Importance of P450 reductase activity in determining sensitivity of breast tumour cells to the bioreductive drug, tirapazamine (SR 4233)

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Summary P450 reductase (NADPH:cytochrome P450 reductase, EC 1.6.2.4) is known to be important in the reductive activation of the benzotriazene-di-N-oxide tirapazamine (SR 4233). Using a panel of six human breast adenocarcinoma cell lines we have examined the relationship between P450 reductase activity and sensitivity to tirapazamine. The toxicity of tirapazamine was found to correlate strongly with P450 reductase activity following an acute (3 h) exposure under hypoxic conditions, the drug being most toxic in the cell lines with the highest P450 reductase activity. A similar correlation was also observed following a chronic (96 h) exposure to the drug in air but not following acute (3 h) exposure in air. We have also determined the ability of lysates prepared from the cell lines to metabolise tirapazamine to its two-electron reduced product, SR 4317, under hypoxic conditions using NADPH as an electron donor. The rate of SR 4317 formation was found to correlate both with P450 reductase activity and with sensitivity to tirapazamine, the highest rates of SR 4317 formation being associated with the highest levels of P450 reductase activity and the greatest sensitivity to the drug. These findings indicate a major role for P450 reductase in determining the hypoxic toxicity of tirapazamine in breast tumour cell lines.

Keywords: P450 reductase; hypoxia; bioreductive drugs; tirapazamine; breast cancer

The presence of regions of low oxygen tension in a variety of human solid tumours is now well established (Gatenby et al., 1988; Hockel et al., 1991; Vaupel et al., 1991; Lartigau et al., 1992) and this hypoxia can predispose to failure of some treatments with radiotherapy (Gatenby et al., 1988; Hockel et al., 1993; Okunieff et al., 1993). The resistance of hypoxic cells to radiation has long been recognised in experimental systems (Moulder and Rockwell, 1987; Rockwell and Moulder, 1990), and methods designed to overcome this resistance have included the development of hypoxic cell radiosensitisers (Adams, 1976; Stratford, 1992) and more recently the combination of nicotinamide and carbogen (Chaplin et al., 1991). An alternative strategy has been to develop agents that selectively kill hypoxic cells (Adams and Stratford, 1986; Stratford et al., 1986; Zeman et al., 1986; Kennedy, 1987). This approach involves the exploitation of various biochemical processes that can result in selective reductive activation of drugs at low oxygen tensions (Kennedy, 1987; Workman, 1992; Workman and Stratford, 1993). In principle, this concept was first proposed in the context of quinone bioreductive drugs, with the notion that the low levels of oxygen in solid tumours could allow reductive activation of a drug to give a product which was more toxic than the parent compound (Lin et al., 1972). Subsequently, agents have been identified that are substantially more toxic to hypoxic compared with oxic cells and this is the basis for their tumour selectivity (Adams and Stratford, 1986, 1994; Stratford and Stephens, 1989.) Classes of compound that are in, or are about to enter, clinical trial as bioreductive drugs include (1) RB6145, the lead compound from a series of dual-function, alkylating nitroimidazoles (Cole et al., 1990, 1991, 1992; Jenkins et al., 1990) (2) quinones such as the mitomycin C analogue porfiromycin (Rockwell et al., 1988) and the indoloquinone EO9 (Hendriks et al., 1993); and (3) the benzotriazene-di-N-oxide tirapazamine (SR4233, WIN-59075) (Brown and Lemmon, 1990; Brown, 1993).

As indicated above, the major rationale for the development of bioreductive drugs has been the presence of tumour hypoxia. However, for these agents to be effective they require metabolic activation, catalysed by the cellular complement of reductase enzymes. These can include isozymes of

the P450 system, cytochrome P450 reductase, cytochrome b_5 reductase, xanthine oxidase etc (Walton et al., 1989; Workman, 1992; Hodnick and Sartorelli, 1993). Therefore, it has been proposed that hypoxic cells could be more effectively targeted if differences in the levels of reductases in various cell types were taken into account in order to direct appropriate agents to particular human tumours based on their enzymology (Workman and Walton, 1986; Workman and Stratford, 1993; Workman, 1994).

The potential usefulness of the 'enzyme-directed' approach to bioreductive drug development has been very clearly demonstrated with the mitosense EO9. The toxicity of this agent in air is highly dependent on the cellular expression of the obligate two-electron reductase, DT-diaphorase, (Robertson et al., 1992, 1994; Plumb et al., 1994; Smitskamp-Wilms et al., 1994). Under hypoxic conditions, reduction of EO9 can also be catalysed by cytochrome P450 reductase (Bailey et al., 1994) an enzyme which has been shown to play an important role in the metabolism of 2-nitroimidazoles (Walton and Workman, 1987) and N-oxides such as tirapazamine (Walton et al., 1989).

