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NITROIMIDAZOLE BIOREDUCTIVE METABOLISM

QUANTITATION AND CHARACTERISATION OF MOUSE TISSUE BENZNIDAZOLE NITROREDUCTASES *IN VIVO* AND *IN VITRO*

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Abstract—We have investigated the nitroreduction of the 2-nitroimidazole benznidazole (BENZO) to its corresponding amine by murine normal tissues and tumours. *In vivo* concentrations of BENZO and its amine metabolite were measured by HPLC 3 hr after BENZO, 2.5 mmoles kg^{-1} i.p. This gave plasma and tissue BENZO concentrations of 96–160 $\mu g\ ml^{-1}$ or g^{-1}. Mouse plasma, KHT and RIF-1 tumour BENZO amine concentrations were very low (0.3–1.4 $\mu g\ g^{-1}$); kidney and EMT6 tumours had intermediate levels; and liver contained very high amine levels (~50 $\mu g\ g^{-1}$). Three per cent of the BENZO dose was recovered as amine in the 24 hr urine, compared to 5% for the parent compound. Nitroreduction to the amine was demonstrated with liver and tumour preparations under N_2 *in vitro*. The reaction was highly dependent on NADPH, and inhibited extensively in air. With liver microsomes and whole homogenates 2 and 3 moles respectively of BENZO were consumed per mole of amine formed. Inhibitor studies showed that NADPH: cytochrome P-450 (cytochrome *c*) reductase and cytochrome P-450 were both involved in BENZO reduction, predominantly at early and late reduction steps respectively. Aldehyde oxidase contributed to the cytosolic nitroreduction. Purified buttermilk xanthine oxidase also reduced BENZO to its amine under anaerobic conditions *in vitro*, but very inefficiently. The apparent K_m and V_{max} for BENZO amine production by whole liver homogenates were 0.148 mM and 1.45 nmole $min^{-1}\ mg^{-1}$ protein respectively. Tumour homogenates were less active than liver; e.g. V_{max} for the KHT tumour was 6–10-fold lower.

Nitroimidazoles are widely used in medicine to treat a variety of anaerobic bacterial and protozoal infections [1], and they are undergoing clinical development as radio- and chemosensitizers in cancer therapy [2]. The 2-nitroimidazole benznidazole (*N*-benzyl-(2-nitroimidazoyl) acetamide; Ro 07-1051; Radanil; BENZO; Fig. 1a) is extensively used in South America for the treatment of the *Trypanosome cruzi* infection Chagas' disease in man, which afflicts 1 in 10 people in Argentina alone [3]. Chemosensitization studies in our unit have shown that BENZO enhances the response of murine KHT tumours to the antitumour chloroethylnitrosourea CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) at clinically achievable exposures of BENZO [4]. Phase 1 and 2 studies have been completed successfully [5, 6] and this combination is now being evaluated further in a national multicentre MRC trial for recurrent brain tumours.

Reduction of the nitro moiety to the corresponding amine has been implicated in the cytotoxicity, neurotoxicity and chemosensitization of nitroimidazoles, probably through the formation of reactive intermediate species [7–9]. A number of enzymes have been shown to be involved in nitroreduction including cytochrome P-450, NADPH:cytochrome P-450 (cytochrome *c*) reductase, xanthine oxidase, aldehyde oxidase and DT-diaphorase [10, 11].

Unpublished studies have established the presence of the amine metabolite of BENZO (*N*-benzyl-(2-amino-1-imidazoyl)acetamide; Ro 11-1721; Roche; BENZO amine, Fig. 1b) in the urine of rats, dogs, monkeys and humans (see [12]). Using loss of parent drug as an analytical method, Toranzo *et al.* [13] demonstrated BENZO nitroreduction by rat and human faecal contents under anoxic conditions *in vitro*. Moreno *et al.* [14] employed electron spin resonance and superoxide formation to observe reduction to the nitro radical anion by rat liver microsomes under oxic conditions *in vitro*. Subsequently, Masana *et al.* [15] used protein adduct formation and parent drug loss to monitor anoxic nitroreduction *in vitro* in an initial characterisation of BENZO nitroreductases of rat liver. In the present work we have employed our novel high-performance liquid chromatography (HPLC) technique for the simultaneous assay of BENZO and BENZO amine [16], to demonstrate for the first time the nitroreduction

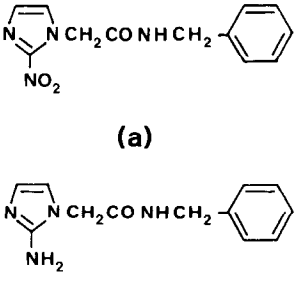

Fig. 1. Structures, (a) BENZO, (b) BENZO amine.

of BENZO in tumour and normal tissues *in vivo*. We have also investigated the kinetics of BENZO amine formation by these tissues *in vitro* and characterised the enzymes involved. Although a previous study, measuring parent drug loss, has shown an overall four electron stoichiometry for reduction of BENZO by purified buttermilk xanthine oxidase [17], we now show that this enzyme is capable of catalysing full six electron addition to the amine.

