased the levels of CYP3A-related proteins (around 7-fold) and this was accompanied by a similar increase in the erythromycin *N*-demethylase activity of the hepatic microsomal fraction. Despite these impressive elevations, the inhibition experiments with erythromycin and cyclosporin showed that Cyp3a isoforms (constitutive or inducible) were probably not involved in SR 4233 reduction. Members of the Cyp2b subfamily have also been shown to be inducible by dexamethasone in mice but not rats [17]. Western immunoblot analysis confirmed that CYP2B-related proteins were elevated 2-fold following dexamethasone treatment. Again this elevation in immunodetectable protein was reflected in an increase in a prototype CYP2B-related activity, benzphetamine *N*-demethylase [41]. This increase paralleled more closely the acceleration of SR 4233 metabolism to SR 4317 observed in dexamethasone-induced mouse hepatic microsomes compared with controls. The involvement of CYP2B- and CYP3A-like isoforms was examined further by determining the effect of SR 4233 on the aerobic N-demethylation by dexamethasone-induced microsomes of benzphetamine and erythromycin, respectively. Despite its relative high K_m as a substrate for microsomal reduction [5], 5 mM SR 4233 was able to inhibit N-demethylation normally associated with CYP2B but not CYP3A isoforms. Together with the significant inhibition afforded by metyrapone and the CYP2B-specific 4-29-5 mAb in induced microsomes, these data are collectively consonant with the major involvement of inducible Cyp2b enzymes in the reduction of SR 4233 to SR 4317. Members of the Cyp2c subfamily (which may also catalyse weakly benzphetamine oxidation [53]) appear to play a more minor role.

Cytochrome P450A enzymes have been shown to display differing reducing capacities for other substrates such as chromium (VI), azo, nitro and halogenated compounds [54, 55]. One-electron reduction of the model quinone 2,3,5,6-tetramethylbenzoquinone was shown recently to be catalysed by both cytochrome P450 and cytochrome P450 reductase from rat liver [56]. Moreover, of particular interest to the present study with benzotriazines, *N*-alkylaminobenzotriazoles have been shown to act as inhibitors of rabbit pulmonary cytochrome P450 metabolism, including specifically CYP2B-dependent reactions [57].

There are certain caveats which relate to the use of diagnostic inducers, inhibitors and antibodies, especially in relation to species differences [18]. Relatively few studies have been carried out in mice and we have had to make a number of inferences based on information in the rat. However, it was important for us to obtain data in the mouse to aid interpretation of toxicity and efficacy studies which have been primarily conducted in this species. Further complications relate to the relatively limited amount of data on the use of diagnostic reagents for anaerobic reduction *vis-à-vis* aerobic oxidation reactions [54] and the direct reduction of SR 4233 by cytochrome P450 reductase [4, 12]. Nevertheless, we believe that the present results: (1) demonstrate clear confirmation of the involvement of the cytochrome P450 superfamily in the bioreductive metabolism of SR 4233; and (2) provide a useful initial characterization of the particular isoforms which may be involved, particularly members of the Cyp2b and Cyp2c but not Cyp3a subfamilies.

Although it is clear that the above-mentioned cytochrome P450 isozymes are likely to participate to a significant extent in the reduction of SR 4233, it remains to be established whether such metabolism constitutes an activating or detoxifying pathway. This is highly relevant to both the antitumour and normal tissue effects mediated by SR 4233. Given the documented qualitative and quantitative differences in drug metabolism between species, it is now also important to define the principal human SR 4233-metabolizing enzymes. Both cytochrome P450 [58, 59] and cytochrome P450 reductase [4] have been found in nuclei and may thus participate in the reductive bioactivation of the drug to a reactive nitroxide or similar radical in close proximity to the target macromolecule, DNA. Although early data from chemical carcinogenesis models in experimental animals suggested that both cytochrome P450 and its reductase are down-regulated in tumour tissue [60], more recent biopsy analyses have demonstrated that significant levels of CYP2C [60] and CYP3A [61, 62] enzymes do persist in human breast and colorectal cancers and that isoform CYP1A1 can be detected in lung cancers [63]. It is also of interest to the present study that elevated levels of CYP2B enzymes have been noted in nitrosamine-induced preneoplastic hepatic lesions in the rat [64]. However, several reports have suggested that the levels and activities of human CYP2B enzymes are normally very low in human liver and the oxidation of pentoxyresorufin and benzphetamine may thus be mediated predominantly by members of the CYP3A subfamily [65] which are expressed at relatively high levels in normal liver and extrahepatic tissues [66]. Studies are in progress in our laboratory to elucidate the role of cytochrome P450s and other enzymes in the activation and detoxification of SR 4233 in human tumours. Characterization of the major SR 4233 activating and detoxifying reductases in human normal and tumour tissues should aid the interpretation of ongoing clinical trials. We have proposed that tumour "enzyme profiling" could then be used in combination with measurement of tumour oxygen levels to facilitate the selection of patients more likely to respond to the drug [67]. Identification of key SR 4233 reductases may also ultimately lead to the development of improved second generation benzotriazine-*N*-oxides through an "enzyme-directed" approach [67, 68].

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