In previous studies on the metabolism of tirapazamine using mouse and rat liver microsomes (Walton et al., 1989, 1992; Lloyd et al., 1991) and tumour cell lysates (Wang et al., 1993) it was shown that both cytochrome P450 and cytochrome P450 reductase contribute to the overall reduction of tirapazamine to its two-electron reduced product SR 4317. Cytochrome P450 is dependent upon the presence of cytochrome P450 reductase for its catalytic activity (Peterson and Prough, 1986). Hence, P450 reductase will play both direct and indirect roles in the reduction of tirapazamine. Moreover, tirapazamine is reduced by purified rat liver cytochrome P450 reductase (Walton et al., 1989, Cahill and White, 1990) leading to the production of strand breaks in co-incubated plasmid DNA (Fitzsimmons et al., 1994). Therefore, the aim of this work was to assess the likely importance of P450 reductase activity for determining the sensitivity of tumour cells to tirapazamine. To do this we have measured P450 reductase activity in a panel of six human breast adenocarcinoma cell lines, and compared this with their sensitivity to tirapazamine under hypoxic and aerobic conditions. Given the role of P450 reductase in reducing tirapazamine, we have also determined the ability of the different cell lines to metabolise tirapazamine to its reduced product SR 4317 under hypoxic conditions.

Materials and methods

Chemicals

Tirapazamine, SR 4317 and SR 4330 were obtained from Sterling Winthrop Ltd or synthesised in house using previously described methods (Seng and Ley, 1972). NADPH was purchased from Boehringer Mannheim (Lewes, UK), HPLC-grade methanol was purchased from Merck (Lutterworth, UK). All other reagents were of analytical grade and were purchased from Sigma (Poole, UK). Tissue culture medium was obtained from ICRF (Clair Hall Labs, UK) and fetal calf serum from Sigma.

Cells and culture

Table I lists the six human breast tumour cell lines used in this work. All cell lines were maintained in exponential growth phase in RPMI-1640 medium (except for SKBr-3 cells which were maintained in E4 medium), supplemented with 2 mM glutamine and 10% (v/v) fetal calf serum. MCF-7 (EP) cells are early passage cells (passage 60–70) from the original strain established by Soule et al. (1973). In one set of experiments, indicated by footnote d in Table I, a late passage strain of MCF-7 cells was used (Newcastle Strain, MCF-7 (LP), passage > 300). Measurements of P450 reductase activity were made in each strain and showed no significant difference.

Drug sensitivity

Dose–response curves were determined using the MTT proliferation assay, which is based on the ability of viable cells to convert a soluble tetrazolium salt, MTT, into purple formazan crystals (Mossman, 1983). The optical density of the dissolved crystals is proportional to the number of viable cells, although this varies between cell lines as the conversion of MTT to formazan depends on the level of mitochondrial dehydrogenase activity in each cell line (Carmichael et al., 1987). The conditions for carrying out the assay have been described elsewhere (Robertson et al., 1994; Carmichael et al., 1987) and require plating $1 \times 10^3 - 5 \times 10^3$ cells (depending on cell line) into each well of a 24-well glass dish 3 h before exposure to tirapazamine for 3 h at 37°C in either air or hypoxia. The cells were then washed free of drug and allowed to grow for 4 days in 0.4 ml of fresh medium. Alternatively, chronic (96 h) aerobic exposures were carried out in a 96-well-plate format. After 4 days, MTT was added (0.2 mg ml^{-1} medium) and cells incubated for a further 4 h. Culture medium and unconverted MTT were removed and the formazan crystals dissolved in 0.2 ml dimethyl-sulphoxide (DMSO). An aliquot of 25 μl of glycerine buffer, pH 10.5 (Plumb et al., 1989) was then added and the optical density at 540 nm measured on a multiwell spectrophotometer. Values of IC$_{50}$, the concentration of tirapazamine required to reduce optical density by 50% compared with the untreated controls, were used as the measure of cellular sensitivity to a given treatment. The IC$_{50}$ values quoted in Table I are the means of at least three independent experiments conducted on different days.

