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Enzymology of the Reduction of the Novel Fused Pyrazine Mono-N-Oxide Bioreductive Drug, RB90740 Roles for P450 Reductase and Cytochrome b_5 Reductase

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ABSTRACT. RB90740 is the lead compound in a series of fused pyrazine mono-N-oxide bioreductive drugs. These agents have potential application in cancer therapy, since they are more toxic to hypoxic than to aerobic cells as a consequence of their bioactivation by cellular reductase enzymes within the hypoxic regions of a tumour. In this study, mouse liver microsomes have been used to characterise the enzymology of the reductive activation of RB90740. Under hypoxic conditions, the reduction of RB90740 to its stable 2-electron reduced product RB92815 was supported by both NADH and NADPH, the former supporting a rate approximately 80% of the latter. Combining the two cofactors had no additive effect. Neither carbon monoxide nor metyrapone inhibited reduction of RB90740, indicating that P450 isozymes were not involved in the reduction of this compound. 2'AMP, an inhibitor of P450 reductase, did not inhibit formation of RB92815, whereas DPIC, another inhibitor but with a different mode of action, inhibited both the NADH and NADPH-dependent reduction of RB90740. Similarly, two selective inhibitors of NADH:cytochrome b_5 reductase, *p*HMB and PTU, completely inhibited both the NADH and NADPH-dependent reduction of RB90740. Our findings implicate P450 reductase, cytochrome b_5 reductase, and cytochrome b_5 in the activation of this compound. However, there is no clear relationship between the intracellular levels of P450 reductase and cytochrome b_5 reductase and the hypoxic toxicity of RB90740, which implies that other factors, in addition to drug activation, play a major role in controlling the toxicity of this particular bioreductive drug. BIOCHEM PHARMACOL 51;6:829–839, 1996.

KEY WORDS. bioreductive drug; P450 reductase; cytochrome b_5 reductase; hypoxia; tumour; pyrazine mono-N-oxide

The relatively poor vasculature of solid tumours gives rise to populations of cells that are in an hypoxic environment. Since the presence of molecular oxygen is a prerequisite for the efficacy of radiation in treating tumours, therapeutic failure can be attributed to the presence of such cells. This is because the cells remain viable and, upon reoxygenation, re-enter cycle, thus becoming foci for tumour regrowth. Bioreductive drugs are targeted at the hypoxic regions of tumours, since they are activated primarily under these conditions. Classes of bioreductive agents include the "dual-function" alkylating nitroimidazoles, the lead compound of which is RB6145, the prodrug of RSU 1069 [1]; quinones, such as mitomycin C [2], its analogue porfiromycin [3], and the indoloquinone EO9 [4]; and N-oxides such as the benzotriazene-di-N-oxide, tirapazamine (SR 4233, WIN 59075) [5] and the anthraquinone-N-oxide AQ4N [6].

The cellular activation of bioreductive drugs depends upon the presence of both hypoxia and appropriate reductase enzymes. The reduced product of a bioreductive drug is more toxic than the parent compound, but is generated by reductase enzymes only in regions of low oxygen tension. Thus, tumour selectivity is achieved by virtue of the fact that hypoxic regions occur only in these tissues. The metabolic activation of bioreductive drugs is mediated by a variety of reductase enzymes, including P450 isoenzymes, NADPH:cytochrome P450 oxidoreductase (EC 1.6.2.4) (P450 reductase), NADH:cytochrome b_5 oxidoreductase (EC 1.6.2.2) (cytochrome b_5 reductase), xanthine oxidase, etc.

In this paper we are concerned with metabolic activation of RB90740, a heterocyclic mono-N-oxide. To date the only heterocyclic N-oxide with proven bioreductive efficacy is the di-N-oxide, tirapazamine. The di-N-oxide functionality has been shown to be essential for the cytotoxicity of tirapazamine; the mono-N-oxide reduction product derived from it, SR 4317, being devoid of biological activity [7]. Several analogues of tirapazamine have been synthesised and evaluated [7, 8], but tirapazamine remains the lead compound.

The cytotoxicity of tirapazamine is due to the formation of an oxidising nitroxide radical, formed by one-electron reduction of the parent compound under hypoxic conditions [9], which results in both single- and double-strand breaks in plasmid DNA [10, 11]. Formation of the radical has been shown to be catalysed by P450 reductase [9], and, more recently, the intracellular activity of P450 reductase activity has been

† *Abbreviations*: PTU, 5-propyl-2-thiouracil; *p*HMB, *p*-hydroxymercuribenzoate; and DPIC, Diphenyldiiodonium chloride.

