N-Hydroxylation of dapsone by multiple enzymes of cytochrome P450: implications for inhibition of haemotoxicity

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- 1 The adverse reactions associated with the administration of dapsone are believed to be caused by metabolism to its hydroxylamine. Previous reports suggest that CYP3A4 is responsible for this biotransformation [1].
- 2 Data presented in this paper illustrate the involvement of more than one cytochrome P450 enzyme in dapsone hydroxylamine formation using human liver microsomes. Eadie-Hofstee plots demonstrated bi-phasic kinetics in several livers. No correlation could be established between hydroxylamine formation and CYP3A concentrations in six human livers ($r = -0.47$; $P = 0.34$).
- 3 Studies with low molecular weight inhibitors illustrate the importance of CYP2C9 and CYP3A in dapsone N-hydroxylation.
- 4 Differential sensitivity of dapsone N-hydroxylation to selective CYP inhibitors indicated that the contribution of individual CYP enzymes varies between livers. Selective inhibition ranged from 6.8 to 44.1% by 5 μM ketoconazole, and from 24.0 to 68.4% by 100 μM sulphaphenazole. The extent of inhibition, by either ketoconazole or sulphaphenazole was dependent on the CYP3A content of the liver.
- 5 The levels of expression of these cytochrome P450 enzymes may be an important determinant of individual susceptibility to the toxic effects of dapsone, and may influence the ability of an enzyme inhibitor to block dapsone toxicity *in vivo*. Because of the inability to produce complete inhibition, selective CYP inhibitors are unlikely to offer any clinical advantage over cimetidine in decreasing dapsone hydroxylamine formation *in vivo*.

Keywords dapsone hydroxylamine metabolism inhibition cytochrome P450 toxicity

Introduction

Dapsone is used in the treatment of a wide variety of ailments, including leprosy [2] and in the prophylaxis of malaria [3]. However, concerns over resistance and, more importantly, severe adverse reactions have limited the use of the drug. Despite these problems, the relative importance of sulphone and sulphonamide drugs has increased over recent years due to the emergence of the human immunodeficiency virus (HIV). Dapsone and several sulphonamide drugs such as sulphamethoxazole and sulphadiazine, have proved effective in the treatment of the opportunistic infections, *Pneumocystis carinii* pneumonia (PCP) and *Toxoplasma gondii* in AIDS

patients [4, 5]. However, there is a higher incidence of adverse reactions to these antimicrobial agents in HIV-infected patients [6–8] when compared with non-infected individuals, which limits the usefulness of the drugs in these patients.

Dapsone (4,4' diaminodiphenyl sulphone) displays two types of toxicity, both of which are thought to be mediated by N-oxidation [9, 10]. A dose-dependent toxicity towards erythrocytes is observed in all individuals, but is only a clinical problem at high doses ($>100 \text{ mg day}^{-1}$) [11], in patients suffering from a deficiency in glucose-6-phosphate dehydrogenase [12], or in AIDS patients [13]. A more serious idiosyncratic reaction which involves white cells, resulting in agranulo-

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cytosis, fever and rashes, occurs in a small proportion of the population (<1:2000) [14, 15]. Stevens-Johnson syndrome has also been reported in patients on dapsone therapy [16]. The haemotoxicity of dapsone is mediated by the hydroxylamine metabolite, which is capable of being co-oxidized with haemoglobin (Hb) in the red blood cell to produce nitroso dapsone (Figure 1) and methaemoglobin (Met-Hb) [9, 10]. The nitroso compound can then be reduced back to the hydroxylamine by the action of glutathione, so that a futile oxidation-reduction cycle exists in which the cell uses oxygen to deplete glutathione and NADPH [17]. Dapsone-induced toxicity to white cells is poorly understood, but is also thought to be a consequence of hydroxylamine

formation, possibly involving further oxidation to other intermediates within the target cell [10].

An obvious approach to completely block hydroxylamine-induced toxicity is by chemical modification of the drug. However, recent structure-activity studies from our laboratory have shown that the extensive *N*-hydroxylation of dapsone, noted both *in vitro* and *in vivo*, is a function of the chemical properties of the *p*-sulphone group [18], which is essential for pharmacological activity [19].

Recent studies have illustrated well the potential clinical benefits associated with the administration of cytochrome P450 (CYP) inhibitors on the reduction of dapsone-induced methaemoglobinaemia [20, 21].