Cell lysates

Cells in exponential growth phase were washed twice with phosphate-buffered saline (PBS) and harvested using a sterile cell scraper. Following centrifugation at 800 r.p.m. for 8 min, pellets were taken and washed in ice-cold hypotonic nuclear buffer A (10 mM Hepes/potassium hydroxide pH 7.4, 1.5 mM magnesium chloride, 10 mM potassium chloride, 0.05 mM DTT). Following repelleting, cells were suspended in 1.5 ml of nuclear buffer A and allowed to stand for 10 min at 4°C. Suspensions were sonicated using an MSE Soniprep 150 for 3×5 s at a nominal frequency of 23 kHz and an oscillation amplitude of between 5 and 10 μm. Samples were placed on ice between each sonication. The suspensions were allowed to stand on ice for a further 10 min, and then centrifuged at 7800 g for 15 min at 4°C. The resulting lysate was removed and stored in liquid nitrogen until required. The protein concentration of the cell lysates was determined using the Bio-Rad protein dye assay (Bradford, 1976) using high-grade bovine serum albumin (BSA) as the standard.

NADPH:P450 reductase activity

P450 reductase activity was determined spectrophotometrically as the NADPH-dependent reduction of cytochrome c. Each incubation comprised 400 μl of the cytochrome c (final concentration 50 μM), 100 μl of 10 mM potassium cyanide acetonitrile (final concentration 1 mM) and 100–300 μg lysate protein (50–100 μl volume) made up to 0.98 ml with 100 mM phosphate buffer, pH 7.6. The reaction was equilibrated to 37°C and initiated by addition of 20 μl 10 mM NADPH to the test cuvette (final concentration 200 μM) and the rate of reduction of cytochrome c was monitored at 550 nm for 3 min against a blank without NADPH. Initial rates of reaction were based on an extinction coefficient of 21 mM^{-1} cm^{-1} calculated and expressed as nmol cytochrome c reduced per min per mg lysate protein.

Western blot analysis

Samples of cells harvested for enzyme assays were washed in PBS buffer containing 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamide, 50 μg ml^{-1} leupeptin and 50 μg ml^{-1} soyabean trypsin inhibitor. Cells were lysed in 1 ml 2% w/v sodium dodecyl sulphate (SDS), plus inhibitors in PBS at 65°C for 5 min, then suspensions passed up and down a fine-gauge needle in order to break up the DNA. Samples were stored at − 20°C. Proteins were subsequently resolved by 7.5% SDS-polyacrylamide gel electrophoresis, and proteins on the gel were electrophoretically transferred overnight to a nitrocellulose hybridisation transfer membrane. The membrane was washed with blocking buffer (20 mM Tris-HCL (pH 7.5), 0.9% sodium chloride, 0.5% Tween 20, 1% low fat Marvel), and incubated for 60 min with specific NADPH:P450 reductase rabbit antibody (dilution 1:500, supplied by Professor CR Wolf, University of Dundee, UK). After washing, horseradish peroxidase-conjugated goat anti-rabbit antibody (dilution 1:5000) was added and incubated for 30 min. All antibodies were diluted in blocking buffer. The membrane was developed using an enhanced chemiluminescence Western blotting detection kit (Amersham, UK).

Metabolism of tirapazamine by cell lysates

Incubations were carried out under nitrogen at 37°C in 4 ml amber glass vials (Chromacol, Welwyn Garden City, UK) sealed with Subaseal (Aldrich, Gillingham, UK). The 500 μl incubation volume comprised 100 μl of cell lysate (maximum final protein concentration of 1.5 mg ml^{-1}), 100 μl of NADPH (5 mM dissolved in incubation buffer, giving a final incubation concentration of 1 mM), 20 μl of tirapazamine (50 mM dissolved in DMSO to give a final incubation concentration of 2 mM) and 280 μl of incubation buffer (0.2 M potassium phosphate buffer, pH 7.4). After preincubation under nitrogen for 10 min, the reaction was started by addition of tirapazamine using a Hamilton syringe inserted through the Subaseal. Reactions were stopped after 40 min by transferring 2×200 μl aliquots of the incubate into polypropylene vials containing 50 μl of internal standard [4-nitroquinoline N-oxide: 0.4 mg ml^{-1} in 20% (v v) ethanol] and 400 μl of methanol. Samples were vortexed vigorously for 2 min, centrifuged for 5 min at 3000 r.p.m. and 200 μl aliquots of the supernatants injected onto the HPLC system for analysis. Three lysate preparations for each cell line were incubated, each in duplicate, with duplicate analyses. Formation of SR 4317 was linear for at least 40 min and up to a protein concentration of 1.5 mg ml^{-1}, determined using

SkBr3 lysates, which have the highest P450 reductase activity amongst the panel of cell lines.

