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THE METABOLISM OF ZIDOVUDINE BY HUMAN LIVER MICROSOMES *IN VITRO*: FORMATION OF 3′-AMINO-3′-DEOXYTHYMIDINE

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Abstract—The characterization of the enzymatic step(s) involved in the reduction of 3′-azido-3′-deoxythymidine (zidovudine)(ZDV) to 3′-amino-3′-deoxythymidine (AMT) was pursued. AMT formation by human liver microsomes was NADPH dependent, enhanced under anaerobic conditions, and increased by flavin adenine dinucleotide (FAD) and FMN. Carbon monoxide inhibited AMT formation by up to 80%. The effect of theophylline (CYP1A substrate), tolbutamide (CYP2C substrate), chlorzoxazone, thiobenzamide, *p*-nitrophenol, mercaptoethanol, isoniazid (CYP2E substrates), cortisol (CYP3A substrate), ketoconazole, itraconazole, fluconazole, cimetidine, miconazole (CYP inhibitors), methimazole (flavin-containing mono-oxygenase inhibitor), chloramphenicol (undergoes nitroreduction), allopurinol (xanthine oxidase inhibitor) and dicoumarol (DT-diaphorase inhibitor) on AMT formation were studied to see if the reduction reaction was mediated by a particular isozyme. The greatest inhibition was observed with ketoconazole (concentration producing 50% inhibition = 78.0 μM). At this concentration ketoconazole acted as a non-selective inhibitor of several CYP isozymes. Overall, these data suggested that ZDV reduction was probably mediated by both cytochrome P450 isozymes and NADPH-cytochrome P450 reductase. Formation of AMT, as measured by intrinsic clearance (Cl_{int}), was significantly increased in microsomes from rats pre-treated with phenobarbitone, dexamethasone and clofibrate (inducers of CYP2B, CYP3A and CYP4A, respectively). Pre-treatment of rats with β-naphthoflavone and ethanol (CYP1A and CYP2E1 inducers, respectively) had no effect on AMT formation.

Key words: AMT formation; CYP isozymes; inhibition

ZDV† undergoes extensive hepatic metabolism to an ether glucuronide [1–5] with the metabolite being rapidly excreted in urine [6]. A second metabolite (AMT) has also been identified in studies with human liver [7], human gastrointestinal bacteria [8], rat liver microsomes and hepatocytes [7, 9], and in the plasma of rhesus monkeys [10]. Recently, AMT has also been detected in the plasma of patients who received an i.v. infusion of [^{3}H]ZDV [11]. Although a minor metabolite, AMT is of importance because studies using human haematopoietic progenitor cells have demonstrated that it is 5–7-fold more toxic to human colony-forming units granulocyte-macrophage and burst-forming units erythroid than ZDV [7]. In addition, both AMT and ZDV have been shown to decrease the rate of globin gene transcription in K-562 leukemic cells with a corresponding decrease in haemoglobin synthesis [12]. Since the major adverse effect in patients receiving ZDV is haematological toxicity resulting in anaemia and granulocytopenia, it is important to get as much information as possible on the metabolic pathway involved in AMT formation. To date the enzyme(s) responsible for reducing ZDV to AMT have not been rigorously identified although there is evidence of the involvement of both cytochrome P450 reductase and cytochrome P450 [7, 13, 14]. Therefore the main aim of this study was to examine AMT formation in human liver and identify any inhibitors of this reaction. In addition, the non-enzymatic formation of AMT was investigated using the reducing agents dithiothreitol, glutathione and bovine serum albumin. Finally, rats were pre-treated with five specific inducers of cytochrome P450 isozymes [β-naphthoflavone (CYP1A), phenobarbitone (CYP2B), ethanol (CYP2E1), dexamethasone (CYP3A1) and clofibrate (CYP4A)] to determine if hepatic metabolism was inducible.

MATERIALS AND METHODS

Chemicals. ZDV and methyl[^{3}H]ZDV (9.1 Ci/mmol) were gifts from the Wellcome Research Laboratories (Beckenham, U.K.), as was the standard AMT. β-NADPH, reduced form (sodium salt), dithiothreitol, reduced form glutathione, BSA, FMN (sodium salt), FAD (disodium salt), cortisol, dapsone, miconazole, tolbutamide, theophylline, thiobenzamide, *p*-nitrophenol, chlorzoxazone, mercaptoethanol, isoniazid, chloramphenicol, allopurinol, dicoumarol, methimazole, phenobarbitone

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† Abbreviations: AMT, 3′-amino-3′-deoxythymidine; Cl_{int}, intrinsic clearance; FMN, flavin mononucleotide; IC_{50}, concentration producing 50% inhibition; K_m, affinity constant; MZP, *meta*-azidopyrimethamine; V_{max} maximal velocity; ZDV, 3′-azido-3′-deoxythymidine (zidovudine).

(disodium salt) and absolute ethanol were purchased from Sigma (Poole, U.K.). Ketoconazole and itraconazole were gifts from Janssen (Beerse, Belgium), fluconazole was a gift from Pfizer Central Research (Sandwich, U.K.) and cimetidine was obtained from Aldrich (Dorset, U.K.). Stock solution (1 mM $MgCl_2$) Tris(hydroxymethyl)-methylamine and EDTA were purchased from BDH (Poole, U.K.). HPLC grade acetonitrile, methanol and other laboratory reagents were obtained from Fison PLC (Loughborough, U.K.). All other chemicals were of the highest grade possible.

Human liver samples. Samples of histologically normal livers were obtained from kidney transplant donors, with consent to removal of the liver being obtained from the donors' relatives. Liver samples were transferred, on ice, to the laboratory within 30 min of death where they were sectioned into 10–20 g portions, placed in plastic vials and immediately frozen in liquid nitrogen at −196°. Liver samples were stored at −80° until required.