Figure 1 Proposed mechanisms of bioactivation and detoxification of dapsone. (GSH: glutathione; GSSG: oxidized glutathione; NAT: N-acetyl transferase; GT: glucuronyl transferase; UDPGA: UDP glucuronic acid.)

Cimetidine has been shown to decrease, but not abolish, the haemotoxicity of dapsone by partial inhibition of *N*-hydroxylation. It has been suggested that CYP3A is responsible for hydroxylamine formation [1]. The purpose of this study, therefore, was to investigate the potential of ketoconazole, a potent inhibitor of CYP3A, to block the bioactivation of dapsone.

Methods

Chemicals

Dapsone (4,4' diaminodiphenyl sulphone, DDS), isoniazid, quinidine hydrochloride, diethyldithiocarbamate sodium and reduced nicotinic adenine dinucleotide phosphate (NADPH) were obtained from Sigma Chemicals (Poole, Dorset, UK). Ketoconazole was provided by Janssen (Beerse, Belgium), furafylline was a gift from Parke Davis Pharmaceutical Division (Michigan, USA), and sulphaphenazole was supplied by Ciba-Geigy Pharmaceuticals (Basle, Switzerland). Dapsone hydroxylamine was synthesized by the method of Uetrecht *et al.* [22]. All h.p.l.c. solvents were of analytical grade and were acquired from Fisons plc (Loughborough, UK). All other chemicals were purchased from BDH Chemicals Ltd (Poole, Dorset, UK).

Human liver samples

Samples were from histologically normal livers which had been removed and transferred to the laboratory within 30 min of death. The liver was sliced into 10–20 g portions, placed in vials and frozen in liquid nitrogen. These samples were stored at -80°C until the preparation of microsomes. Ethical approval was granted and consent was obtained from the donors' relatives before removal of the liver.

Preparation of microsomes

The frozen liver was thawed and minced in ice cold 0.067 M phosphate buffer (pH 7.5) containing 1.15% KCl (w/v). The livers were then homogenized using a motor driven Polytron homogenizer. The homogenates were centrifuged at 10 000 *g* for 20 min at 4°C to remove mitochondria, nuclei and cell debris. The supernatants were decanted off and centrifuged at 105 000 *g* for 60 min at 4°C to produce the microsomal pellet. The pellets were resuspended in KCl phosphate buffer and centrifuged for a further 60 min at 105 000 *g*. The washed microsomal pellets were then reconstituted in 0.067 M phosphate buffer (pH 7.5). Protein was assayed by the method of Lowry *et al.* [23], using bovine serum albumin as the protein standard, and cytochrome P450 content was determined by the method of Omura & Sato [24]. The microsomes were stored at -80°C until required.

Microsomal incubations

Dapsone was incubated for 1 h at 37°C with microsomal protein (1 mg ml $^{-1}$), HEPES (*N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethane sulphonic acid]) buffered salt solution (pH 7.4) and NADPH (1 mM) to give a final volume of 100 μl . The reaction was terminated by the addition of methanol (200 μl), and the protein was left to precipitate overnight. Samples were centrifuged at 13 000 *g* for 5 min before injection of the supernatant onto the h.p.l.c. In order to prevent further spontaneous oxidation of the hydroxylamine, ascorbic acid (500 μM) was added to incubations before initiating the reaction and again on termination of the reaction. All of the incubations were performed in triplicate and under linear conditions with respect to time and protein concentration.

Kinetic studies

Kinetic studies were performed using microsomes prepared from six different human livers. Dapsone concentrations ranged from 10 to 1000 μM . Data from kinetic experiments were analysed by Grafit (version 3.01) by an iterative non-linear least squares algorithm, using initial estimates of K_m and V_{max} obtained from Eadie-Hofstee plots. One- and two-site models were fitted to the data and best fit was assessed by the significance of the *F* ratio.

Inhibition studies

The following compounds were tested for their ability to inhibit dapsone *N*-hydroxylation: furafylline (CYP1A2 inhibitor; 0–30 μM); sulphaphenazole (CYP2C inhibitor; 0–250 μM); quinidine (CYP2D6 inhibitor; 0–10 μM); ketoconazole (CYP3A inhibitor; 0–50 μM); isoniazid (CYP2E1 inhibitor; 0–500 μM) and diethyldithiocarbamate (DDC) (CYP2E1 inhibitor; 0–500 μM). Furafylline and DDC were pre-incubated with the microsomal suspension and NADPH for 15 min prior to the addition of dapsone and ascorbic acid. A dapsone concentration of 100 μM was used for all inhibition experiments.