HPLC

Concentrations of SR 4317 and SR 4330 in incubation samples were determined by isocratic reverse phase HPLC (Walton and Workman, 1990). Chromatography was performed using a Waters μBondapak phenyl 4 μm radial compression cartridge in a Waters radial compression module (Waters Chromatography, Watford, UK) and protected with a phenyl guard column. The mobile phase consisted of 32% methanol in water delivered at a flow rate of 3 ml min^{-1}. Detection was at 267 nm. Approximate retention times under these conditions were 2.7, 4.6, 5.2 and 8.4 min for tirapazamine, SR 4330, SR 4317 and 4-nitroquinoline N-oxide (internal standard) respectively. Concentrations of metabolites were calculated from peak height ratios and comparison with calibration curves (0–500 μM) prepared by spiking lysate preparations with known amounts of metabolite.

Statistical analysis

The data were analysed using the standard model for a linear functional relationship with sampling errors in both variables. The data were logarithmically transformed and the pooled variance of each data set was calculated. It is assumed that the random sampling errors are normally and independently distributed with zero means and variances inversely proportional to the sample size. For statistical analysis of any two data sets, the model is fitted to the observations by the method of weighted least squares, each sample mean being weighted in direct proportion to the sample size. The statistical goodness-of-fit for any two data sets was tested by calculating the weighted mean-square deviation of the observations for mean x_n and mean y_n from the fitted model, and comparing this mean-square with the pooled variance within samples by a variance–ratio test. The statistical significance of the estimate of the slope of the straight line of best fit was tested by a Student's t-test.

Results

The toxic effect of tirapazamine on the breast cancer cell lines was determined in two sets of experiments. Firstly, by exposing cells to drug for 3 h under aerobic or hypoxic conditions and secondly, by growing cells in air for 96 h in the presence of tirapazamine. Cytotoxicity was measured by the MTT assay and typical survival curves, derived from individual experiments are given in Figure 1. The data illustrate the large increase in toxicity that occurs when drug exposure is under hypoxic compared with aerobic conditions and also show that the absolute potency of the drug in air and hypoxia can vary between cell lines. From such curves values of IC$_{50}$ can be obtained. Mean values derived from at least six independent, replicate experiments which update our

previously published data (Patterson et al., 1994) are given in Table I. (Cell lines are listed in order of sensitivity to tirapazamine under hypoxic conditions). Also included in the table are values of differential toxicity. This is the ratio of IC$_{50}$ values obtained following 3 h exposure to tirapazamine in air vs hypoxia. Each cell line is more sensitive to tirapazamine under hypoxia, with values of differential toxicity covering a 5- to 6-fold range from 13 to 69. There is no relationship between the value of differential toxicity and drug potency under either aerobic or hypoxic conditions.

The level of NADPH-dependent P450 reductase activity, measured in lysates from up to four separate cultures of each cell line, is given in Table I. The enzyme activity in the breast cancer cell lines covers a 6-fold range. This contrasts with the activity of the reductase DT-diaphorase in these cell lines, which varies by a factor of 260, the highest expressor being ZR-75 and the lowest MDA-231 cells (Robertson et al., 1994). We have shown previously that the toxicity of tirapazamine in breast and lung cancer cells following a 96 h aerobic exposure does not depend on DT-diaphorase activity (Patterson et al., 1994). However, as can be seen in Figure 2, there is a clear relationship between P450 reductase activity and tirapazamine toxicity in breast cancer cells following chronic (96 h) exposure in air. The highest toxicity (lowest value of IC$_{50}$) occurs in the cell line with highest activity of P450 reductase (SKBr3). The value of slope derived from these data is − 1.6 ± 0.3 (P = 0.006).

The importance of P450 reductase in the activation and toxicity of tirapazamine in the breast cancer cells has been characterised further by carrying out acute (3 h) exposure to drug under aerobic and hypoxic conditions. The dependence of IC$_{50}$ on P450 reductase activity is shown in Figure 3. Under hypoxic conditions there is a highly significant relationship between intracellular enzyme activity and drug tox-
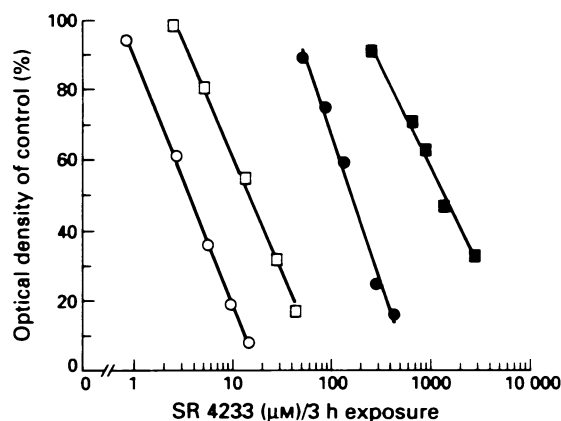

Figure 1 Examples of individual survival curves derived by the MTT assay for SKBr3 cells (circles) and ZR-75 cells (squares) exposed to tirapazamine for 3 h under aerobic (closed symbols) or hypoxic (open symbols) conditions.