Preparation of microsomes. Frozen liver samples were partially thawed in 1/15 M phosphate buffer (pH 7.5) containing 1.15% KCl (w/v) and 0.2 mM EDTA, roughly chopped with scissors and ground with an ultra-turrax for 20 sec. A 25% homogenate was then produced using a motor driven Teflon in glass homogenization device. Washed microsomes were prepared by the classical differential centrifugation technique. Microsomal protein yield was determined by the method of Lowry *et al.* [15] with BSA as the protein standard. Cytochrome P450 content was determined by the method of Omura and Sato [16].

Animals. Male Wistar rats weighing 200–300 g were administered either a specific inducer of a cytochrome P450 isozyme or the corresponding vehicle (control animals). The inducers were β-naphthoflavone (60 mg/kg/day in corn oil), clofibrate (500 mg/kg/day in corn oil), phenobarbitone (80 mg/kg/day in 0.9% saline) and dexamethasone (100 mg/kg/day in PEG: 0.9% saline at a ratio of 75:25), each administered for 3 days i.p., and ethanol (5% v/v) administered for 9 days in drinking water. Control animals received nothing or 0.9% saline, corn oil or PEG: saline (75:25) which were administered 2 mL/kg/day for 3 days i.p. Following treatment all animals were starved for 24 hr prior to sacrifice. The rats were killed by cervical dislocation and the livers removed immediately. Washed microsomes were prepared by the classical differential centrifugation technique.

Assay conditions. The reduction of ZDV to AMT by human liver microsomes was assayed in 1.5 mL eppendorf microcentrifuge tubes that typically contained the following: $MgCl_2$, 5 mM; Tris–HCl, pH 7.5, (10 mM); [^{3}H]ZDV, (0.1 μCi, 500 μM). Incubation variables were NADPH concentration (0–10 mM), incubation time (0–75 min) and human microsomal protein content (0–7 mg). The final incubation volume was 200 μL. Duplicate tubes were also incubated under an atmosphere of nitrogen to create anaerobic conditions. Reactions were terminated by boiling for approximately 30 sec, followed by centrifugation at 12,000 *g* to remove all particulate matter. Aliquots (100 μL) were then analysed by HPLC with on-line radiometric detection. When linear conditions for the formation of AMT had been established (human microsomal protein 1–6 mg, incubation time 30 min, NADPH concentration 5 mM) incubations were performed with a range of ZDV concentrations (0–20 mM) in order to generate the kinetic parameters K_m and V_{max}. The following compounds were tested for inhibition of AMT formation: cortisol, dapsone, miconazole, tolbutamide, theophylline, thiobenzamide, *p*-nitrophenol, chlorzoxazone, mercaptoethanol, isoniazid, chloramphenicol, allopurinol, dicoumarol, methimazole, ketoconazole, itraconazole, fluconazole and cimetidine (100 and 500 μM). Ketoconazole, itraconazole, fluconazole and methimazole were further tested over a wide range of concentrations.

The reduction of ZDV to AMT by rat liver microsomes was determined to be linear up to 1.5 mg of protein and a 30 min incubation time with an NADPH concentration of 10 mM not rate limiting. Kinetic parameters for the reduction of ZDV were determined using 30 min incubations containing [^{3}H]ZDV (0.05–20 mM; 0.1 μCi), $MgCl_2$ (5 mM), Tris–HCl (10 mM, pH 7.5), microsomal protein (1.2 mg) and NADPH (10 mM) in a final volume of 200 μL. Incubations were terminated by the addition of trichloroacetic acid (20% w/v; 100 μL) and protein free supernatants following centrifugation analysed by HPLC.

K_m and V_{max} for both human and rat liver microsomal formation of AMT were determined using an iterative programme (ENZPACK) based on regression analysis of the Eadie–Hofstee linear derivative plot of velocity (V) vs velocity/substrate concentration (V/S). Cl_{int} of ZDV by rat liver microsomes was calculated as V_{max}/K_m. Statistical comparisons were made by Student's non-paired *t*-test with a significance level of 5%.

Incubations with FAD, FMN and carbon monoxide. To determine the effect of the flavins FAD and FMN the standard assay procedure was used with the addition of FAD (5 mM) or FMN (5 mM) or a combination. Incubations were performed in the presence and absence of carbon monoxide.

HPLC analysis. Samples were analysed by HPLC using a Spectra Physics pump (SP8800) connected to a Radial-PaK LC cartridge (10 cm × 8 mm i.d., μBondpak C_{18}, 10 μ, Waters Associates, Milford, MA, U.S.A.) housed in a Z-module radial compression system (Waters Associates) with a C_{18} precolumn. Samples were eluted at a flow rate of 1 mL/min using a mobile phase comprising of 15:85 mixture of acetonitrile: 0.1% (v/v) ammonium phosphate buffer (pH 2.7). Metabolism of the parent compound to its catabolites was measured by on-line radiometric flow detection using a A250 FLO-ONE detector (Canberra-Packard) with Flo-Scint A (Canberra-Packard). The retention times of authentic AMT and ZDV were 5 and 15 min, respectively (Fig. 1).