MATERIALS AND METHODS

Chemicals. Benznidazole (BENZO) and Ro 07-0602 (1-(2-nitroimidazoyl-1-yl)-3-*n*-butoxypropan-2-ol) were supplied in powder form by Roche Laboratories (Welwyn Garden City, U.K.). Benznidazole amine (BENZO amine) was supplied as the hydrochloride salt by Hoffmann La Roche (Basle, Switzerland). NADPH, NADH, hypoxanthine, allopurinol, menadione, *N*-methylnicotinamide and purified xanthine oxidase (from buttermilk, grade 1) were purchased from Sigma Chemical Company (Poole, Dorset, U.K.). Thallium trichloride tetrahydrate ($TlCl_3.4H_2O$) was obtained from Ventron Alfa Products (Karlsruhe, F.R.G.). Zero grade nitrogen (<5 vpm O_2) was supplied by British Oxygen Company (London, U.K.) and carbon monoxide (CO) was purchased from British Drug House (Poole, U.K.).

Mice and tumours. Adult BALB/c mice were obtained from OLAC (Southern) Ltd. (Bicester, U.K.) and adult inbred C3H/Km mice were supplied from our own breeding colony. Mice were allowed laboratory chow and water *ad lib.* and were used at 25–35 g body wt. KHT, RIF-1 (in C3H/Km mice) and EMT6 tumours (in BALB/c mice) were grown in the gastrocnemius muscle of the hind leg as described by Twentyman *et al.* [18]. In some experiments EMT6 tumours were grown intradermally (i.d.) in the flank [19]. Tumours were used when the mean diameters measured 10–12 mm.

High-performance liquid chromatography. Concentrations of BENZO and BENZO amine in biological material were determined by reverse-phase HPLC essentially as described previously [16]. Briefly, analyses were performed at ambient temperature using a modular HPLC system (Waters Ass., Milford, MA). The equipment consisted of two Model 6000A chromatography pumps, a Model 710B Automated Sample Processor (WISP), Model 440 and 441 fixed wavelength u.v. absorbance detectors and a Z-module. Separations were carried out on Waters Rad-Pak reverse-phase μ Bondapak octadecylsilane (C18) columns (8 mm × 10 cm, 10 μm diameter beads). The mobile-phase consisted of 25% acetonitrile (HPLC grade S, Rathburns, Scotland) in 0.2 M glycine/hydrochloric acid buffer, pH 2.5, containing 5 mM octanesulphonic acid (Fisons, Loughborough, U.K.). The column was eluted isocratically at a flow rate of 3.5 ml min^{-1} and the absorbance monitored at 313 nm for BENZO and at 229 nm for BENZO and BENZO amine. All buffers were thoroughly degassed before use. BENZO and its amine metabolite were identified by co-chromatography with authentic material. Quantitation was by peak-height ratio with reference to standard curves which were linear over the range 0.5–100 μg ml^{-1} and 0.5–1000 μg ml^{-1} for BENZO amine and BENZO respectively. Using an injection volume of 20 μl, the lower limit of detection was 0.2 μg ml^{-1} and 0.5 μg g^{-1} for plasma and tissue homogenates respectively, which corresponds to an on-column detection of 2–5 ng. Run times were <9 min.

Drug administration and sample preparation. BENZO was administered as a suspension in 50% polyethylene glycol (MW 400) in Hanks' buffered salt solution (HBSS), and injected i.p. in a volume of 10 ml kg^{-1} at a dose of 650 mg kg^{-1} (2.5 mmol kg^{-1}). Blood and tissue samples were collected 3 hr after drug administration.

Whole blood was obtained under diethyl-ether anaesthesia by cardiac puncture into heparanised syringes. Plasma was obtained by centrifugation at 3000 *g* for 15 min in a refrigerated (4°) Du Pont Sorvall RC-5B Superspeed centrifuge, and stored at −20° for up to 2 weeks prior to analysis. Plasma was treated with 2 or 4 vol. of methanol containing internal standard Ro 07-0602 (20 mg l^{-1}), thoroughly mixed and then centrifuged at 4000 *g* for 20 min in a refrigerated (−10°) Du Pont Sorvall RC-5B Superspeed centrifuge. Supernatants were evaporated to dryness *in vacuo* using a Savant Speed Vac concentrator coupled to a Model 100A Refrigerated Condensation Trap (Savant, Farmingdale, NY). Residues were resuspended in 100 μl of running buffer, and 20–45 μl was injected into the HPLC for analysis.

Tumor and other tissues were excised rapidly, snap frozen at −70° in dry-ice and all samples were stored at −20° for up to 2 weeks before analysis. Tissue homogenates, 33% w/v in distilled water, were prepared in all-glass or Teflon–glass homogenizers and treated as for plasma. All samples were handled at 4°.

Urinary excretion. Groups of five to six mice were contained in a Urimax metabolism cage and urine was collected frozen on dry-ice for 24 hr after a single i.p. dose of 2.5 mmol kg^{-1} BENZO.

Enzyme preparation. Whole liver, KHT and EMT6 tumour homogenates were used as an enzyme source for the *in vitro* nitroreduction studies. These were prepared as either 33% or 50% w/v homogenates in 83 mM sodium phosphate buffer (pH 7.4) for liver and tumour tissues respectively. All samples were handled on ice.

Hepatic microsomal and cytosolic enzyme fractions were prepared according to the method of Wolpert *et al.* [20]. Briefly, livers were homogenised in 3 vol. 20 mM Tris–HCl buffer, containing 1.15% potassium chloride, pH 7.4. The liver homogenates were centrifuged at 3000 *g* for 15 min and the supernatant decanted and centrifuged at 10,000 *g* for a further 10 min. The resulting supernatant was centrifuged at 100,000 *g* for 1 hr and the microsomal pellet and cytosolic supernatant recovered. The former was washed and subsequently stored in 83 mM phosphate buffer, pH 7.4, and the latter dialysed overnight against 200 vol. of 20 mM Tris–HCl buffer containing 1.15% KCl, pH 7.4. Microsomes and cytosol were stored at −70° for up to 6 weeks prior to use [21].