Table I Human breast cancer cell lines: response to tirapazamine under aerobic or hypoxic conditions, activity of NADPH-dependent P450 reductase and ability to metabolise tirapazamine to the two-electron reduced product SR 4317 under hypoxic conditions

	IC$_{50}$μM[a]				NADPH:P450 reductase activity nmol substrate reduced min^{-1} mg^{-1} lysate protein	SR 4317 formation velocity	Western blot densitometry values (arbitrary units)[c]
Cell line	Aerobic exposure (96 h)	Aerobic exposure (3 h)	Hypoxic exposure (3 h)	Differential toxicity[b]			
SKBr 3	10.4 ± 0.52	167.2 ± 48.2	5.1 ± 2.0	32.8	39.8 ± 3.2	22.4 ± 3.4	3187 ± 495
MDA-468	18.1 ± 7.0	180.9 ± 76.6	7.3 ± 2.1	24.9	20.5 ± 1.8	11.0 ± 1.9	1372 ± 251
T47D	20.9 ± 5.3	301.3 ± 67.8	12.7 ± 2.0	23.7	16.1 ± 3.2	13.2 ± 2.7	1081 ± 253
MCF-7 (EP)	24.7 ± 9.9[d]	546.0 ± 61.3	15.0 ± 3.9	36.4	16.1 ± 1.2	10.2 ± 0.9	960 ± 136
ZR-75-1	26.6 ± 3.6	1295 ± 502	18.9 ± 2.4	68.5	10.8 ± 1.8	9.1 ± 1.4	473 ± 120
MDA-231	35.4 ± 4.1	307.8 ± 70.4	23.8 ± 4.0	12.9	6.9 ± 1.8	3.7 ± 0.7	231 ± 56

[a]Values ± s.d. updated from Patterson et al. (1994) with data being derived from up to five additional experiments on each cell line. [b]Ratio of IC$_{50}$ values for 3 h drug exposures under aerobic vs hypoxic conditions. [c]Data derived from Western blots of three separate lysates of each cell line. [d]Data from MCF-7 (LP), NADPH:P450 reductase activity = 18.7 ± 3.4 nmol cytochrome c reduced min^{-1} mg^{-1} protein.
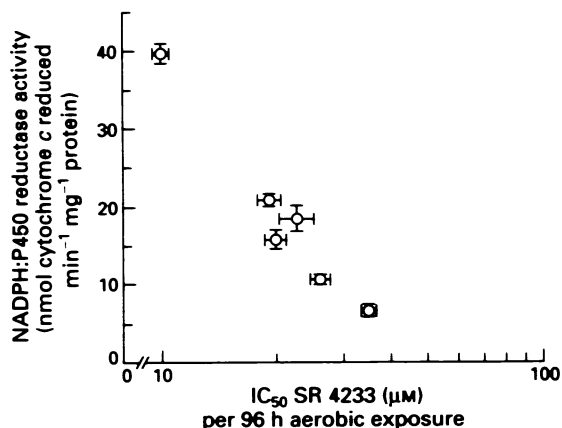
Figure 2 Dependence of IC_{50} values, derived by MTT assay of human breast cancer cells following 96 h exposure to tirapazamine in air, on NADPH cytochrome P450 reductase activity. Bars indicate standard errors.
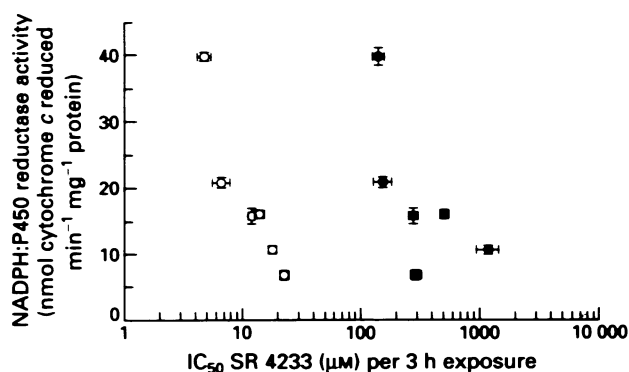

Figure 3 Dependence of IC_{50} values of human breast cancer cells exposed to tirapazamine for 3 h under aerobic (●) or hypoxic (○) conditions on NADPH cytochrome P450 reductase activity. Bars indicate standard errors.

icity (slope value $= -1.10 \pm 0.12$; $P < 10^{-10}$). In contrast, aerobic toxicity at 3 h does not show such a dependency (slope value $= -1.34 \pm 0.84$; $P = 0.19$).