Incubations with reducing agents. Incubations of dithiothreitol (10 mM) and reduced form glutathione (10 mM) with [^{3}H]ZDV (0.1 μCi; 500 μM), $MgCl_2$ (5 mM) and Tris–HCl (10 mM) were performed at

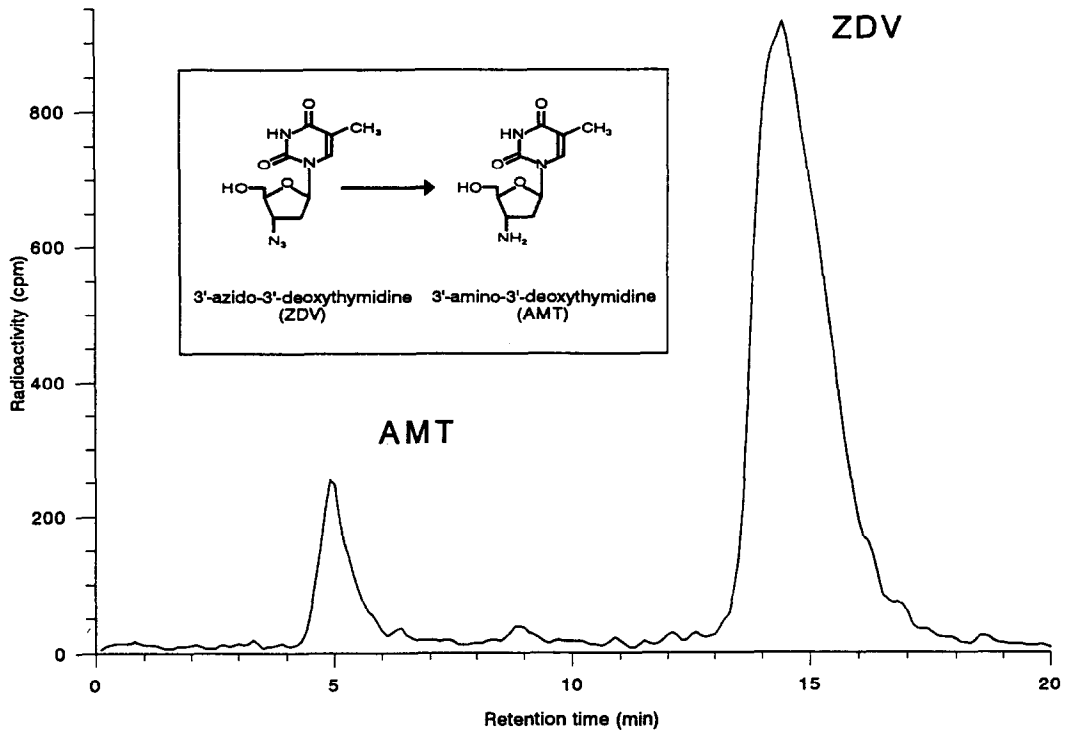

Fig. 1. HPLC radiochromatogram illustrating the formation of AMT. Incubation conditions as described in the text.

37°. At various time points aliquots were removed and analysed by HPLC to determine the extent of ZDV reduction to AMT. Incubations were also performed with BSA (0–5 mg/incubation).

RESULTS

All of the human livers used in this study were obtained from patients who had normal case histories and to our knowledge had not received drugs known to be enzyme inducing agents or inhibitors prior to death.

AMT was formed in microsomal incubations under both aerobic and anaerobic conditions (Fig. 2). Metabolite formation was shown to be linear to at least 5 mg microsomal protein (Fig. 2a) when the incubation time (1 hr), NADPH (5 mM) and ZDV (0.1 μCi; 500 μM) were fixed. With fixed protein (3 mg), NADPH and ZDV, metabolite formation deviated from linearity beyond 30 min (Fig. 2b). An NADPH concentration of 5 mM was non rate-limiting (3 mg protein, 30 min incubation, ZDV, 0.1 μCi; 500 μM) (Fig. 2c).

Having defined the optimal conditions for AMT formation, it was attempted to generate kinetic parameters using a ZDV concentration range of 0.1–20 mM under aerobic conditions. Non-linear Eadie–Hofstee plots resulted (Fig. 3b), indicating the involvement of two (or possibly more) components in the reduction of ZDV. Based on a two-enzyme fit, K_m and V_{max} values were generated. The K_m values were 0.69 ± 0.40 (mean ± SD; N = 4) and 9.23 ± 4.6 mM, and the V_{max} values were 7.6 ± 3.1 and 38.6 ± 24.1 nmol/mg/hr for the high and low affinity components, respectively.

AMT formation was NADPH dependent (Fig. 4), and the addition of FAD (5 mM) or FMN (5 mM) enhanced the formation by approximately 3-fold from 0.54 ± 0.20 (mean ± SD; N = 3) to 1.66 ± 0.86 and 1.62 ± 0.83 nmol/mg/hr, respectively. Carbon monoxide inhibited AMT formation by up to 80% (Fig. 4).

The effect of theophylline (CYP1A substrate), tolbutamide (CYP2C substrate), chlorzoxazone, thiobenzamide, *p*-nitrophenol, mercaptoethanol, isoniazid (CYP2E substrates), cortisol (CYP3A substrate), ketoconazole, cimetidine, miconazole (CYP inhibitors), chloramphenicol (underwent nitroreduction), allopurinol (xanthine oxidase inhibitor) and dicoumarol (DT-diaphorase inhibitor) on AMT formation are shown in Table 1. Only dicoumarol and thiobenzamide were non inhibitory. Cytochrome P450 substrates and inhibitors produced variable degrees of inhibition. However, since ketoconazole produced the greatest inhibition at 100 μM, a more comprehensive range of concentrations was investigated (10, 20, 50, 100, 200 and 500 μM) and comparative data from the azoles itraconazole and fluconazole obtained (Fig. 5). The IC_{50} value was 78.0 μM (data not shown).

The reduction of ZDV to AMT by dithiothreitol was rapid with an 80% conversion observed at 5 hr.