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shown to correlate with the hypoxic cytotoxicity of tirapazamine in a panel of human breast cancer cell lines [12]. The role of P450 in the reduction of tirapazamine is less clear. Although the work of Walton *et al.* (1991) [13] suggests that P450 can catalyse the formation of the stable 2-electron reduced product, SR 4317, other workers [9, 14] have shown that P450 is not important in formation of the radical. This suggests that P450 may form SR 4317 by a direct two-electron reduction, without forming the toxic radical. Similarly, DT-diaphorase, an O_2-independent obligate 2-electron reducing enzyme, has also been shown to convert tirapazamine directly to SR 4317, bypassing the radical, and essentially detoxifying the drug [15]. Certainly, it has been shown that DT-diaphorase activity is not an important contributory factor in determining the hypoxic cytotoxicity of the drug *in vitro* [16].

There are few examples of biologically active aromatic mono-N-oxides. However, we have recently synthesised and evaluated a novel series of fused pyrazine mono-N-oxides that do have bioreductive activity [17, 18]. RB90740 (1,2-dihydro-8-(4-methylpiperazin-1-yl)-4-phenylimidazo[3,2-e]pyrazine 5-oxide) (Fig. 1) is the lead compound in this series. It is up to 20-fold more toxic under hypoxic than under aerobic conditions in some cell lines, although the magnitude of the differential (i.e. the ratio of drug concentrations required to give the equivalent amount of cell killing under hypoxic and aerobic conditions) does vary among cell types [18].

RB90740 undergoes reduction in hypoxic conditions to form RB92815, a stable two-electron reduced product. The toxicity of RB92816 (the hydrochloride salt of RB92815) is very similar under aerobic and hypoxic conditions, and is less toxic than that of the parent compound under hypoxic conditions [18]. This indicates that a one-electron reduced radical intermediate is likely to be responsible for mediating the toxicity of the parent compound under hypoxic conditions, as is the case with tirapazamine. Studies in Chinese hamster V79 cells indicate that the cytotoxicity of RB90740 is a consequence of single-strand, and possibly some double-strand, breakage in DNA, which is observed only under hypoxic conditions, cytotoxicity being greatest in DNA repair-deficient cell lines [18].

Little is known about the enzymology of the reduction of RB90740. From the original data it is clear that DT-diaphorase is not involved in the reductive activation of RB90740, since no correlation was observed between toxicity and cellular DT-diaphorase activity [19], a finding that is not surprising considering that DT-diaphorase is an obligate 2-electron donor. However, the CHO-MMC^R cell line, which has lower levels of cytochrome P450 reductase than wild type CHO-K1 cells [20], was found to be almost twice as resistant to RB90740 under hypoxia, indicating that P450 reductase may be involved in the bioreductive activation of RB90740 [18].

The aim of the current study was to elucidate which enzyme(s) catalyse the activation of RB90740 to its one- and two-electron reduced products, with a view both to explaining the wide variation in tumour cell sensitivity to this compound, and to predicting tumour cell sensitivity, based on enzyme profile. This will enable the suitability of the "enzyme-directed" approach to the development of RB90740 to be assessed. These enzymology studies were performed in uninduced mouse liver microsomes, which are rich in a number of membrane-bound reductase enzymes, and the results compared with levels of reductase activity in the different human tumour cell lines used for chemosensitivity testing.

MATERIALS AND METHODS

Chemicals and Reagents

RB90740, RB92816, and RB91720 (1,2-dihydro-8-(piperidin-1-yl)-4-phenylimidazo [1,2-a] pyrido [3,2-e] pyrazine 5-oxide bishydrochloride; internal standard for HPLC assay) were synthesized in-house [17]. PTU, *p*HMB, 2′AMP, NADH, and cytochrome c were all purchased from the Sigma Chemical Co. (Poole, U.K.). NADP, NADPH, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (grade II suspension) were purchased from Boehringer Mannheim (Lewes, U.K.), and DPIC from Lancaster Synthesis (Morecombe, U.K.). Zero