Further evidence for a relationship between P450 reductase activity and acute hypoxic drug toxicity is provided by a Western blotting experiment in which the levels of P450 reductase protein in the cell lines were measured using a specific NADPH-dependent P450 reductase antibody. This is shown in Figure 4. An equivalent quantity of protein from each cell line was loaded onto the gel in lanes from left to right in rank order of their sensitivity to tirapazamine. It is apparent that the levels of P450 reductase protein decrease from left to right, i.e. the lowest level of protein occurring in the cell line with the greatest resistance to tirapazamine under hypoxic conditions. Values of the densitometry readings from these Western blots are given in Table I. When compared with values of enzyme activity in each cell line there is a strong correlation (slope ratio $= 2.43 \pm 0.32$, $P < 10^{-5}$).

P450 reductase has previously been shown to play an important role in the metabolism of tirapazamine (Walton et al., 1989, 1992; Lloyd et al., 1991; Wang et al., 1993). Furthermore, the rate of metabolism has been linked to cytotoxic efficiency under anaerobic conditions (Biedermann et al., 1991). In order to determine whether P450 reductase is a major factor contributing to the metabolism of tirapazamine in breast cancer cells, we have measured the rate at which cell lysates reduce tirapazamine to its two-electron reduced product, SR 4317 under hypoxic conditions. The structures of these compounds are given in Figure 5. No formation of the deoxygenated four-electron reduced product, SR 4330, was detected in these experiments. These rates of formation of SR 4317 are given in Table I and a plot of P450 reductase
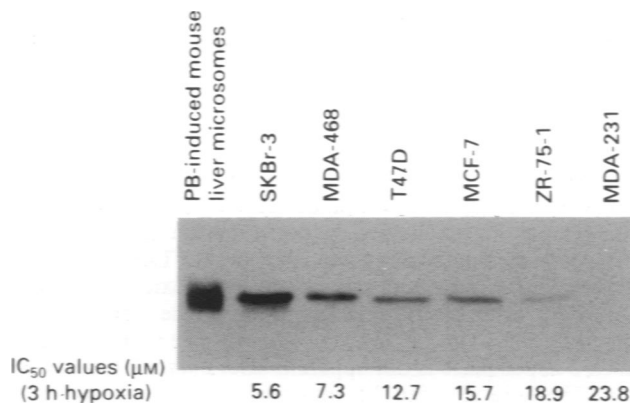

Figure 4 Western immunoblot of the relative NADPH cytochrome P450 reductase expression in breast cancer cells ranked in order of in vitro hypoxia sensitivity to tirapazamine. Gels were loaded with equal protein concentrations of lysates from each cell line. Microsomes derived from livers in phenobarbital-treated mice were used as a positive control (67 μg of protein in 100 μl). Phenobarbital was given at 80 mg kg^{-1} i.p. four times daily and mice sacrificed 24 h after the last injection (Walton et al., 1992).
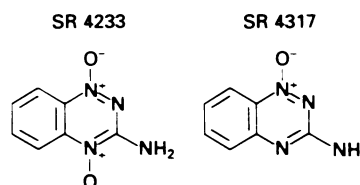

Figure 5 Structure of tirapazamine (SR 4233) and its two-electron reduced product SR 4317.
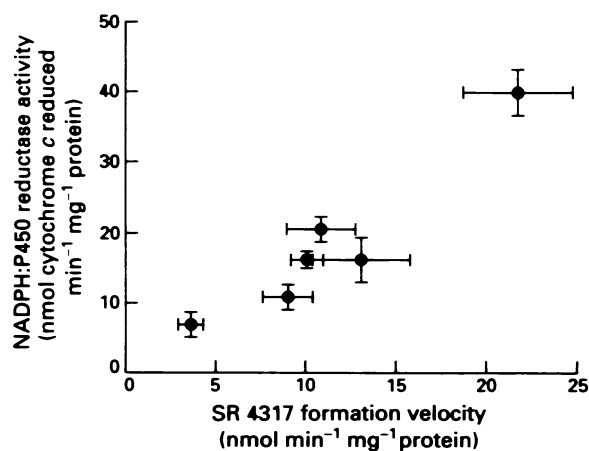

Figure 6 Dependence on NADPH cytochrome P450 reductase activity for the ability of breast cell lysates to convert tirapazamine to SR 4317 under hypoxic conditions. Bars indicate standard errors.

activity in the breast cancer cell lines vs the rate of SR 4317 formation catalysed by lysates of each cell line, with NADPH provided as the electron source, is given in Figure 6. It can be seen from these data that there is a strong correlation (slope value $= 1.10 \pm 0.23$; $P = 0.009$) between P450 reductase activity and SR 4317 formation, with higher values of enzyme activity resulting in greater rates of metabolism. When similar experiments were carried out in air, no metabolism was detected.