Incubation conditions. These were essentially as

described by McManus *et al.* [22] for bioreductive activation of MISO. All anaerobic incubations were carried out in a final volume of 3 ml in specially adapted 25 ml conical flasks, with agitation at 150 oscillations min^{-1} in a waterbath at 37°. The incubation mixtures were pregassed for 5–7 min with humidified zero grade nitrogen, which had been further deoxygenated by passage through a 15% w/v alkaline solution of pyragallol at a flow rate of 50 ml min^{-1}. Oxygen inhibition of the reaction was achieved by allowing air free access to the flasks. Inhibition by CO was achieved by replacing the nitrogen gas with humidified CO at a flow rate of 40–50 ml min^{-1}. The NADPH:cytochrome P-450 reductase inhibitor $TlCl_3$ was added in sodium phosphate buffer (50 μl) to give a final concentration of 0.2 mg ml^{-1}. Standard incubations contained: 83 mM sodium phosphate buffer (pH 7.4); 0.9 mM NADPH and NADH; and enzyme source which was either 200 μl 33% w/v, 800 μl 50% w/v whole homogenate, or 2 mg ml^{-1} microsomal protein. Reactions were initiated by addition of BENZO in dimethylsulphoxide (DMSO 50 μl) to give a final substrate concentration of 0.0833–1 mM. Cytosolic enzyme activity was assayed using either 500 μl 33% w/v whole liver homogenates or 7–8 mg ml^{-1} cytosolic protein, and purified buttermilk xanthine oxidase was used at a concentration of 1 U (Sigma) per assay. NADPH and NADH were omitted and replaced by the appropriate substrate. The aldehyde oxidase substrate *N*-methylnicotinamide and inhibitor menadione were added in sodium phosphate buffer (50 μl) and DMSO (50 μl) to give final concentrations of 2.5 mM and 10 μM respectively. The xanthine oxidase substrate hypoxanthine and inhibitor allopurinol were each added in 50 μl of DMSO to give final concentrations of 0.5 mM and 0.3 mM respectively. All incubations for cytosolic enzyme activity were carried out at 1 mM BENZO substrate concentration.

For all assays, samples (100 or 200 μl) of the incubation mixture were removed at 3–5 consecutive time points out to a final incubation time of 15–20 min for kinetic studies or 25–30 min for stoichiometric studies. The reaction was stopped by the addition of methanol containing internal standard and samples were treated as for plasma before HPLC analysis. Incubation conditions were non-limiting with respect to cofactor concentration and N_2 flow rates. Progress curves for amine production were linear up to 25 min. Reaction rates for amine formation were linear with protein concentration over the range used for all studies. For example with microsomal preparations this was between 1–3 mg ml^{-1}.

Protein assay. Protein concentrations were determined using the method of Lowry *et al.* [23] with bovine serum albumin as a standard.

Statistics. The parameters K_m and V_{max} were determined by least-squares linear regression analysis of Lineweaver–Burk plots using the GLIM statistical programs of the Royal Statistical Society of London.