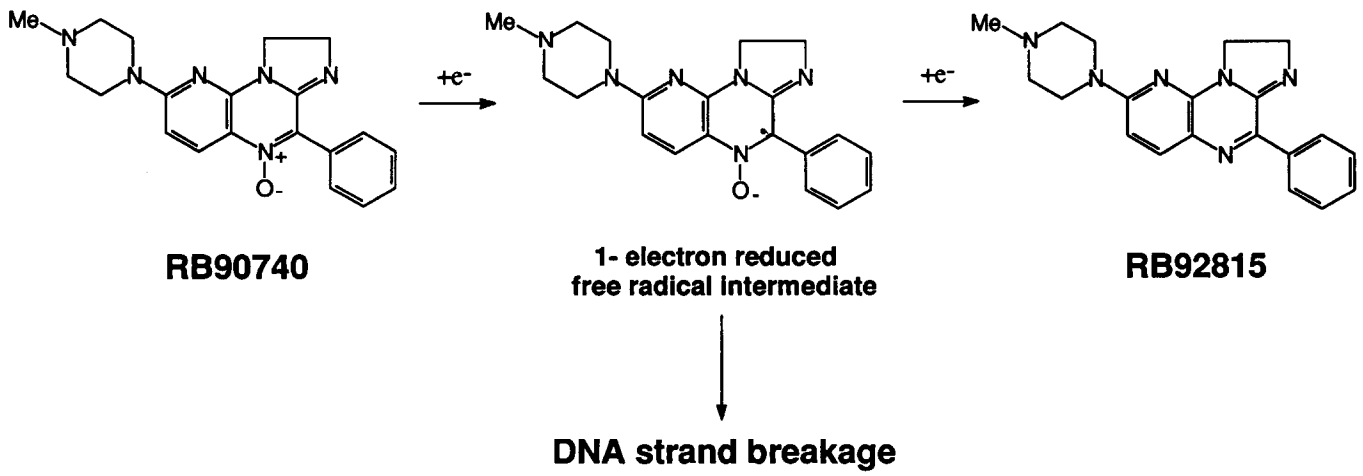

FIG. 1. The structure of RB90740, and its 2-electron reduced product RB92815. The active intermediate is believed to be a carbon-centred 1-electron reduced radical [18] that induces DNA strand breakage.

grade nitrogen was obtained from the British Oxygen Co. Carbon monoxide was obtained from the Aldrich Chemical Co. (Gillingham, U.K.) Anti-P450 antibodies and purified rat P450 reductase were gifts from Prof. C. R. Wolf (Ninewells Hospital and Medical Centre, Dundee). All other reagents were of analytical reagent grade.

Preparation of Liver Microsomes

Livers were obtained from female C3H mice and stored in liquid nitrogen until use. Microsomes were prepared by differential centrifugation using the method of Shaw *et al.* [21]. Microsomal pellets were resuspended in 0.25M potassium-phosphate buffer (pH 7.25) containing 30% glycerol (v/v), and stored as aliquots in liquid nitrogen. Microsomal protein concentration was measured by the Pierce BCA assay [22] using bovine serum albumin as the standard.

Incubations

All incubations were performed in 4-mL amber glass vials (14 mm diameter, 7 mm depth of liquid) at 37°C in a shaking water bath. The incubation mixture (0.5 mL) consisted of 0.1 mL microsomal suspension (diluted with incubation buffer to achieve a final incubation protein concentration of 0.8–1 mg/mL), 0.1 mL RB90740 (1 mM, dissolved in 4% (v/v) ethanol and stored at −20°C), 0.2 mL incubation buffer (0.2 M potassium phosphate buffer, pH 7.4), and 0.1 mL of either an NADPH-generating system, NADPH (1 mM final concentration), or NADH (1 mM). The NADPH-generating system consisted of 2 μmol glucose-6-phosphate, 0.2 μmol NADP, 0.4 U glucose-6-phosphate dehydrogenase, and 2 μmol $MgCl_2$ dissolved in incubation buffer, and was prepared immediately prior to use. The following concentrations of inhibitors were used: metyrapone, 1 mM and 5 mM; 2′AMP, 25 mM; DPIC, 1 mM; *p*-HMB, 0.1 mM; and PTU, 60 mM. The methods used for determining appropriate concentrations of the various inhibitors are described in the results section. Inhibitors were added in a small volume of buffer or appropriate solvent.

With the exception of the RB90740, the reagents were added to the vials, which were then sealed with Subaseals (Aldrich Chemical Co., Gillingham, Dorset, U.K.) and gassed across the liquid surface for 10 minutes with nitrogen passed through an Oxy-Trap™ (Alltech Associates Inc., Carnforth, U.K.). Reactions were started by addition of RB90740 using a Hamilton syringe inserted through the Subaseal. The reaction was stopped after 45 minutes by transferring two aliquots (200 μL) of the incubate to polypropylene vials containing 50 μl NaOH (2M), 1 mL ethyl acetate, and the internal standard (RB91720, 20 μL, 200 ng/mL). Samples were vortexed for 5 seconds and then centrifuged for 5 minutes at 6000 rpm. The supernatants were transferred to clean polypropylene tubes and dried under vacuum using a speed vac sample concentrator. The residues were reconstituted in mobile phase (300 μl) and 50 μl aliquots injected onto the HPLC for analysis. The HPLC analysis of RB90740 and RB92816 has been described previously [23]. Briefly, separation was achieved using a Waters (Millipore U.K. Ltd., Watford, U.K.) μbondapak C_{18} 10μ radial compression cartridge held in a Waters radial compression module (RCM) and protected by a Waters C_{18} guard column. The mobile phase consisted of 66% buffer (0.26 g/L potassium dihydrogen orthophosphate, 0.2 g/l heptane sulfonic acid, 0.2% (v/v) triethylamine adjusted to pH 5.7 with 20% (v/v) orthophosphoric acid), and 34% acetonitrile, at a flow rate of 2 mL/min. Detection of drug and metabolites was at 450 nm. Calibration standards were prepared by spiking heat-treated microsomes, which were diluted to a similar protein concentration as the samples, with known amounts of RB92815. Each run also included RB90740 controls in which 200 μM RB90740 was extracted from a microsomal suspension to measure any background concentrations of RB92815.