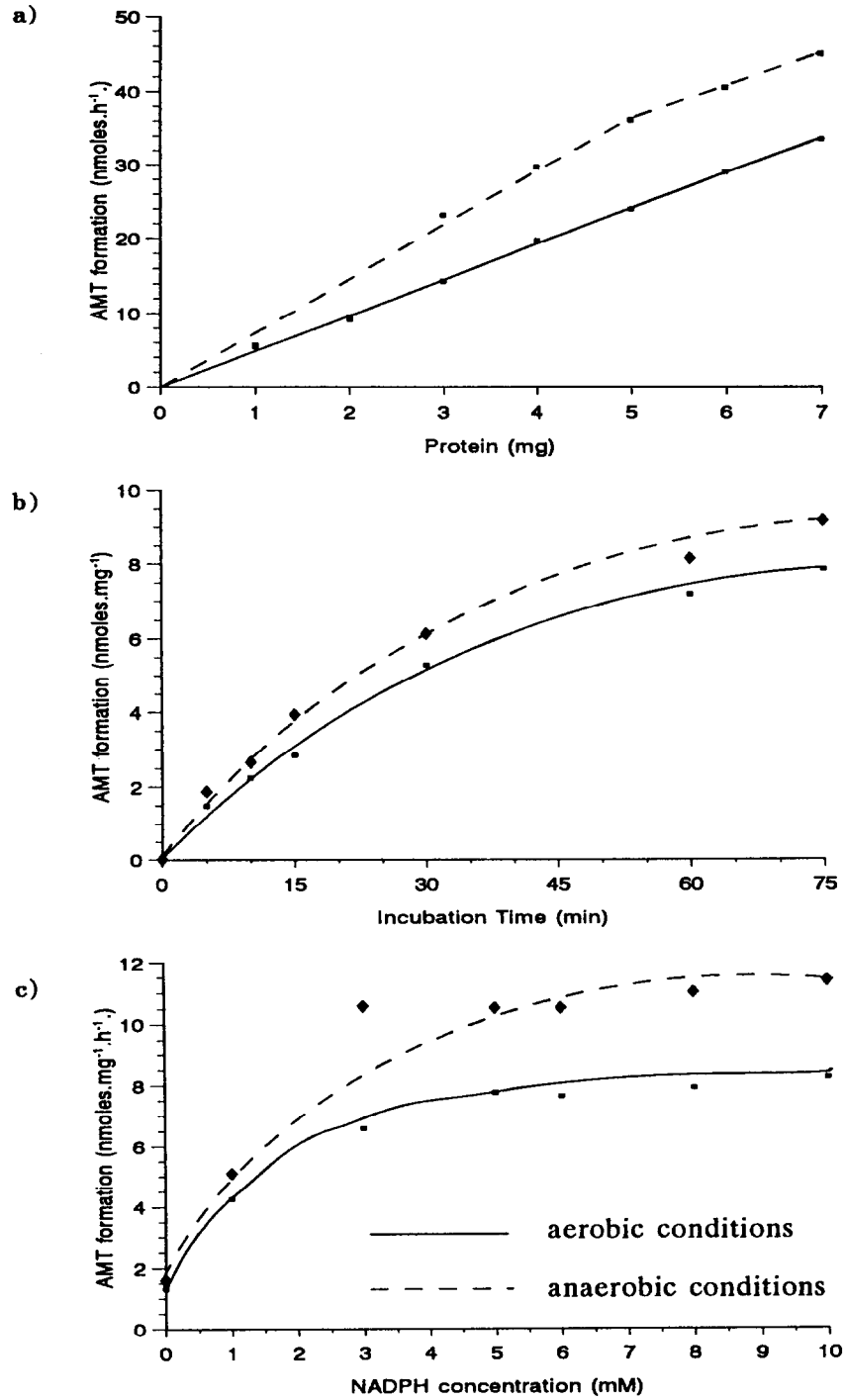

Fig. 2. Linearity studies to determine the optimum incubation conditions for AMT formation by human liver microsomes. (a) Protein; (b) time; (c) [NADPH]. Each point is the mean from three livers.

Reduced form glutathione was not as efficient as dithiothreitol (6% conversion in 24 hr). No reduction of ZDV to AMT was observed in studies with BSA.

Administration of β-naphthoflavone, phenobarbitone, dexamethasone and clofibrate to rats resulted in a significant increase in total microsomal cytochrome P450 levels, whereas the administration of ethanol had no effect on total cytochrome P450 (Table 2).

As with human liver microsomes, formation of AMT by rat liver microsomes resulted in non-linear Eadie–Hofstee plots (data not shown), indicating