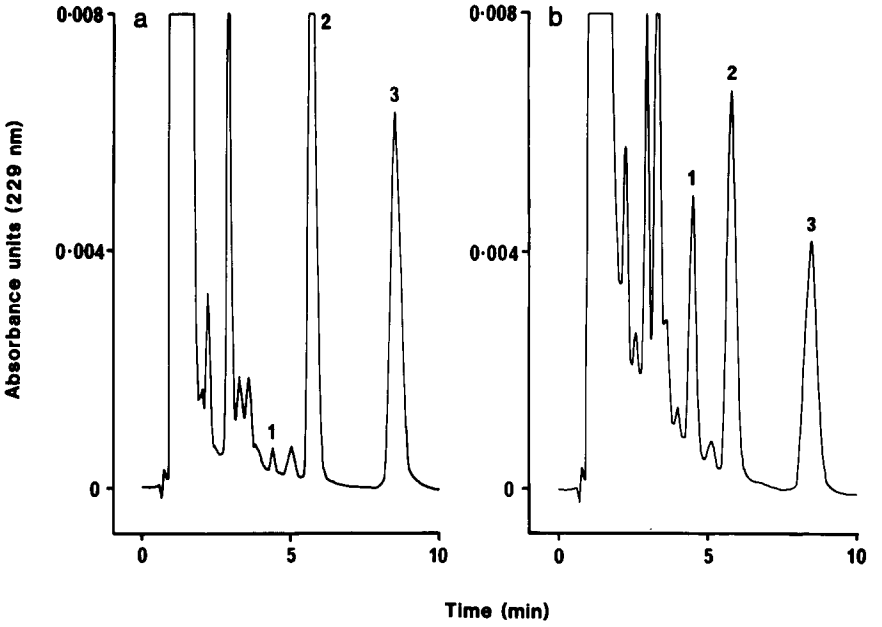

Fig. 2. HPLC chromatograms of resuspended dried methanolic extracts of: (a) an EMT6 leg tumour (BENZO amine; 1.4 μg g^{-1} homogenate, 4.44 μg g^{-1} tumour and BENZO; 54.7 μg g^{-1} homogenate, 164 μg g^{-1} tumour); and (b) a C3H/Km mouse liver (BENZO amine; 21.8 μg g^{-1} homogenate, 65.4 μg g^{-1} liver and BENZO; 37.7 μg g^{-1} homogenate, 113 μg g^{-1} liver). Samples taken 3 hr after 2.5 mmol kg^{-1} BENZO i.p. Peaks 1, 2 and 3 correspond to BENZO amine, BENZO and internal standard (Ro 07-0602, 20 mg l^{-1}) respectively. Chromatographic conditions: column, μ Bondapak C18 Rad-Pak (10 cm × 0.8 cm i.d.: particle size 10 μm); mobile phase, 25% acetonitrile in 0.2 M glycine/hydrochloric acid buffer, pH 2.5, containing 5 mM octanesulphonic acid; flow rate, 3.5 ml min^{-1}; column pressure, 10 MPa or 1500 psi; temperature, ambient; detection, u.v. absorbance at 229 nm; sample volume, 20 μl; chart speed, 10 mm min^{-1}.

RESULTS

High-performance liquid chromatography (HPLC)

Figure 2 shows HPLC analysis of BENZO and BENZO amine in resuspended dried methanol extracts of EMT6 leg tumour (a) and liver (b), both 3 hr after 2.5 mmol kg^{-1} BENZO i.p. For clarity only the traces at 229 nm are shown. In both chromatograms peaks representing BENZO and the internal standard are clearly distinguishable (peaks 2 and 3 respectively). The EMT6 tumour chromatogram contains an obvious peak corresponding to the amine, but this is much larger in the liver chromatogram (peak 1). Figure 2b also shows a number of minor peaks which were not present in control liver samples and which may represent additional hydrophilic metabolites or metabonates.

BENZO amine production in vivo

Figure 3 shows the *in vivo* concentrations of BENZO and its amine metabolite in tissues from C3H/Km and BALB/c mice 3 hr after an i.p. dose of 2.5 mmol kg^{-1} BENZO. These conditions were chosen on the basis of previous work [24] which showed that peak plasma BENZO concentrations were reached at 60 min and maintained for at least 6 hr, thus facilitating good tissue exposure to the parent drug.

The plasma and tissues studied had mean BENZO concentrations between 96–160 $\mu g\ ml^{-1}$ or g^{-1}, giving tissue/plasma ratios from 61% to 102%. In both strains of mice very little amine was present in plasma (~0.5 $\mu g\ ml^{-1}$). The two C3H/Km mouse tumours, RIF-1 and KHT, contained ~1.2 and ~0.60 $\mu g\ g^{-1}$ BENZO amine respectively, concentrations similar to those in plasma. In contrast, EMT6 tumours from BALB/c mice contained ~4 $\mu g\ g^{-1}$ BENZO amine, 5–8 times greater than plasma. Intermediate levels of BENZO amine were present in kidney, with the highest (~50 $\mu g\ g^{-1}$) occurring in liver of both mouse strains. These liver amine concentrations represented up to 68% of the total detectable drug related material in this tissue. Interestingly, no amine was detectable in the brain (<0.2–0.3 $\mu g\ g^{-1}$).

Urinary excretion

The 24 hr urinary recoveries for two independent experiments in C3H/Km mice were 5.2 and 4.8% for BENZO and 3.4 and 3.2% for BENZO amine. Corresponding values for two experiments in BALB/c mice were 3.8 and 3.4% for BENZO and 2.7% and 2.7% for BENZO amine.

In vitro *nitroreduction*

Using whole liver and tumour preparations we have clearly demonstrated the reduction of BENZO

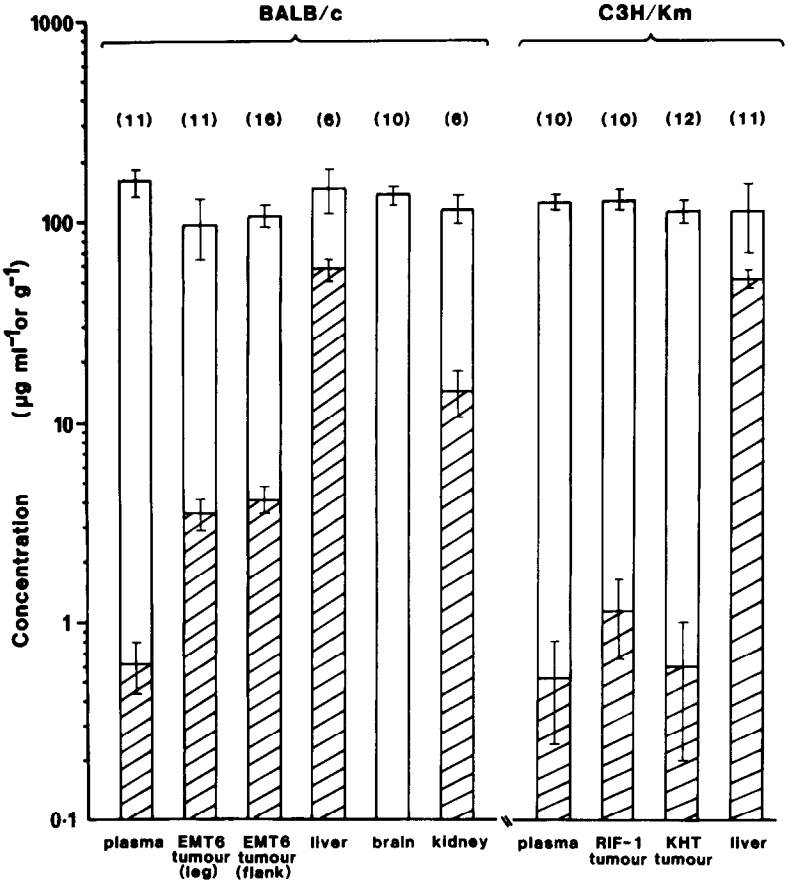

Fig. 3. Concentrations of BENZO (□) and BENZO amine (▨) 3 hr after 2.5 mmol kg^{-1} BENZO i.p., in normal and tumour tissues from C3H/Km and BALB/c mice. Histograms show mean ± 2 SE for (N) determinations. Results are from one to three independent experiments. Please note logarithmic scale.