Measurement of Enzyme Activities

Methods for the preparations of cell lysates [12] and enzyme activities have been described elsewhere. DT-diaphorase activity was measured as the dicoumarol inhibitable NADH-dependent reduction of cytochrome c [24]; Cytochrome b_5 reductase activity was measured as the *p*HMB-inhibitible NADH-dependent reduction of cytochrome c [25] and P450 reductase activity as the NADPH-dependent reduction of cytochrome c [26; Chinje *et al.*, unpublished data].

RESULTS

Preliminary Experiments

The reduction of RB90740 by mouse liver microsomes was measured by HPLC as the formation of the stable 2-electron reduced product, RB92815. Preliminary experiments showed that formation of RB92815 was low in air and did not occur in the presence of heat-denatured microsomes, or in the absence of cofactor (NADH or NADPH), indicating that the reduction of RB90740 is an enzyme-mediated process. Using an NADPH-generating system as the electron source, formation of RB92815 was linear for at least 45 minutes, and up to a final microsomal protein concentration of approximately 1 mg/mL at the substrate concentration of 200 μM. These were selected as standard conditions for all subsequent incubations. Formation of the N-demethylated metabolite observed *in vivo* [22] was very low in air, and did not occur under nitrogen. No other metabolites were observed.

The formation of RB92815 proceeded at a rate of approximately 1 nmol/min/mg protein in the presence of either NADPH (1 mM) or an NADPH-generating system. This rate was found to be highly reproducible between experiments using microsomes from the same batch or from different batches (data not shown). Reduction of RB90740 was supported by either NADH or NADPH as the electron donor (Table 1), the former supporting RB92815 formation at a rate approximately 80% of that supported by the latter. Incubation of RB90740 with microsomes in the presence of equimolar concentrations of both NADH and NADPH did not have any additive effect, the overall rate of RB92815 formation in the presence of both cofactors being similar to that observed for NADPH alone.

TABLE 1. Rates of reduction of RB90740 by mouse liver microsomes in the presence of various enzyme inhibitors and cofactors. Rates represent mean ± SD formation velocity of RB92815 (nmol/min/mg protein) $n = 4$ incubations. All incubations were performed under nitrogen unless otherwise indicated.

Cofactor	Inhibitor	Concentration of inhibitor	Rate of RB92815 formation
NADPH	none	—	1.02 ± 0.16
NADH	none	—	0.72 ± 0.11
NADPH + NADPH	none	—	0.99 ± 0.18
NADPH	carbon monoxide	100%	1.01 ± 0.06
NADPH	metyrapone	1 mM	0.86 ± 0.30
NADPH	metyrpaone	5 mM	1.49 ± 0.03
NADPH	anti P450 reductase antibodies		0.58 ± 0.09
NADPH	2′ AMP	25 mM	1.54 ± 0.18*
NADH	2′ AMP	25 mM	0.58 ± 0.03
NADPH	DPIC	1 mM	n.d.
NADH	DPIC	1 mM	n.d.
NADPH	*p*HMB	0.1 mM	n.d.
NADH	*p*HMB	0.1 mM	n.d.
NADPH	PTU	60 mM	0.13 ± 0.03 (control: 1.45 ± 0.12)
NADH	PTU	60 mM	n.d.

* Identical to control rate, which was high in this series of experiments.

Involvement of Cytochrome P450 in RB92815 Formation

To determine whether the cytochrome P450 isozymes are involved in the formation of RB92815, microsomes were incubated with RB90740 and an NADPH-generating system either under carbon monoxide, or in the presence of 1 and 5 mM metyrapone (Table 1). The rate of formation of RB92815 in the presence of 1 mM metyrapone was similar to that observed in controls, but was increased by 49% in the presence of the higher concentration (5 mM). The rate of formation of RB92815 in the presence of carbon monoxide was significantly higher than in controls run concomittantly (1.01 ± 0.06 vs 0.86 ± 0.09 nmol RB92815 formed/min/mg protein), but were within the range normally expected in control incubations. These data indicate that cytochrome P450 isozymes do not have a direct involvement in the formation of RB92815, and suggest that inhibition of P450 may in fact stimulate reduction of RB90740.