H.p.l.c. and LC-MS analysis

Supernatants were analysed by h.p.l.c. with ultra-violet (u.v.) detection. Aliquots of supernatant (50 μl) were injected onto a μ -Bondapak 10 C $_{18}$ column (30 cm $\times$ 3.9 mm) supplied by HPLC Technology (Macclesfield, Cheshire, UK). The mobile phase consisted of distilled water, acetonitrile, glacial acetic acid and triethylamine in the ratio of 80:20:1:0.1 v/v. Chromatography was performed isocratically at a flow rate of 1 ml min $^{-1}$ using a SP8800 pump (Spectra-Physics, San Jose, California, USA). U.v. absorbance was monitored at 254 nm on an SP100 wavelength detector (Spectra-Physics). The peak areas were calcu-

lated using a Spectra-Physics Chromjet Integrator. An internal standard was not necessary due to a good correlation between radiometric and u.v. analysis of hydroxylamine formation ($r=0.988$).

LC-MS analysis was performed using a μ -Bondapak 10 C₁₈ column (30 cm $\times$ 3.9 mm). Samples were eluted with a mobile phase consisting of ammonium formate (6 mM, pH 3.5) and acetonitrile (80:20 v/v) at a flow rate of 1 ml min⁻¹. The mobile phase was delivered by two Jasco PU980 pumps (Jasco Corporation, Tokyo, Japan). Eluate passed through a Jasco u.v.-975 absorbance detector (254 nm), a Valco tee-union stream splitter (Phase Separations, Deeside, Clwyd, UK) and a fused silica capillary (75 μ m i.d., 150 cm length) to the electrospray probe and interface of a QuattroII tandem quadrupole mass spectrometer (Fisons Biotech MS, Manchester, UK). Nebulizing and drying gas (nitrogen) was delivered at 12 l h⁻¹ and 280 l h⁻¹, respectively. The analysis was performed under the following conditions; capillary flow of *circa.* 40 μ l min⁻¹, interface temperature of 60°C, capillary voltage of 4×10^3 V, and a cone voltage of 30–80 V. Compressed centroid spectra were acquired between m/z 100–850. Data were processed via MassLynx 2 software. The identity of the hydroxylamine metabolite was confirmed by co-chromatography with an authentic standard and by liquid chromatography-mass spectrometry (LC-MS). The test material and the authentic standard gave identical spectra (pseudomolecular ion, $[M+1]$ at m/z 265). The inter- and intra-assay variation were 6.3% and 4.6% respectively, at a metabolite concentration of 1 nmol ml⁻¹.

Results

Kinetic studies

The rate of dapsone hydroxylamine formation at 100 μ M displayed a relatively low variation between the different livers (105.5–126.0 pmol mg⁻¹ min⁻¹), despite a three-fold variation in CYP3A content. There was no correlation between hydroxylamine formation and CYP3A concentrations [25] in the six livers studied ($r = -0.47$; $P = 0.34$). Liver L19, which had the lowest CYP3A content, produced the greatest turnover to the hydroxylamine. Eadie-Hofstee plots were constructed for all of the livers. Four of the six livers displayed bi-phasic kinetics (Figure 2), suggesting the involvement of more than one enzyme in dapsone metabolism.

Inhibition studies

L20, which had an intermediate CYP3A concentration, was chosen initially for these studies. Furaflavone and quinidine did not markedly inhibit (<8%) hydroxylamine formation, indicating that CYP1A2 and CYP2D6 do not play a significant role in this biotransformation. However, the addition of ketoconazole and sulphaphenazole did produce a decrease in metabolism to the

Figure 2 Eadie-Hofstee plot for dapsone hydroxylamine formation in one human liver (L19) (Assuming two enzyme model: $V_{\max 1} = 116.1$ pmol mg⁻¹ min⁻¹, $K_{m1} = 52.6$ μ M; $V_{\max 2} = 478.6$ pmol mg⁻¹ min⁻¹, $K_{m2} = 881.8$ μ M).

hydroxylamine (inhibition = 23.3% at 5 μ M ketoconazole, and 48.7% at 100 μ M sulphaphenazole), indicating a role for CYP3A and CYP2C9.