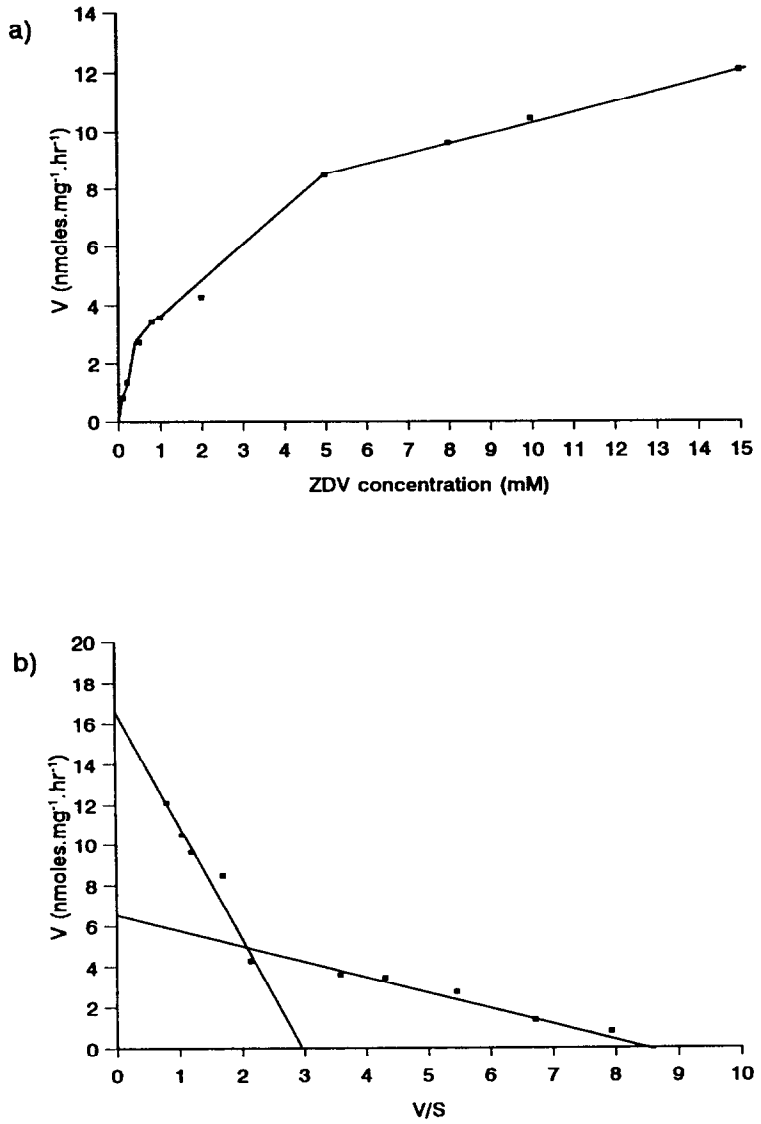

Fig. 3. Plots to determine K_m and V_{max} values for AMT formation in microsomes from human liver LXXII. (a) Velocity vs [ZDV] (mM); (b) Eadie–Hofstee plot.

the involvement of more than one site. As the high affinity, low capacity component was thought to be most relevant clinically and in terms of cytochrome P450 involvement, K_m, V_{max} and Cl_{int} were calculated for this site based on substrate concentrations of 0.05–5 mM.

Each group of animals administered an inducer was compared with the corresponding control group administered vehicle alone. There was no alteration in Cl_{int}, K_m or V_{max} when animals were administered β-naphthoflavone or ethanol. However, administration of phenobarbitone resulted in an increased Cl_{int} (71.2 ± 4.8 vs 30.9 ± 8.9 μL/mg/hr; $P < 0.001$; mean ± SD; N = 4) as did administration of dexamethasone (145.5 ± 24.4 vs 52.8 ± 5.7 μL/mg/hr; $P < 0.05$; N = 4) and clofibrate (82.0 ± 16.7 vs 49.8 ± 9.9 μL/mg/hr; $P < 0.05$) (Table 2).

For rats administered phenobarbitone there was a significant decrease in K_m (1.20 ± 0.18, N = 4 vs 2.03 ± 0.77 mM, N = 5; $P < 0.05$) when compared to control animals, whereas although administration of dexamethasone resulted in an 8-fold increase in V_{max} (564.8 ± 149.0, N = 4 vs 72.5 ± 11.1 nmol/mg/hr, N = 4; $P < 0.05$) this was accompanied by a 3-fold increase in K_m(3.89 ± 0.84 vs 1.38 ± 0.20 mM; $P < 0.05$), with an overall 3-fold increase in Cl_{int}.

Kinetic plots of velocity vs substrate concentration for each group of rats illustrated the increase in AMT formation with administration of phenobarbitone, dexamethasone and clofibrate and the lack of effect of β-naphthoflavone and ethanol (Fig. 6).

DISCUSSION

Formation of AMT by human and rat liver

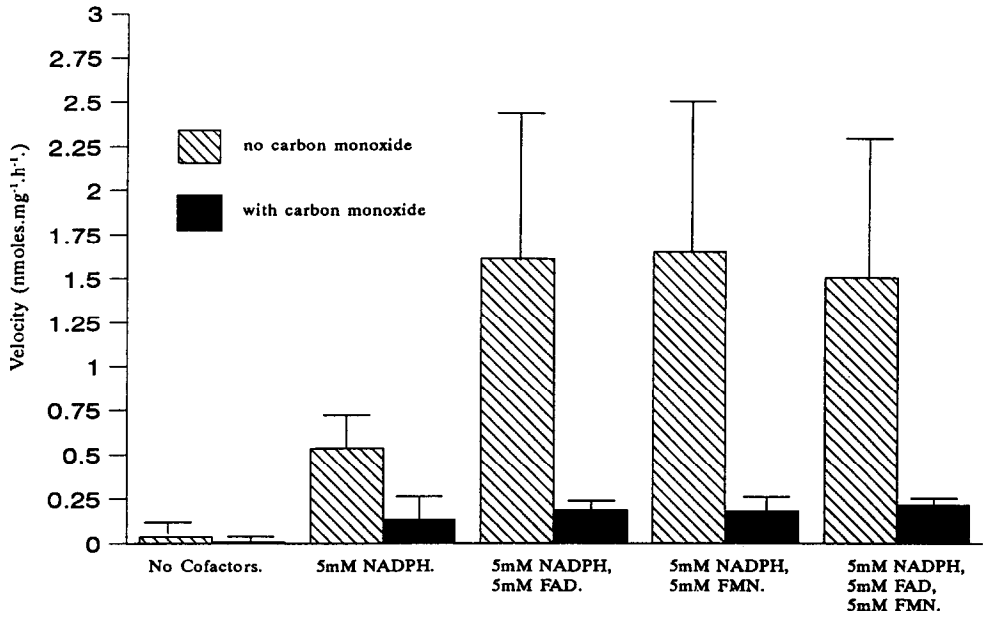

Fig. 4. The effect of FAD, FMN and carbon monoxide on the reduction of ZDV (0.1 μCi; 500 μM) to AMT by human liver microsomes. Each value is the mean ± SD from three determinations.