Involvement of P450 Reductase in the Reduction of RB90740

To determine whether P450 reductase is involved in the formation of RB92815, RB90740 was incubated with microsomes in the presence of NADPH and either anti-rat P450 reductase antibodies, 2′AMP, a reversible inhibitor of P450 reductase [27, 28], or DPIC, an irreversible inhibitor of P450 reductase [29,30]. Preliminary experiments were performed to determine appropriate antibody titres and inhibitor concentrations using the spectrophotometric P450 reductase assay. To determine the titre of antibody required to maximally inhibit P450 reductase 2, 4, 6, 8, and 10 μL aliquots of antibody or preimmune serum were added to 20 μL aliquots of microsomal suspension (approximately 2 mg/mL), mixed, and left on ice for 30 minutes. Ten-μl aliquots of the suspension were then used in the reductase assay. The maximum inhibition of cytochrome c reduction achieved was approximately 85%, in agreement with Walton *et al.* [13]. The titre of antibody achieving this was then scaled up to allow for the protein present in an incubation of microsomes with RB90740, and was found to inhibit RB92815 formation by 50% (Table 1). Inhibitory concentrations of 2′AMP and DPIC were also determined using the spectrophotometric assay. Maximal inhibition of NADPH-dependent cytochrome c reduction by 2′AMP was approximately 75% at a concentration of 10 mM, and by DPIC was 85% at a concentration of 1 mM. Neither agent inhibited the NADH-dependent reduction of cytochrome c, which is a measure of cytochrome b_5 reductase activity.

Initial experiments showed that 25 mM 2′AMP inhibited microsomal RB90740 reduction by 50% when supported by an NADPH-generating system, but not when supported by NADPH alone. Subsequent experiments showed this was because of 2′AMP-inhibiting components of the NADPH-generating system, and that 2′AMP did not inhibit the reduction of RB90740 in the presence of NADPH. DPIC, at a concentration of 1 mM, completely inhibited the NADPH-dependent reduction of RB90740. Although neither inhibitor had any effect on the microsomal NADH-dependent reduction of cytochrome c, both inhibited the NADH-dependent reduction of RB90740, 2′AMP by approximately 40% and DPIC completely (Table 1).

Purified P450 Reductase

The activity of purified rat P450 reductase was determined spectrophotometrically as the NADPH-dependent reduction of cytochrome c. Enzyme aliquots equivalent to the P450 reductase activity of the microsomal preparation were incubated

with RB90740 for 5 minutes (The purified enzyme was found to denature in high phosphate concentration buffer beyond this time point). The purified enzyme reduced RB90740 at a rate of 0.47 ± 0.18 mmol/min ($n = 4$). This rate is equivalent to approximately 50% of that catalysed by an equivalent amount of microsomal reductase activity. Twenty-five mM 2'AMP did not inhibit the reduction of RB90740 catalysed by the purified enzyme.

Involvement of Cytochrome b_5 Reductase in RB92815 Formation

To determine whether cytochrome b_5 reductase is involved in the formation of RB92815, RB90740 was incubated with microsomes and NADH (1 mM) in the presence of selective inhibitors of the enzyme: *p*HMB [31] or PTU [32]. Appropriate inhibitory concentrations of the inhibitors were determined using the spectrophotometric cytochrome b_5 reductase assay [25]. Almost complete inhibition of cytochrome b_5 reductase activity was achieved by 0.1 mM *p*HMB or 25 mM PTU. The latter required coincubation with the microsomes in the absence of NADH for 10 minutes at 37°C to achieve its inhibitory effect. The NADH-dependent reduction of RB90740 was inhibited completely by 0.1 mM *p*HMB (Table 1). Inhibition by PTU was approximately 50% at a 25 mM concentration; complete inhibition was achieved at a 50 mM concentration. *p*HMB and PTU were also tested against NADPH-dependent reductions. *p*HMB did not inhibit the NADPH-dependent reduction of cytochrome c, and yet completely inhibited the NADPH-dependent reduction of RB90740. Fifty mM PTU inhibited the NADPH-dependent reduction of cytochrome c by approximately 50%, and of RB90740 by 91%. The activity of microsomal cytochrome b_5 reductase, measured using the spectrophotometric assay described above, was found to be optimal in a 0.05 M phosphate buffer, pH 6.8 [25]. The NADH-dependent reduction of cytochrome c was 50% higher in this buffer compared with the standard incubation buffer (0.2 M phosphate, pH 7.4). The microsomal reduction of RB90740 was increased by 36% in 0.05 M phosphate/pH 6.8 buffer compared with standard buffer, providing further evidence for a role of cytochrome b_5 reductase in the reduction of RB90740.