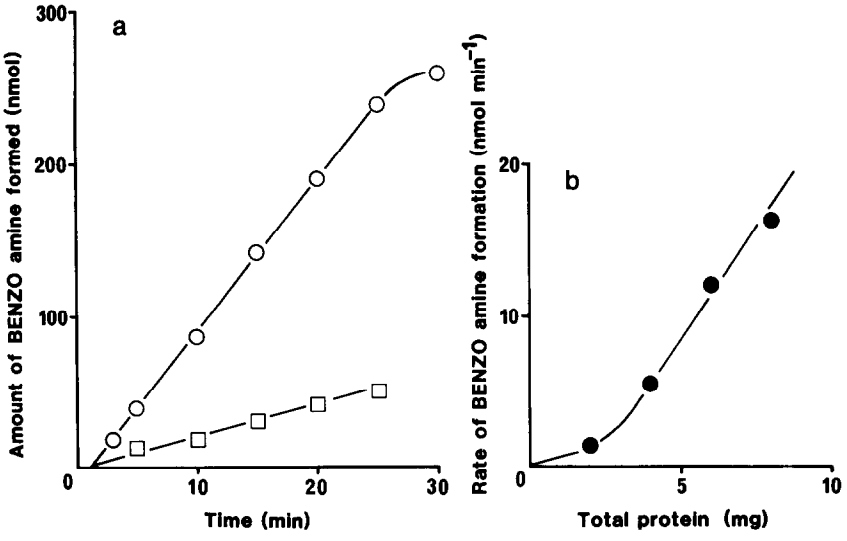

Fig. 4. Nitroreduction of BENZO to BENZO amine under N_2 *in vitro*. (a) Typical progress curves for BENZO amine production by whole liver (○) and whole KHT tumour (□) homogenates. (b) Effect of microsomal protein concentration on the rate of BENZO amine formation. Incubation mixtures contained: either 200 μl 33% w/v whole liver homogenate, 800 μl 50% w/v whole KHT homogenate, or 2 mg ml^{-1} microsomal protein; 0.9 mM NADPH and NADH; and 83 mM sodium phosphate buffer, pH 7.4. The final volume was 3 ml. For other details see Methods.

to its amine metabolite under anoxia *in vitro* (Fig. 4). Progress curves were linear for up to 20–25 min, during which substrate depletion did not exceed 40% (Fig. 4a). Figure 4b shows that reaction rates increased linearly with microsomal protein in the range 1–3 mg ml^{-1}, but were non-linear at lower protein concentrations. Cofactor concentrations were shown to be non-limiting.

Characterisation of liver BENZO nitroreductases

In view of the high levels of amine occurring in liver *in vivo* we attempted to characterise some of the hepatic enzymes responsible for this reaction. Table 1 summarises the cofactor requirements and the effects of air, CO (a specific inhibitor of cytochrome P-450 [25]) and $TlCl_3$ (an inhibitor of NADPH: cytochrome P-450 (cytochrome *c*) reductase [26]) on the BENZO amine formation activity of whole liver homogenates and microsomes *in vitro*. At least 85% of the total homogenate BENZO nitroreductase activity was associated with the microsomal fraction. With microsomes the reaction was inhibited completely in air and by $TlCl_3$, and by about 90% in CO. Whole homogenates were inhibited somewhat less in air and CO.

BENZO amine production was critically depen-

Table 1. Characterisation of BENZO nitroreductases in C3H/Km mouse liver preparations under anoxic conditions *in vitro**

Conditions	BENZO nitroreductase activity (% rate of amine production in complete system)	
	Whole homogenate	Microsomes
Complete system (N_2)†	100	100
$-N_2$ + CO	28, 32	8, 12
$+TlCl_3$	ND, ND	0, 0
$-N_2$ + air	3.9, 12	0, 0
+NADPH − NADH	103, 91, 125	108, 99
−NADPH − NADH	3.6, 2.6	0, 0
Boiled preparations	0, 0	0, 0

* Incubation mixtures contained: either 200 μl 33% w/v whole liver homogenate or 2 mg ml^{-1} microsomal protein; 1 mM BENZO; 0.9 mM NADPH and NADH; and 83 mM sodium phosphate buffer, pH 7.4. The final volume was 3 ml. For other details see Methods. Replicate results determined in separate experiments.

† Rates of amine production in complete liver homogenate and microsomal systems were 0.897 ± 0.176 and 2.53 ± 0.260 nmoles min^{-1} mg^{-1} protein (mean ± 2 SE, N = 6) respectively. ND Not determined.