Table 1. Inhibition of AMT formation by substrates and inhibitors of cytochrome P450 isozymes and other enzymes

Compound	Concentration (μM)	AMT formation (nmol/mg/hr)	Activity remaining (%)
Control	0	0.88 ± 0.28	100
Theophylline	100	0.63 ± 0.24	71
(CYP1A substrate)	500	0.51 ± 0.06	58
Tolbutamide	100	0.79 ± 0.26	89
(CYP2C substrate)	500	0.69 ± 0.18	78
Cortisol	100	0.79 ± 0.22	90
(CYP3A substrate)	500	0.73 ± 0.30	83
Ketoconazole	100	0.42 ± 0.11	49
(CYP inhibitor)	500	0.33 ± 0.14	38
Cimetidine	100	0.65 ± 0.31	74
(CYP inhibitor)	500	0.54 ± 0.14	62
Miconazole	100	0.72 ± 0.31	82
(CYP inhibitor)	500	0.62 ± 0.27	70
Chloramphenicol	100	0.62 ± 0.21	71
(nitroreduction)	500	0.61 ± 0.26	69
Allopurinol	100	0.69 ± 0.33	78
(XO inhibitor)	500	0.67 ± 0.26	77
Dicoumarol	100	0.85 ± 0.42	97
(DT-Diaphorase inhibitor)	500	0.92 ± 0.36	105
Control	0	1.07 ± 0.15	100
Chlorzoxazone	25	0.90 ± 0.26	84
(CYP2E1 substrate)	100	0.82 ± 0.30	77
	200	0.80 ± 0.08	75
	500	0.74 ± 0.21	70
Thiobenzamide	100	0.97 ± 0.10	91
(CYP2E1 substrate)	500	1.03 ± 0.13	96
p-Nitrophenol	100	0.88 ± 0.05	82
(CYP2E1 substrate)	500	0.60 ± 0.04	56
Mercaptoethanol	100	0.96 ± 0.14	89
(CYP2E1 substrate)	500	0.80 ± 0.29	74
Isoniazid	100	0.96 ± 0.14	89
(CYP2E1 substrate)	500	0.63 ± 0.38	59

Values for microsomal AMT formation represent the means (±SD) of determinations from three livers.

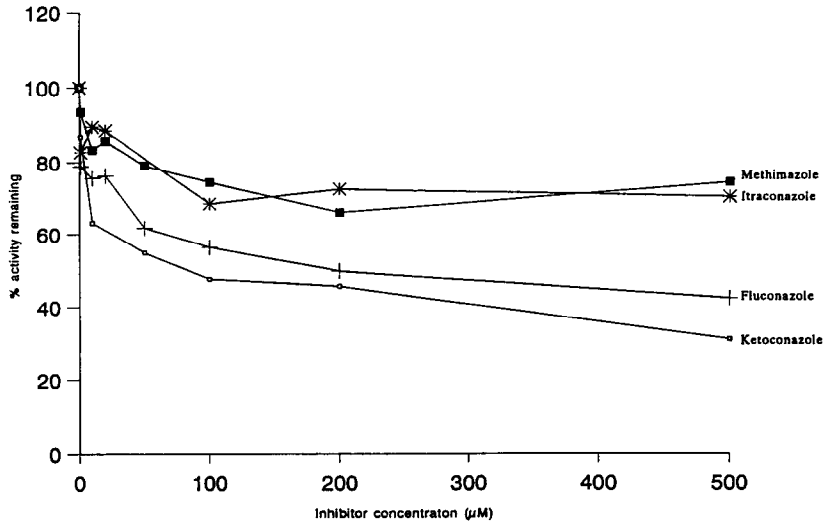

Fig. 5. The inhibitory effect of ketoconazole, fluconazole, itraconazole and methimazole on the reduction of ZDV (0.1 μCi; 100 μM) to AMT by human liver microsomes. Each point is the mean value from three livers.

Table 2. The effect of selective inducers of various isozymes of cytochrome P450 on rat liver microsomal cytochrome P450 content and on K_m, V_{max} and Cl_{int} for AMT formation

Group	Cytochrome P450 (nmol/mg)	K_m (mM)	V_{max} (nmol/mg/hr)	Cl_{int} (μL/mg/hr)
Control	0.97 ± 0.28	1.95 ± 0.24	99.0 ± 15.7	51.5 ± 11.3
Ethanol-induced	1.14 ± 0.06	1.82 ± 0.20	97.3 ± 24.9	52.9 ± 8.4
Saline control†	1.08 ± 0.36	2.03 ± 0.77	61.3 ± 23.5	30.9 ± 8.9
Phenobarbitone-induced	1.86 ± 0.18†	1.20 ± 0.18*	84.8 ± 8.7	71.2 ± 4.8‡
Corn oil control	0.70 ± 0.13	2.03 ± 0.36	103.5 ± 40.3	49.8 ± 9.9
Clofibrate-induced	1.52 ± 0.34†	1.85 ± 0.29	151.5 ± 37.6	82.0 ± 16.7*
β-naphthoflavone-induced	1.58 ± 0.20‡	1.85 ± 0.30	97.5 ± 12.0	53.6 ± 3.7
PEG/saline control	1.04 ± 0.12	1.38 ± 0.20	72.5 ± 11.1	52.8 ± 5.7
Dexamethasone-induced	2.65 ± 0.42‡	3.89 ± 0.84*	564.8 ± 149.0*	145.5 ± 24.4*

Control groups for each inducer were administered vehicle alone. Data expressed as means ± SD, N = 4 except †N = 5.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$ significantly different compared to corresponding control group administered vehicle alone, Student's non-paired *t*-test.

microsomes appeared to be a complex process involving at least two components, as indicated by the non-linear nature of Eadie–Hofstee and Lineweaver–Burk plots. It is not known whether both these components were enzymatic. Azides are susceptible to chemical reduction and AMT was reduced by dithiothreitol and to a smaller extent by glutathione.