DISCUSSION

The "enzyme-directed" approach to bioreductive drug development [33, 34] has as its basis the variation in the ability of different tumour types to respond to bioreductive compounds, combined with knowledge of the levels of various reductase enzymes in these cell lines/tumours. This approach involves both the rational design of compounds as targets for activation by specific enzymes and enzyme profiling of both tumour tissue and surrounding healthy tissue to define likely targets for drug activation. For example, levels of DT-diaphorase have been shown to vary 10,000-fold in a panel of 23 tumour cell lines [24], and also to be elevated in tumour tissue compared with surrounding noncancerous tissue [15]. The aerobic toxicity of the indoloquinone EO9 correlates highly with intracellular DT-diaphorase activity [24, 35]. Therefore, EO9 should be targeted at tumours with high levels of DT-diaphorase, and would be expected to be of little or no therapeutic benefit when used as a single agent to treat tumours with low DT-diaphorase levels. Knowledge of the structure activity characteristics of DT-diaphorase should enable the rational design of analogues of EO9 for targeting at DT-diaphorase rich tumours.

The cytotoxicity of tirapazamine is mediated by a one-electron reduced radical, which is formed by P450 reductase [9]. The sensitivity of a panel of human breast cell lines to tirapazamine correlates well both with P450 reductase activity, and with the ability of lysates from these cells to convert tirapazamine to SR 4317 [12].

In contrast to the mono-N-oxide derivative of tirapazamine (*viz.* SR 4317), which has no biological activity [36], RB90740 has been shown to have greater toxicity towards hypoxic than aerobic cells. The magnitude of the hypoxic differential (the ratio of drug concentrations required to produce toxicity under aerobic versus hypoxic conditions) in a panel of 7 human tumour cell lines varied between 3.5 and 19 [18]. The basis for this variation may have its origin in the inherent sensitivity of cells to DNA damage, their ability to activate the compound, or a combination of these factors.

Although it is clear that the toxicity of RB90740 is likely to be mediated by a radical species, the enzymology of this process is largely unknown. A possible role for P450 reductase is suggested by indirect evidence from a pair of CHO cell lines with differing P450 reductase activities [18]. Findings in the current study provide direct evidence for a role of P450 reductase in the activation of RB90740, but in conjunction with cytochrome b_5 reductase. The most well-known role for P450 reductase is as a component of the cytochrome P450 monooxygenase system, involved in oxidative metabolism, in which it facilitates the transfer of electrons from NADPH, a 2-electron donor, to P450, a 1-electron acceptor [27]. This is achieved by the presence of FAD and FMN contained in the reductase enzyme. However, under anaerobic conditions, P450 reductase can transfer electrons directly from NADPH to a substrate. Indeed, P450 reductase has been shown to be involved in the activation of a number of bioreductive drugs in addition to tirapazamine: Mitomycin C [37, 38], EO9 [39], and the model 2-nitroimidazole, benznidazole [40]. Our investigations using specific inhibitors with differing modes of action suggest that the role of P450 reductase in the reduction of RB90740 may be indirect. 2'AMP, which inhibits the enzyme by competitive binding at the NADPH binding site, did not inhibit the reduction of RB90740 either by microsomes or by purified P450 reductase in the presence of NADPH (inhibition of the RB90740 reduction observed in the presence of the NADPH-generating system arose as a consequence of inhibiting the glucose dehydrogenase, presumably by binding at the NADPH-binding site on this enzyme, and thus preventing a supply of reducing equivalents). In contrast, DPIC, which inhibits the enzyme by irreversibly inactivating the FMN moiety [29, 30], completely inhibited the reduction of RB90740. These findings suggest that P450 reductase does play a role in

the reduction of RB90740, but that the electrons are not necessarily derived directly from NADPH. The flavin moieties of the enzyme are likely to be involved in transferring electrons to the substrate, but presumably these electrons are derived indirectly, rather than directly from NADPH.

Cytochrome b_5 reductase is a component of the cytochrome b_5 electron transport system involved in fatty acid and lipid metabolism [41], in which it transfers electrons from NADH to cytochrome b_5. Cytochrome b_5 reductase and cytochrome b_5 are also involved in some P450-mediated reactions [42]. The role of cytochrome b_5 reductase in activating bioreductive drugs has been studied little to date. Studies using purified cytochrome b_5 reductase have shown that the enzyme can activate mitomycin C [43], and can reduce adriamycin [44] and bleomycin [45] to form toxic species. However, studies with purified enzyme are not always representative of a whole cell or *in vivo* situation, especially when one considers that P450 reductase and cytochrome b_5 reductase are usually associated with P450 and cytochrome b_5 in the endoplasmic reticulum. In the present study, selective cytochrome b_5 reductase inhibitors, *p*HMB and PTU, were used to investigate the role of this enzyme in the reduction of RB90740. *p*HMB completely inhibited NADH-dependent reduction of RB90740. PTU partially inhibited the reduction of RB90740 at a concentration that completely inhibited the cytochrome b_5 reductase activity measured spectrometrically, and totally inhibited RB90740 reduction at a 50 mM concentration. Interestingly, *p*HMB also inhibited totally the NADPH-dependent reduction of RB90740, even though it was found that it did not inhibit the NADPH dependent reduction of cytochrome c. Similarly, PTU inhibited the NADPH-dependent reduction of RB90740 almost completely. However, this inhibitor partially inhibited the NADPH-dependent reduction of cytochrome c, indicating that some selectivity may have been lost at this higher concentration. Nevertheless, the effects of *p*HMB clearly indicate that the reduction of RB90740 is a complex process that involves cytochrome b_5 reductase. Further support for a role of this enzyme stems from the observation that the rate of reduction of RB90740 increases at pH 6.8, compared with pH 7.4. A pH range of 6.6–6.8 has been also been shown to be optimal for the cytochrome b_5 reductase-catalysed reduction of cytochrome c [25], mitomycin C [43], and adriamycin [41]. This observation is particularly interesting when one considers that the hypoxic regions of tumours are known to have a lower pH than surrounding oxygenated tissue [47].