In order to investigate inhibition further, the experiments with ketoconazole and sulphaphenazole were repeated in the remaining five livers. The livers display a differing sensitivity to the inhibitors (Figures 3 and 4, Table 1). Ketoconazole (CYP3A inhibitor) had a greater inhibitory effect on the metabolism of dapsone by L36 (inhibition at 5 μ M = 44.1%), compared with all the other livers (Figure 3, Table 1). However, sulphaphenazole (a selective CYP2C inhibitor), displayed a relatively low inhibitory potential towards L36 (inhibition at 100 μ M = 24.0%), and the greatest inhibitory effect was observed with L19 (inhibition at 100 μ M = 68.4%) (Figure 4, Table 1). A single liver (L22) was chosen for

Table 1 Effect of selective P450 inhibitors on the metabolism of dapsone by human liver microsomes *in vitro*. (Values shown are mean of triplicate determinations; s.d. values <10%)

	CYP3A content (relative intensity)	% Inhibition of dapsone hydroxylamine formation	
		Ketoconazole (5 μ M)	Sulphaphenazole (100 μ M)
L19	930	15.9%	68.4%
L18	1028	6.8%	60.7%
L22	1469	27.7%	49.8%
L20	1671	23.3%	48.7%
L7	1882	29.8%	40.5%
L36	3073	44.1%	24.0%

Figure 3 Differential sensitivity of six human livers (● L36; ▲ L7; ○ L20; ■ L22; □ L19; △ L18) to the inhibitory effect of ketoconazole on dapsone hydroxylamine formation *in vitro*. (Data points shown are mean values of triplicate incubations; s.d. values <10%).

dual inhibition studies. A combination of both ketoconazole (50 μM) with sulphaphenazole (250 μM) produced significantly ($P < 0.001$; unpaired Student's *t*-test) greater inhibition ($95.5 \pm 0.8\%$; mean $\pm$ s.d.; $n = 4$ replicate incubations) than either sulphaphenazole ($50.5 \pm 7.6\%$) or ketoconazole ($68.4 \pm 3.4\%$) alone (Figure 5). CYP3A concentrations correlate highly with inhibition at 5 μM ketoconazole ($r = 0.95$; $P < 0.01$). A negative correlation was achieved between CYP3A concentrations and inhibition at 100 μM sulphaphenazole ($r = -0.97$; $P < 0.001$; Figure 6).

Isoniazid, a substrate and inhibitor of CYP2E1, was also studied. Partial inhibition was observed only at relatively high isoniazid concentrations (inhibition at 100 μM [L19] = 28.6%, [L20] = 18.1%, [L36] = 26.0%), and displayed little variation between the different livers. DDC also caused partial inhibition of dapsone *N*-hydroxylation in L20 (inhibition at 100 μM = 29.1%).

Discussion

The level of expression of various hepatic cytochrome P450 enzymes may be an essential determinant of dapsone toxicity, which is dependent on the balance between *N*-oxidation, *N*-glucuronidation and also possibly the glutathione status of the patient [26–28]. Evidence obtained in this *in vitro* study clearly indicates the role of more than one hepatic CYP enzyme in dapsone *N*-hydroxylation.

Figure 4 Differential sensitivity of six human livers (□ L19; △ L18; ■ L22; ○ L20; ▲ L7; ● L36) to the inhibitory effect of sulphaphenazole on dapsone hydroxylamine formation *in vitro*. (Data points shown are mean values of triplicate incubations; s.d. values <10%).

Figure 5 Inhibition of hydroxylamine formation by sulphaphenazole alone, and by ketoconazole in the presence of sulphaphenazole (250 μM) using a single human liver (L22). (Data points shown are mean values of quadruplicate incubations; s.d. values <10%).

In contrast to earlier work by Fleming *et al.* [1], we were unable to show a correlation between dapsone *N*-hydroxylation and CYP3A levels in a panel of six human livers. More importantly, biphasic kinetics were observed for this biotransformation in several of these livers. Thus, dapsone *N*-hydroxylation is not catalysed exclusively by CYP3A4 and cannot be regarded as a specific probe for this enzyme. Indeed, Kinirons *et al.*

Figure 6 Relationship between inhibition of dapsone hydroxylamine formation by sulphaphenazole at 100 μM and CYP3A content in six different human livers. (Data points shown are mean values of triplicate incubations; s.d. values <10%).