Table 2. K_m and V_{max} for the nitroreduction of BENZO to its amine by whole tissue homogenates under anoxic conditions *in vitro**

Sample	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1} protein)
Liver	0.148 ± 0.0525	1.45 ± 0.217
KHT tumour	1.78 ± 1.42	0.160 ± 0.0968

* Values are mean ± 2 SE for three separate determinations.
Parameters were determined by least-squares linear regression analysis of Lineweaver–Burk plots. Incubation conditions: either 200 μl 33% w/v whole liver homogenate or 800 μl 50% w/v whole KHT tumour homogenate; 0.9 mM NADPH and NADH; and 83 mM sodium phosphate buffer, pH 7.4. The final volume was 3 ml. For other details see Methods.

dent on reduced nicotinamide adenine dinucleotide cofactors, particularly NADPH (Table 1). Nitroreduction rates similar to those in the complete system occurred in the presence of NADPH alone. The rate was considerably reduced with NADH alone, but the dependence on NADPH was greater in microsomes than in whole homogenates.

Comparative nitroreduction kinetics in liver and tumour

In order to compare the nitroreductase activities of liver, KHT and EMT6 tumour tissues, BENZO amine formation rates were measured *in vitro* at 1 mM BENZO substrate concentration using standard incubation conditions. The relative tissue homogenate activities were liver > KHT tumour > EMT6 tumour, the actual rates being 0.855 ± 0.260, 0.047 ± 0.017 and 0.011 ± 0.003 nmol min^{-1} mg^{-1} protein respectively (mean ± 2 SE, for 3 independent determinations). The comparative abundance of nitroreductase activity in liver preparations is consistent with the *in vivo* biodistribution of the amine metabolite described above. However, in contrast to the *in vivo* results, KHT tumour preparations were 4–5 times more active than EMT6 *in vitro*.

The kinetics of BENZO amine formation were characterised in more detail for liver and KHT tumour homogenates using Lineweaver–Burk plots. The results (Table 2) confirmed the high activity of liver, which had a V_{max} approximately 10-fold higher than that for KHT tumour. In addition, the K_m for liver was 12 times lower than the tumour value.

Stoichiometry in whole liver and microsomes

We determined the stoichiometry of the nitroreduction of BENZO to BENZO amine by liver whole homogenates and microsomes. This was done by allowing BENZO (0.23 or 0.4 mM) to be reductively metabolised by ~50% during a 25–30 min incubation, and comparing the rates of BENZO loss and amine formation. Both were linear during this period. In homogenates ~3 moles of BENZO were consumed in the formation of 1 mole of amine metabolite, whereas in microsomes the stoichiometry was about 2:1 (Table 3). The effects of inhibitors were also investigated. With microsomes in the presence of $TlCl_3$ no amine was formed and in addition no BENZO loss occurred, indicating that the initial step in nitroreduction was inhibited. CO inhibited microsomal amine formation by 94%, whereas BENZO loss was inhibited by only 57%. The stoichiometry was altered so that 14 moles of BENZO were consumed for each mole of amine produced, demonstrating that CO is a more potent inhibitor of the latter steps in BENZO nitroreduction.

Characterisation of cytosolic nitroreduction

BENZO amine was formed by whole liver homo-

Table 3. Stoichiometry of BENZO nitroreduction by C3H/Km mouse liver nitroreductases under anoxic conditions *in vitro**

Preparation	Conditions	Rate of amine formation (nmol min^{-1} mg^{-1} protein)	Rate of BENZO loss (nmol min^{-1} mg^{-1} protein)	Stoichiometry (amine formed: BENZO loss)
Homogenate†	Control	0.329 ± 0.097	0.985 ± 0.378	1:2.99
Microsomes‡	Control	2.22, 1.73	3.88, 3.55	1:1.7, 1:2.1
	$+TlCl_3$	0, 0	0, 0	0, 0
	$-N_2 + CO$	0.11, 0.13	1.67, 1.48	1:16, 1:11

* Incubation conditions: either 200 μl 33% w/v whole liver homogenate or 2 mg ml^{-1} microsomal protein; 0.9 mM NADPH and NADH; and 83 mM sodium phosphate buffer, pH 7.4. BENZO substrate concentrations were 0.23 mM for homogenates and 0.4 mM for microsomes. The final volume was 3 ml. For other details see Methods.
† Values represent mean ± 2 SE for three determinations.
‡ Replicate values were determined in separate experiments.

Table 4. Characterisation of the *N*-methylnicotinamide-dependent BENZO nitroreductase activity in C3H/Km mouse liver cytosol under anoxic conditions *in vitro**

	Rate of amine production (pmol min^{-1} mg^{-1} protein)	
	Liver homogenate	Cytosol
Control	85.3, 64.2	30.8, 18.5
Menadione	55.7, 50.8	15.6, 6.96
% Inhibition	35, 21	49, 62

* Incubation mixtures contained: either 500 μl 33% w/v whole liver homogenates or 9 mg ml^{-1} cytosolic protein; 2.5 mM N-methylnicotinamide; 10 μM menadione as indicated; 1 mM BENZO; and 83 mM sodium phosphate buffer, pH 7.4. The final volume was 3 ml. For other details see Methods. Values are for two independent experiments.

genates and cytosol in the presence of *N*-methylnicotinamide, a specific substrate for aldehyde oxidase [11], and the reaction was inhibited by 21–62% when menadione, a specific inhibitor of aldehyde oxidase [20], was included (Table 4). Although reduction to BENZO amine was seen with whole liver homogenates and cytosol using hypoxanthine as substrate, consistent inhibition by the xanthine oxidase inhibitor allopurinol [20, 27] could not be demonstrated. However, both BENZO loss and amine formation were observed using purified buttermilk xanthine oxidase in the presence of hypoxanthine, and both were inhibited completely by allopurinol (Fig. 5). Amine formation was, however, very inefficient, with about 20 moles of BENZO consumed for each mole of amine formed.