Table 1 shows that, although both NADH and NADPH support the reduction of RB90740 at similar rates, there is no additive effect when the drug is incubated with equimolar concentrations of the two cofactors. This indicates that the cofactors are acting as alternative electron donors for the same enzyme system. Cytochrome b_5 can be reduced by both NADH and NADPH via cytochrome b_5 reductase and P450 reductase, respectively. Therefore, either cofactor can serve as the electron donor for electron transfer reactions involving this cytochrome [27]. It would therefore appear that a combination of cytochrome b_5 reductase, cytochrome b_5, and P450 reductase are involved in catalysing the reduction of RB90740. The components of this system cannot be inhibited selectively, indicating a complex interaction of electron flow through the enzymes. Figure 2 shows a stylised interpretation of this data. Electrons are derived from either NADH via cytochrome b_5 reductase, or from NADPH via P450 reductase. If either pathway is inhibited, then no reduction of RB90740 is observed. Cytochrome b_5 is likely to be intimately involved in the electron transfer processes.

DT-diaphorase is also known to be able to accept electrons from both NADH and NADPH. However, DT-diaphorase is largely a cytosolic enzyme, and this is highly unlikely to be involved in the reduction of RB90740 in these studies. We confirmed that the microsomal preparation used contained no DT-diaphorase activity (dicoumarol inhibited less than 3% of the total reduction of cytochrome c measured using the DT-diaphorase assay). Furthermore, dicoumarol did not inhibit the NADPH-dependent reduction of RB90740, and inhibited the NADH-dependent reduction by only 25% (results not shown). The latter is probably attributable to inhibition of cytochrome b_5 reductase [43]. It may be considered feasible that DT-diaphorase could have a role in detoxifying RB90740 by a direct 2-electron reduction, as reported for tirapazamine [15]. However, the cytotoxicity data presented in Table 2 suggest that this is not the case, since the aerobic cytotoxicity of RB90740 is the same in the MDA468 and H322 cell lines, and yet the DT-diaphorase activity in these two cell lines differs by some 800-fold.

The role of P450 in the reduction of RB90740 appears largely indirect rather than direct. The lack of synergism between NADH and NADPH indicates that P450 itself is not involved in the reduction of RB90740 [27]. This is supported further by the fact that classical RB90740 inhibitors, carbon monoxide and metyrapone, had no inhibitory effect on the reduction of RB90740. Carbon monoxide is a general P450 inhibitor, which works by binding at the haem moeity of the one electron reduced enzyme. Although it is possible that RB90740 may have been reduced before the P450, this seems unlikely, since the drug was added following 10 minutes' pre-incubation of the microsomes with CO to ensure that binding of CO would occur. Thus, lack of inhibition of RB90740 by CO indicates that P450 is not directly involved in the reduc-

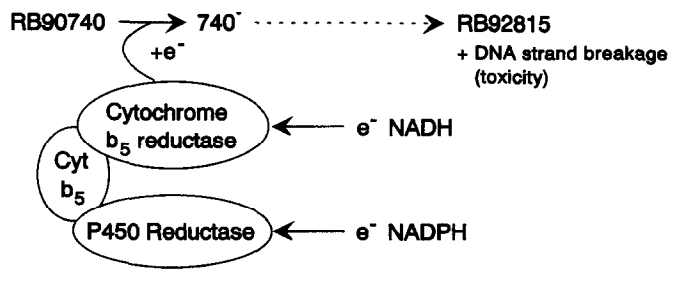

FIG. 2. A schematic representation of the electron transfer processes involved in the bioreduction of RB90740. Electrons are derived from NADH via cytochrome b_5 reductase and from NADPH via P450 reductase. Electron transfer almost certainly involves cytochrome b_5. Inhibitors of either reductase appear to prevent electron transfer through the system.

TABLE 2. Cytotoxicity of RB90740 and enzyme activities in human cancer cell lines. Cytotoxicity values are given as the IC_{50} (mM), that is, the concentration of RB90740 reducing optical density to 50% that of untreated controls using the MTT assay [18]. P450 reductase, cytochrome b_5 reductase, and DT-diaphorase activities are expressed as nmol cyt c reduced/min/mg protein. Values are from Chinje *et al.* (1994, unpublished data), Barham *et al.*, 1995 [25] and Patterson *et al.* 1994 [16], respectively.