[29], failed to show a correlation between the *N*-hydroxylation of dapsone and either the erythromycin breath test or urinary 6 β -hydroxycortisol excretion, both of which are regarded as probes for CYP3A enzymes.

To determine the enzymatic nature of the biotransformation, the effect of low molecular weight inhibitors on the *in vitro* *N*-hydroxylation was investigated. The purpose of this was twofold: first, to gain further insight into the nature of the enzymes involved in the bioactivation of dapsone and second, to establish whether a suitable effective inhibitor exists which could be used *in vivo* to block dapsone toxicity completely.

A minor degree of inhibition was noted with furafylline and quinidine, indicating that neither CYP1A2 nor CYP2D6 are involved significantly in the biotransformation. Inhibition was observed with ketoconazole and sulphaphenazole, which would suggest a role for both CYP3A and CYP2C9 in dapsone *N*-hydroxylation. A notable feature observed with both these inhibitors was the lack of complete inhibition (Figures 3 and 4). Furthermore, the maximum inhibition seen with sulphaphenazole, which is regarded as a relatively specific inhibitor of CYP2C9 [30, 31], was variable between livers, as was the inhibition produced by ketoconazole, a selective inhibitor of CYP3A at low concentrations [32, 33]. These observations probably reflect inter-liver variation in the expression of the two sub-families of enzymes which has been well documented elsewhere

[34–36]. We also found that a combination of ketoconazole (50 μM) and sulphaphenazole (250 μM) produced a greater degree of maximal inhibition than either agent alone (>90%; Figure 5).

Inhibition of hydroxylamine formation by 5 μM ketoconazole correlated well with CYP3A content ($r=0.95$). Furthermore, a negative correlation was established between inhibition with sulphaphenazole (100 μM) and CYP3A content ($r=-0.97$; Figure 6). Extrapolation of the data indicates that sulphaphenazole is only capable of producing 80% inhibition at zero CYP3A levels. This provides evidence for the possible involvement of another enzyme, in addition to CYP3A and CYP2C9.

Inhibition was observed with isoniazid, a substrate and inhibitor of CYP2E1 [37], but only at relatively high concentrations. This is consistent with a role for CYP2E1 in dapsone *N*-hydroxylation, as suggested in a preliminary report by Mitra *et al.* [38]. However, its precise contribution can only be determined once the selectivity of inhibitors for human CYP2E1 have been clearly defined [39].

The contribution of CYP enzymes is likely to be a function of substrate concentration. In this study dapsone concentrations of 100 μM were used to allow a comparison with previous work [1]. However, only low concentrations of dapsone are achieved *in vivo* (<10 μM) [21] and it is possible this may alter the contribution of individual CYP enzymes. Further work to develop more sensitive assay systems is required to address this issue.

One approach to decrease the toxicity associated with dapsone is the co-administration of a CYP inhibitor. Cimetidine appears promising in this respect. Clearance of dapsone to the dapsone hydroxylamine glucuronide and observed levels of methaemoglobin were significantly reduced in volunteers given cimetidine (400 mg three times daily) [20]. Cimetidine also decreased methaemoglobinaemia in patients during chronic therapy [21]. However, cimetidine failed to abolish methaemoglobin formation completely, which is probably a function of its short half-life *in vivo* [40] compared with dapsone, and its relative lack of potency as an enzyme inhibitor. Cimetidine was, however, found to produce >90% inhibition of dapsone *N*-hydroxylation *in vitro* [41] and is therefore a more potent inhibitor of the biotransformation than either ketoconazole or sulphaphenazole. This may reflect its relative non-selective inhibition of CYP enzymes, widely reported in both *in vitro* [42] and *in vivo* studies [43].

In conclusion, it would appear that the ability of CYP inhibitors to reduce dapsone bioactivation *in vivo*, will depend on a number of factors including both the selectivity and potency of the inhibitor, and the level of expression of CYP enzymes in the liver of the patient. From a clinical point of view, the *in vitro* and *in vivo* [44] data suggest that neither selective CYP3A inhibitors nor selective CYP2C inhibitors offer any advantage over cimetidine in this respect, as they cannot produce complete inhibition. This lack of complete inhibition of bioactivation is of particular concern with regard to hypersensitivity reactions, which unlike methaemoglobinaemia, are not simple dose-dependent phenomena

because of the ability of the immune system to respond to very low levels of chemical antigen [45].

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