DISCUSSION

We have used a novel HPLC technique to demonstrate that mouse tissues can convert BENZO to its amine metabolite *in vivo* and *in vitro*. Efficient conversion to the amine was seen in both systems. As with other nitrocompounds [20, 28] full six electron reduction of 2-nitroimidazoles is thought to produce the stable terminal amine metabolite via the nitroso (2 electron), hydroxylamino (4 electron) and other intermediates. For some nitrocompounds relatively stable intermediates are formed [29, 30] whereas for others, including MISO, these could not be identified [31–36]. Several undergo reductive activation to products that generate covalent adducts in protein [37, 22], breaks in DNA [38], and/or potentially cytotoxic fragments [39]. These reactive species may arise from decomposition of unstable reduction intermediates such as the hydroxylamino [40] and other moieties [41], and various reduction products have been implicated in the cytotoxicity, carcinogenicity and neurotoxicity [7–9]. This type of reaction is inhibited by oxygen, through its combination with the one-electron reduction product, the nitro anion radical, generating superoxide and parent compound in a futile cycle [42, 43]. Oxygen may also compete for cytochrome P-450 [25], and/or reoxidise labile intermediates [28, 44].

Several enzymes have been implicated in nitroreduction, the major hepatic microsomal enzymes being NADPH: cytochrome P-450 (cytochrome *c*) reductase and cytochrome P-450 [10, 28, 36]. Our *in vitro* nitroreduction studies with BENZO showed liver to be very active. Reduction of BENZO to BENZO amine was highly dependent on NADPH and an anaerobic atmosphere, as originally noted for

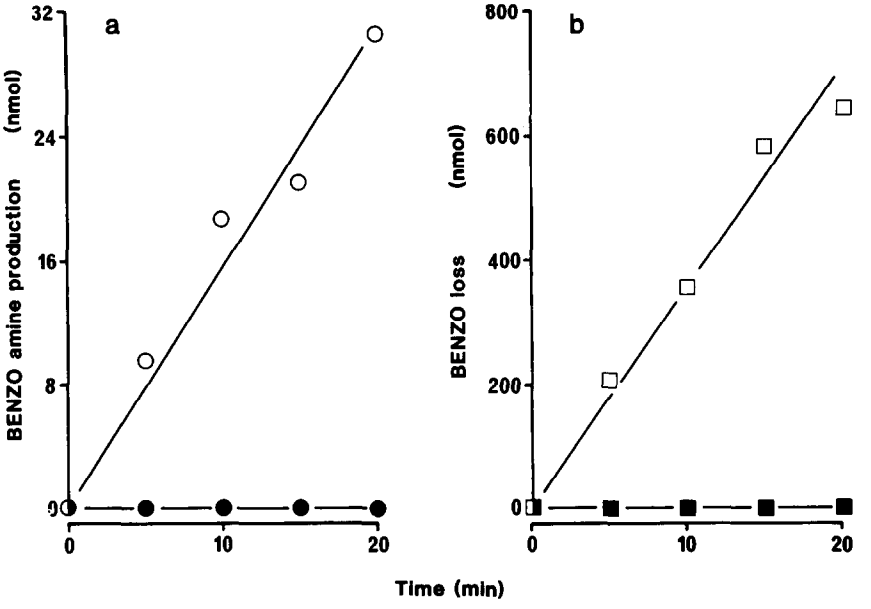

Fig. 5. Reduction of BENZO by purified buttermilk xanthine oxidase (Sigma) and the effects of allopurinol. Progress curves show (a) BENZO amine formation and (b) BENZO disappearance in the presence of hypoxanthine alone (0.5 mM, open symbols), and hypoxanthine with allopurinol (0.3 mM, closed symbols). Incubation mixtures contained: 1 U (Sigma) buttermilk xanthine oxidase; 0.5 mM hypoxanthine; 0.3 mM allopurinol as indicated; 1 mM BENZO; and 83 mM sodium phosphate buffer, pH 7.4. The final volume was 3 ml. For other details see Methods.

other compounds [45]. At least 85% of the activity in whole liver homogenates was microsomal. Amine production was inhibited extensively by $TlCl_3$, an inhibitor of NADPH: cytochrome P-450 reductase, and by the cytochrome P-450 inhibitor CO, implicating both enzymes in the reaction.

Previous work suggested that NADPH: cytochrome P-450 reductase is primarily responsible for the initial one-electron reduction to form the radical nitro anion for BENZO [14] and other nitrocompounds [46]. In support, we have shown that in hepatic microsomes $TlCl_3$ not only inhibited BENZO amine formation, but also parent drug loss. This indicates that the reductase is essential in the first step of BENZO reduction. CO also inhibited BENZO loss, but much less than amine production. This suggests a cooperative interaction of cytochrome P-450 and NADPH cytochrome P-450 reductase in the initial reduction step, as postulated for p-nitrobenzoate [25] and nitrobenzene [47]. The comparatively greater effect of CO on amine formation implies that cytochrome P-450 is more important at later steps, probably the final two electron addition from the hydroxylamine to the terminal amine [25, 47].