Discussion

Previous studies have shown that tirapazamine is reductively metabolised to SR 4317 by purified rat liver NADPH:

cytochrome P450 reductase (Cahill and White, 1990; Fitz-simmons et al., 1994). Lloyd et al. (1991) identified a free radical intermediate formed during microsomal reduction of tirapazamine and demonstrated, following the use of appropriate inhibitors, that P450 reductase was the enzyme responsible for the radical production. In the present work it is demonstrated that NADPH: cytochrome P450 reductase, present in human breast cancer cell lines in vitro, plays a major role in the reduction of tirapazamine and thus in determining the toxicity of the drug. The relationship between P450 reductase activity and drug toxicity is apparent following exposure of cells to tirapazamine under hypoxic conditions (3 h) and following 96 h exposure in air. However, such a dependence is not apparent after only 3 h treatment in air. These results can be interpreted on the basis of the activation toxicity scheme outlined below.
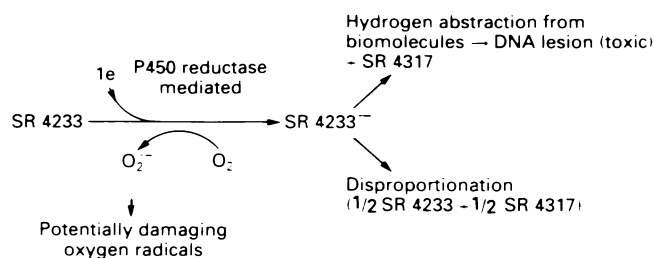
In the presence of P450 reductase, tirapazamine (SR 4233) will be subject to one-electron reduction to give the radical anion (SR 4233⁻), which in its protonated form has been identified as a nitroxide radical (Lloyd et al., 1991). In the absence of oxygen the radical will either disproportionate giving tirapazamine and SR 4317 (which can be regarded as a detoxification reaction) or undergo reaction with biomolecules e.g. hydrogen atom abstraction from DNA (Baker et al., 1988). The latter reaction could yield a DNA radical and SR 4317. There is some evidence to suggest that this latter process is the first step in a type of chain reaction that could result in multiple damaged sites from a single reductive event. Laderoute et al. (1988) demonstrated the existence of a short chain reaction during the radiolytic reduction of tirapazamine in a solution containing formate. P Wardman (personal communication) has suggested that SR 4233⁻-mediated hydrogen atom abstraction from the sugar backbone of DNA will result in strand breakage and the formation of a sugar radical and this radical can subsequently reduce another molecule of tirapazamine etc., thereby amplifying the damage to DNA. It has been suggested that such multiply damaged sites in DNA (which could be produced by the mechanism described by Wardman) could be the critical lesions for tirapazamine toxicity (Brown, 1993). Therefore, if the assumption is made that the breast cancer cell lines have similar abilities to repair damage caused by SR 4233⁻, then the level of damage under hypoxic conditions will be dependent upon the rate at which SR 4233⁻ is formed, i.e. dependent on the cellular activity of P450 reductase. Incidental support for this comes from observations of Keohane et al. (1990) who showed that tirapazamine was less toxic towards a CHO cell line, designated MMCʳ, which is deficient in P450 reductase activity compared with the parental line from which it was derived (CHO-K1).

A correlation between P450 reductase activity and cellular sensitivity to tirapazamine is observed in air following chronic (96 h) but not acute (3 h) exposure to the drug. This difference and the difference between the values of IC₅₀ for acute hypoxic vs aerobic exposure (values of differential toxicity varying from 12.9 to 68.5) are most likely to be explained by the effects of redox cycling which will occur in air. During this process SR 4233⁻ will be formed following one-electron reduction of tirapazamine, catalysed by P450 reductase. In the presence of oxygen the reduced product will be back-oxidised to tirapazamine, with the concomitant production of oxygen radicals. Thus, in air, cellular damage may