It was almost certain that the high affinity, low capacity site of AMT formation was enzymic in nature, with the reduction of ZDV to AMT being NADPH dependent. At a concentration of ZDV associated with this component (0.1 mM) AMT formation was inhibited by carbon monoxide and enhanced by FAD and FMN. The inhibitory potential of carbon monoxide would indicate involvement of cytochrome P450. Placidi *et al.* [14] correlated AMT formation by human liver microsomes with cytochrome P450 levels at 1 mM ZDV. However, the kinetics of ZDV reduction were studied at higher ZDV concentrations (3–20 mM) which would overlook the high affinity, low capacity component involved in this reduction.

The results of the present study show some similarities with those obtained with another azide compound, MZP (reduced to meta-amino-pyrimethamine [17]). MZP was extensively reduced by mouse liver homogenate in an NADPH-dependent fashion under an atmosphere of nitrogen. Mouse liver microsomes catalysed MZP reduction

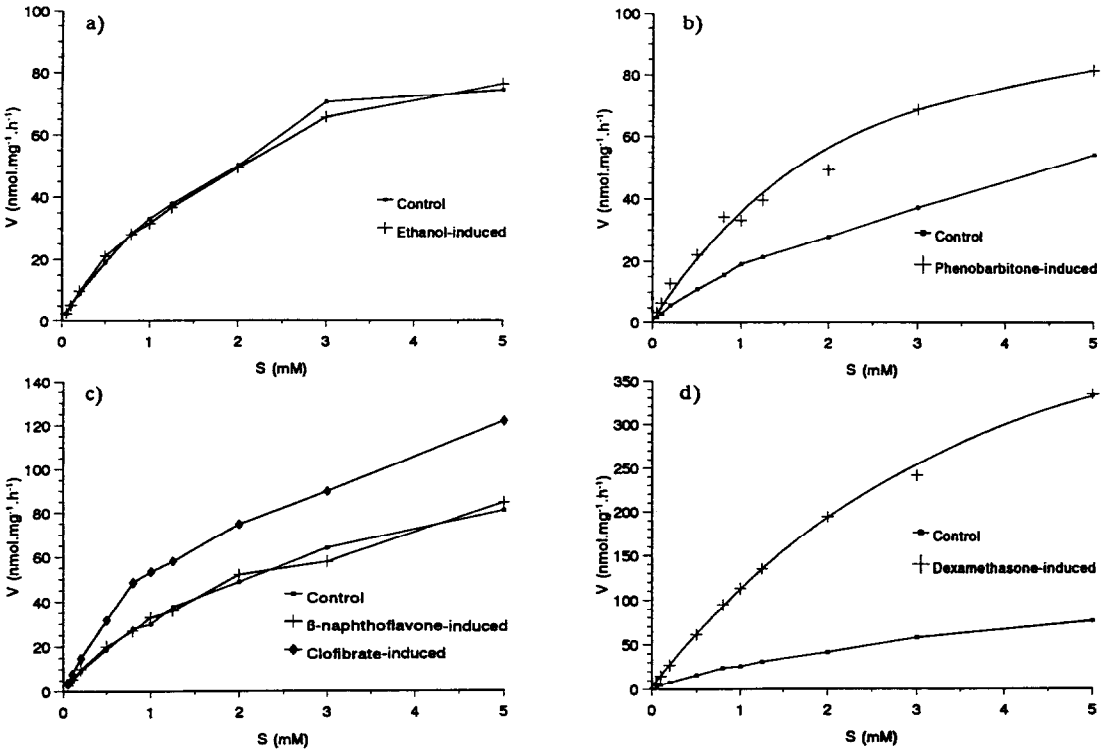

Fig. 6. AMT formation (nmol/mg/hr) in liver microsomes obtained from rats adminstered selective inducers of cytochrome P450 isozymes and the corresponding rats administered vehicle alone. (a) Ethanol induced rats; (b) phenobarbitone induced rats and saline control; (c) β-naphthoflavone and clofibrate induced rats and corn oil control; (d) dexamethasone induced rats and PEG : saline control. Each point represents the mean data from four rats except saline controls (N = 5).

to a lesser extent than homogenate, although full activity was restored when microsomes and cytosol were reconstituted.

Although cytochrome P450 is usually associated with oxidation reactions, its involvement in the reduction of several groups of compounds has been reported. One such group is the azo dyes, which in turn may be divided into two groups depending on the oxygen- and carbon monoxide-sensitivity of the reduction.

Azo dyes which are reduced by an oxygen- and carbon monoxide-sensitive process (S-substrates) have long been recognized as being metabolized at least partially by cytochrome P450 [18]. Although early studies indicated the involvement of both cytochrome P450 and cytochrome P450 reductase in the metabolism of neoprontosil, an S-substrate [19], the reduction of another S-substrate, aramanth, has been determined to be mediated solely by cytochrome P450 [20], a reduction that is stimulated by the addition of FMN [21].