Hepatic cytosolic enzymes also reduce BENZO to its amine *in vitro*. Compared to microsomes, whole liver homogenates were inhibited less in air and CO atmospheres, and were more capable of utilising NADH in place of NADPH. We have shown that aldehyde oxidase is capable of catalysing the reduction of BENZO to BENZO amine. Although we have demonstrated BENZO nitroreduction with the xanthine oxidase substrate hypoxanthine, consistent inhibition by allopurinol was not obtained. Nevertheless purified buttermilk xanthine oxidase reduced BENZO to its amine, and this reaction was abolished by allopurinol. Amine formation was, however, very inefficient with ~20 moles of BENZO consumed per mole of amine formed, as compared to 2 moles for microsomal enzymes. Our results are therefore not inconsistent with the overall four electron stoichiometry for reduction of 2-nitroimidazoles including BENZO [48, 49], but provide the first demonstration of the ability of xanthine oxidase to catalyse full 6 electron reduction with this class of compounds. Recent results have shown that methyl 5-nitro-2-furoate is reduced to its amine by purified buttermilk xanthine oxidase *in vitro* [50].

Our conclusions regarding the enzymes responsible for BENZO reduction in mouse liver agree closely with those of Masana *et al.* [15], who used loss of parent drug and protein adduct formation in an initial characterisation of rat hepatic nitroreductases. Our study differs in providing a more detailed kinetic characterisation, and in employing terminal amine formation as a measured parameter. We also provide stronger evidence in support of the major involvement of NADPH: cytochrome P-450 reductase, as well as on the comparative efficiencies of NADPH: cytochrome P-450 reductase, cytochrome P-450 and xanthine oxidase in catalysing early versus late nitroreduction steps. Based on results measuring O_2 consumption and $O_2^{\cdot-}$ formation to monitor reduction of BENZO to the nitro radical anion in the presence of various inhibitors under oxic conditions, Moreno *et al.* [14] proposed that NADPH: cytochrome P-450 reductase but not cytochrome P-450 to be responsible for the initial reduction step. Both our results and those of Masana *et al.* [15] suggest that cytochrome P-450 may be involved cooperatively under anoxia.

In vivo concentrations of BENZO amine will depend on the nature and quantity of the nitroreductases, and in addition on the concentrations of reducing equivalents and inhibitory oxygen in the tissue. In isolated perfused rat liver, nitroreduction of MISO to MISO amine is highly dependent on oxygen tension [34]. The high concentrations of BENZO amine in liver strongly suggest that it is a major site of nitroreduction *in vivo* [10, 33]. The low amine concentrations in plasma imply that further hepatic metabolism occurs, probably to non-u.v. absorbing fragments as with other nitroimidazoles [12, 39]. The extensive DNA damage detected in kidney, liver, and tumour from mice treated with MISO further suggests that these tissues can reductively activate nitroimidazoles *in vivo* [38].

We found that mouse tumour BENZO amine levels were much lower than liver. This is in contrast to the biodistribution of MISO amine, which was similar in liver and KHT tumour [33]. The comparatively low tumour BENZO amine levels are not attributable to poor supply of BENZO, which was similar in all tissues examined (60–190 $\mu g\ ml^{-1}$ or g^{-1}). To determine whether the tumours possessed lower BENZO nitroreductase activity than liver, we studied the reaction kinetics using tissue homogenates under optimal conditions *in vitro*. In agreement with the *in vivo* biodistribution data, both KHT and EMT6 tumours were considerably less active than liver. In contrast to the *in vivo* data, KHT tumour homogenates exhibited markedly higher rates of amine formation than EMT6 *in vitro*. This suggests that KHT nitroreductase activity is suboptimal *in vivo*, possibly due to inhibitory oxygen concentrations. The greater extent of nitroreduction in EMT6 compared to RIF-1 and KHT tumours *in vivo* may be a consequence of more extensive hypoxia in EMT6 [51].

Michaelis–Menten parameters were obtained for liver and KHT tumour homogenates. V_{max} for BENZO amine formation was 5–10-fold higher in liver and the K_m value 12-fold lower. The enzymes responsible for BENZO nitroreduction in this tumour are likely to be different from the microsomal enzymes predominantly associated with hepatic nitroreduction. Because of their relatively low activities these have not been characterized as yet.

Compared to other tissues examined, brain contained no measurable amine *in vivo*, again in contrast to previous work with MISO [52]. This presumably reflects the inefficiency of nitroreduction, as well as poor uptake due to low plasma levels and the hydrophilic nature of the amine. Thus although prolonged BENZO dosing causes neurotoxicity in man [53] this is unlikely to be due to BENZO amine. Nevertheless evidence has been presented to support the accumulation of ^{14}C-MISO in rat brain tumour compared to normal brain, consistent with nitroreduction to reactive products [54].

BENZO is extensively metabolised in mice with

only 5% and 3% recovery of parent drug and amine metabolite respectively in the 24 hr urine. Urinary recoveries of MISO amine in conventional rats were similar (3–6%), whereas considerably lower amounts occurred in germ-free rats [55, 56]. Consequently, urinary MISO amine production was almost exclusively attributed to the gut flora. This is also an important site of reduction for several other nitrocompounds including BENZO [10, 13, 28]. Nevertheless the present work suggests that mouse tissues do catalyse BENZO nitroreduction, and comparative studies with germ-free mice are underway.

In summary, BENZO is reduced to its amine *in vivo* and *in vitro* by mouse tumour and normal tissues, particularly liver. The microsomal enzymes NADPH: cytochrome P-450 reductase and cytochrome P-450 have both been implicated as major hepatic BENZO nitroreductases, with additional minor activity from the cytosolic enzyme aldehyde oxidase. Purified xanthine oxidase was also shown to reduce BENZO to its amine *in vitro*. In view of the considerable clinical interest in BENZO and the importance of reductive metabolism in nitrocompound activity and toxicity, we are now investigating the metabolism of BENZO to its amine in man.

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