be mediated by both superoxide radicals and SR 4233⁻. Relatively high concentrations of tirapazamine were used during the acute exposure to air, which is likely to result in the formation of sufficiently high concentrations of oxygen radicals to overwhelm cellular antioxidant defence mechanisms. Thus, the variation in cellular sensitivity to tirapazamine following acute exposure in air may well reflect variations in the tolerance of the different cell types both to oxidative damage and to damage by SR 4233⁻, thereby explaining the apparent lack of a relationship between the drug toxicity and P450 reductase activity under these conditions. Evidence suggesting that the type of damage occurring in cells following acute (3 h) exposure to tirapazamine in either air or nitrogen can be different, comes from studies comparing drug toxicity in a V79 cell line and a DNA repair-deficient mutant line derived from it (Keohane et al., 1990). V79 and irs-1 cells have similar sensitivities to tirapazamine in hypoxia whereas, in air irs-1 cells are 10-fold more sensitive (as measured by values of IC₅₀). The irs-1 cells are radiation sensitive and are defective in the fidelity of DNA strand break repair. If it is assumed that the two cell lines have a similar ability to metabolise tirapazamine i.e., similar P450 reductase activities, then the difference in sensitivities between V79 and irs-1 cells observed in air but not in nitrogen would reflect differences in the damage caused under these conditions. That is, in hypoxia, the damage is caused by P450 reductase-mediated formation of tirapazamine radicals which can interact with DNA, whereas, following acute exposures in air, cells are additionally damaged by oxygen radicals. Support for the latter is indicated by the finding that the addition of metal chelators can protect cells against the toxic effects of tirapazamine in air but not in nitrogen (Herscher et al., 1994).

Following chronic (96 h) exposure of cells to tirapazamine in air, a relationship between P450 reductase activity and drug toxicity is observed. However, the concentrations of drug used in these experiments were 10- to 50-fold lower than those used in the acute (3 h) aerobic experiments. Therefore, it might be expected that the concentrations of oxygen radicals formed by redox cycling are likely to be low enough to be accommodated by the cellular antioxidant defence mechanisms. Thus, for this treatment condition, toxicity will be due predominantly to damage caused by SR 4233⁻.

Lysates derived from the breast cancer cells have been assayed for their ability to metabolise tirapazamine to SR 4317 under hypoxic conditions and this is shown to depend on P450 reductase activity (Figure 5). Since conversion of tirapazamine to its one-electron reduced product (SR 4233⁻) is enzyme mediated and, under hypoxic conditions, subsequent conversion to SR 4317 is a chemical process, we believe that measurement of formation of SR 4317 is an indirect measure of 'toxic' radical formation. Thus, it would be expected that formation of SR 4317 by cell lysates should correlate with the IC₅₀ for tirapazamine toxicity under hypoxic conditions, which indeed is the case (slope value = – 0.93 ± 0.30, P = 0.036). Further, this correlation strongly suggests that conversion of tirapazamine to SR 4317 by direct two-electron reduction, e.g. by DT-diaphorase (which will bypass the one-electron reduction product), does not contribute to toxicity (Patterson et al., 1994).

The level of hypoxia (Koch, 1993), the ability of cells to repair DNA damage (Keohane et al., 1990; Biedermann et al., 1991) and the level of cellular reductases, will all influence the therapeutic selectivity of tirapazamine. However, in breast cancer cells the activity of NADPH:cytochrome P450 reductase is important for the hypoxic toxicity of tirapazamine. In the application of these observations it is useful to note that the expression of P450 reductase protein (measured by immunoblot analysis) correlates well with the activity of P450 reductase, indicating that the P450 reductase protein is catalytically active. Thus, enzyme profiling of cells and tissues, together with a measure of tumour hypoxia, may provide a useful screen for predicting the activity of tirapazamine in vivo (Philip et al., 1994; Rampling et al., 1994). The work of Wang et al. (1993) and Biedermann et al.

(1991) has shown that the hypoxic toxicity of tirapazamine correlates with the overall rate of metabolism of the parent drug. In a mouse and a human sarcoma cell line, DT-diaphorase and cytochromes P450 were shown to contribute to the metabolism of tirapazamine under hypoxic conditions but no evidence was provided for the role of P450 reductase in these cells (Wang et al., 1993). Our previous studies on DT-diaphorase clearly indicate that this enzyme does not play an important role in determining sensitivity to tirapazamine in air or hypoxia in the breast cancer cells (Patterson et al., 1994). The role of P450 isozymes is less clear, particularly since cells in culture lose their ability to regulate the expression of P450 genes (Paine, 1990). From studies using chemical and antibody inhibitors in enzyme-induced mouse liver microsomes, it has been suggested that Cyp2b and Cyp2c can contribute to tirapazamine reduction (Riley et al., 1993). However, this has yet to be linked to toxicity. In clinical samples of breast tumour tissue only the presence of CYP1A has been demonstrated unequivocally (Murray et al., 1991), whereas in breast xenografts in mice CYPs 2A, 2B,

2C, 3A and 4A have been detected, but only to a significant extent after enzyme induction (Smith et al., 1993).

In conclusion, we have demonstrated that NADPH:P450 reductase plays a major role in determining the hypoxic toxicity of tirapazamine in breast cancer cells. This provides an example of the enzyme-directed approach to bioreductive drug development, and we are currently evaluating the generality of this approach for tirapazamine in other tumour types.

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