Cell Line	Origin/histology	Aerobic toxicity	hypoxic toxicity	P450 reductas activity	Cytochrome b_5 reductase activity	DT-diaphorase activity
MDA468	breast adenocarcinoma	1.3	0.07	20.9 ± 1.7	38.9 ± 8.3	63
T47D	breast adenocarcinoma	1.5	0.11	17.2 ± 3.3	50.4 ± 6.5	46
H647	lung adenosquamous carcinoma	1.8	0.15	9.1 ± 1.0	49.7 ± 5.0	5140
H460	lung large cell carcinoma	2.8	0.15	17.6 ± 2.3	38.7 ± 4.7	5340
A549	lung adenocarcinoma	2.6	0.18	22.0 ± 2.7	51.6 ± 3.4	5930
H322	lung bronchio-alveola caracinoma	1.3	0.37	18.4 ± 3.2	55.5 ± 38.7	5030

tion process. Further support for this stems from the fact the P450 inhibitor metyrapone did not inhibit the reduction of the RB90740. Metyrapone is thought to preferentially inhibit the phenobarbitone-inducible forms of P450 rather than being a general inhibitor [48]. However, these are the isozymes most likely to be involved in reductive metabolism [49]. Further supporting evidence against a direct role for P450 is provided by the fact that 2'AMP, which inhibits P450 reductase by binding at the NADPH-binding site, did not inhibit the reduction of RB90740. Since P450 is critically dependent upon P450 reductase for its supply of electrons from NADPH, one would expect 2'AMP to inhibit any P450-mediated reductions by virtue of its inhibitory effect on the reductase. Ideally, spectral binding studies could be used to confirm that P450 is not directly involved in the reduction of RB90740. Unfortunately, this compound absorbs strongly at 450 nm, which would interfere with the P450 absorption spectra. Nevertheless, the evidence presented here does suggest that any role of P450 in the reduction of RB90740 is likely to be indirect.

In some situations P450 has been shown to increase the rate at which P450 reductase reduces a compound without having any direct involvement (e.g. mitomycin c) [37]. However, this does not appear to be the case with RB90740. In fact, the rate of reduction of RB90740 was increased by approximately 50% in the presence of 5 mM metyrapone, and may also have been higher in the presence of CO than in controls. This suggests that the rate of reduction of RB90740 is increased by inhibiting P450. We have also found that the rate at which RB90740 was reduced by a human breast cancer cell line, transfected with human P450 reductase to give an intracellular enzyme activity equivalent to that measured in mouse liver microsomes (Patterson and Barham, unpublished data), was inhibited by the addition of a source of P450 *viz* liver microsomes (data not shown). These findings therefore indicate that in the case of RB90740, P450 may have an inhibitory effect on electron transfer, possibly by diverting electrons away from P450 reductase and cytochrome b_5 reductase and, therefore, from the substrate.

The findings of this current study describe a novel situation for the activation of a bioreductive drug, in that the whole enzyme complex or system comprising P450 reductase, cytochrome b_5 reductase, cytochrome b_5 and P450 (indirectly) is involved, rather than individual components. This is in contrast to the activation of the di-N-oxide, tirapazamine, which is largely mediated by P450 reductase. Although the rate at which NADH supports the microsomal reduction of tirapazamine is negligible [46], the possible involvement of cytochrome b_5 and cytochrome b_5 reductase cannot be discounted. In this respect it is interesting to note that the NADPH-dependent formation of tirapazamine radicals is inhibited by *p*HMB [9]. We have also shown that *p*HMB will totally abolish the NADPH-dependent reduction of tirapazamine to SR 4317 by breast tumour cell lysates (Barham and Stratford, unpublished data).

Table 2 shows values for the cytotoxicity of RB90740 in a panel of human cancer cell lines, together with activities of cytochrome b_5 reductase [25], P450 reductase (Chinje and Barham, unpublished data), and DT-diaphorase [16]. No obvious relationship between cytotoxicity and any of the enzyme activities is evident. This is perhaps not surprising, given that the activation of RB90740 involves the interaction of P450 reductase and cytochrome b_5 reductase. This is in contrast to EO9 and tirapazamine, in which clear correlations between cytotoxicity and DT-diaphorase activity and P450 reductase activity, respectively, have been observed. In the case of the latter two drugs, an "enzyme directed" approach to their use in cancer treatment is entirely appropriate, since the activation of the drug can be predicted, at least to some extent, by the enzyme levels in the tumour. However, because the activation of RB90740 is more complex, its development and use as a selective hypoxic cytotoxin will be driven by potency considerations rather than the "enzyme-directed" approach.

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