Other azo dyes are reduced in a reaction that is insensitive to both oxygen and carbon monoxide (I-substrates). Studies involving these substrates have indicated involvement of cytochrome P450 in this reductive process: reduction is inhibited by inhibitors of cytochrome P450 activity, proceeds at a greater rate when cytochrome P450 reductase is incubated with various cytochrome P450 isozymes rather than incubated alone, and is induced by clofibrate, an inducer of cytochrome P450 [22]. That a reductive reaction may be mediated by cytochrome P450 and not inhibited by carbon monoxide is plausible given that carbon monoxide binds to the oxygen binding site of cytochrome P450 and oxygen should not be required for a reductive process.

Cytochrome P450 has been implicated in the reduction of nitrobenzene to aniline [23], of 1-nitropyrene to 1-aminopyrene [24] and of 3-nitrofluoroanthrene [25]. In the case of the latter compound, reduction was inhibited by oxygen, carbon monoxide and SKF-525A. Pre-treatment of rats with phenobarbitone stimulated the reduction which suggested the involvement of CYP2B. Probably the most extensively studied compounds that undergo cytochrome P450-mediated reduction are carbon tetrachloride and halothane [26, 27] with evidence that CYP2E1 catalyses the reduction of CCl_4 [28].

The results of the present study suggested that ZDV reduction was at least partially mediated by isozyme(s) of cytochrome P450. A wide range of substrates and inhibitors of different isozymes were screened and the most marked effect was seen with ketoconazole (IC_{50} value 78.0 μM). It has recently been shown that at low concentrations (around

1 μM), ketoconazole is a strong and selective inhibitor of CYP3A [29] but is a non-specific inhibitor of other isozymes at higher concentrations. All of the CYP2E1 substrates screened (with the exception of thiobenzamine) had some inhibitory potential, but none had a greater propensity for inhibition than the substrates for the other cytochrome P450 isozymes studied, all of which inhibited AMT formation by 20–40% at a concentration of 500 μM.

Ethanol, an inducer of CYP2E1 [30] did not induce microsomal AMT formation when administered to rats in the present study, also indicating that CYP2E1 was not involved to any great extent. There was also no induction of AMT formation following administration of β-naphthoflavone, an inducer of CYP1A [31]. However, phenobarbitone (primarily an inducer of CYP2B [31] and also CYP3A to a lesser degree), dexamethasone (an inducer of CYP3A [32]) and clofibrate (an inducer of CYP4A [31]) induced hepatic microsomal formation of AMT when administered to rats.

It has recently been reported that phenobarbitone is capable of inducing the reduction of ZDV in rats at a single concentration of ZDV [33]. Although dexamethasone may not be entirely selective for CYP3A and has been shown to cause slight induction of CYP2B [32], there was greater induction of AMT formation with dexamethasone than phenobarbitone in the present study. This would indicate that the enhanced AMT formation associated with dexamethasone induction was due to induction of CYP3A rather than CYP2B. Furthermore, in control rat liver microsomes AMT formation was strongly inhibited by ketoconazole (unpublished data) with 50.4 ± 3.0% activity remaining in the presence of 5 μM ketoconazole (mean ± SD; N = 4), a concentration selective for CYP3A [29].

Clofibrate has also been shown to induce CYP2B, CYP2E1 and CYP3A to a lesser extent than CYP4A [31]. It is not clear whether increased AMT formation in microsomes from rats administered clofibrate is due to a specific effect on CYP4A or a combination of effects on CYP2B and CYP3A. The data obtained from rat induction studies would therefore implicate the involvement of CYP2B, CYP3A and possibly CYP4A in AMT formation.

However, cytochrome P450 may not be solely responsible for the reduction of ZDV in human liver microsomes. Formation of AMT was inhibited by up to 80% by carbon monoxide in the presence of NADPH (Fig. 4). It has been postulated that carbon monoxide prevents the transfer of electrons from haem to the reducible substrate in the case of azo dyes, although carbon monoxide insensitivity of the reduction may not exclude the involvement of cytochrome P450 [22]. Alternatively, residual activity after exposure to carbon monoxide may be attributable to the catalytic activity of the NADPH-dependent cytochrome P450 reductase. Purified cytochrome P450 reductase is able to reduce substrates directly, for example 2,3,5,6-tetramethylbenzoquinone [34].

In addition, carbon monoxide was capable of inhibiting FAD/FMN-enhanced NADPH-dependent formation of AMT by human liver microsomes indicating the involvement of cytochrome P450 in flavin-enhanced reduction. This is in contrast to a recent study [33] which suggested that FAD and FMN serve as direct electron donors to ZDV. Stiff *et al.* [35] recently investigated the reductive metabolism of zonisamide, an anticonvulsant, and suggested a number of possible pathways whereby the flavin was involved with either the cytochrome P450, the cytochrome P450 reductase or both in a reaction sequence that would reduce the substrate. Although zonisamide could also be reduced by NADPH and FMN in the absence of enzyme, this was not the case with ZDV (unpublished data).

Given the observed cytotoxicity of AMT [7, 12] and its presence in the plasma of rhesus monkeys and humans administered radiolabelled ZDV [10, 11] it has been postulated that the haematological toxicity associated with ZDV therapy may, at least in part, be attributable to AMT. The aim of the present study was to identify the enzyme(s) involved in this metabolic step with a view to predicting the possible effects of coadministered drugs. Any approach to inhibit the formation of AMT could have clinical benefit. This approach has been successfully utilized with dapsone-dependent methaemaglobinaemia which was reduced by cimetidine [36].

Although the role of cytochrome P450 in ZDV reduction to AMT has been demonstrated, a single isozyme has not been implicated. It would appear that several isozymes may be involved in this metabolic step in both humans and rats. The contribution of cytosolic enzymes and reducing agents to AMT formation needs to be investigated.

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