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Inhibition of dapsone-induced methaemoglobinaemia in the rat

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Dapsone (4,4'diaminodiphenyl sulphone) is the most important component of the multidrug regimen for the treatment of leprosy advised by the World Health Organisation (WHO) [1]. It is used for malarial prophylaxis (with pyrimethamine; [2]), inflammatory disease states such as rheumatoid arthritis [3], and disorders characterized by polymorphonuclear leucocyte infiltration [4]. The drug is also effective in the treatment of *Pneumocystis carinii* in AIDS patients [5]. Dapsone is extensively metabolized in man through N-hydroxylation to DDS hydroxylamine (DDS NOH, [6, 7] and acetylation [8]). Monoacetyl dapsone may also undergo N-hydroxylation to form monoacetyldapsone hydroxylamine [9]. Adverse reactions to dapsone include agranulocytosis [10], methaemoglobinaemia [11] and haemolysis [12]. The two latter reactions are dose dependent and occur to some extent in all patients taking the drug [13]. All three adverse effects have been attributed to the hydroxylamine metabolites of dapsone [14–16]. Within the red cell, DDS NOH mediated oxidation of haemoglobin outstrips the capacity of NADH-dependent methaemoglobin reductase to convert methaemoglobin back to haemoglobin [17]. Impairment of this enzyme function, either genetic or through toxicity, may cause severe methaemoglobinaemia [18]. In addition, due to haemolysis, reduced red cell lifespan is especially marked in individuals with glucose-6-phosphate dehydrogenase deficiency or diminished glutathione reductase activity [13].

The efficacy of dapsone is thought to be due to the parent compound; in fact neither DDS NOH nor monoacetyl dapsone possess anti-bacterial activity [13, 19]. Therefore the aim of this study was to investigate the possibility of selectively inhibiting the formation of the metabolites thought to be responsible for the side effects of dapsone therapy (e.g. methaemoglobinaemia), while not affecting detoxication pathways such as acetylation. If this hypothesis were to be correct, full antibacterial activity could be retained while potentially serious toxicity could be wholly or partly diminished.

Materials and methods

Chemicals. DDS, ketoconazole and methimazole were supplied by the Sigma Chemical Co. (Poole, U.K.). Cimetidine was obtained from Smith, Kline and French Ltd, (Welwyn Garden City, U.K). Piperonyl butoxide was obtained from Pfalz and Bauer (Stamford, CT). All other chemicals were of reagent grade and were obtained from BDH Chemicals Ltd (Poole, U.K.).

Animals. Male Wistar rats (200–250 g) were obtained from the Department of Pharmacology and Therapeutics Breeding Colony (University of Liverpool) and were maintained on a Labsure CRM diet (Biosure Ltd., Cambridgeshire, U.K.) plus water *ad lib.*

Protocol. A first set of animals (groups A–F, N = 10 per group) were dosed with DDS at 33 mg/kg (i.p.) in dimethyl sulphoxide (DMSO 200 μL; groups A, B, C, E, F) or 3.3 mg/kg (group D). One hour prior to dapsone administration five animals from each group were pretreated with either methimazole (60 mg/kg i.p. in saline, 400 μL, group A), ketoconazole (50 mg/kg i.p. in DMSO; group C and D), cimetidine (100 mg/kg, i.p. DMSO, 400 μL, group E) or piperonyl butoxide (1100 mg/kg, i.p. group F). Group B were pretreated with methimazole (60 mg/kg i.p.) 24, 12 and 1 hr prior to the DDS administration (33 mg/kg i.p.). Levels of methaemoglobin were measured in these animals. Administration of DMSO at up to 600 μL did not affect methaemoglobin formation by dapsone, nor did dosage of DMSO alone cause haemoglobin oxidation.

A second set of five groups of animals (N = 6 per group) were treated with dapsone in DMSO as above (33 mg/kg i.p.). One of the groups was pretreated with piperonyl butoxide (1100 mg/kg). Groups 2, 3 and 4 received cimetidine 1 hr prior to dapsone administration, at 10, 50 and 100 mg/kg. Whole blood concentrations of dapsone were measured in all five groups including the fifth control group.

In both sets of rats, animals were bled from the tail vein while anaesthetized with diethyl ether; samples were removed pre-dose, then at 1, 2, 3, 5 and 24 hr. A maximum of 150 μL of blood was removed per sample.

Sample analysis. The methaemoglobin level relative to total haemoglobin in the blood samples was measured using the spectrophotometric technique of Harrison and Jollow [20]. The area under the methaemoglobin time course curve was estimated for each rat from 0–24 hr using the trapezoidal rule [21]. Dapsone was assayed in whole rat blood by the HPLC method of Grossman and Jollow [22] employing UV detection. Dapsone area under the curve was also estimated by the trapezoidal rule. AUC from 24 hr to infinity was calculated by the ratio C_{24}/β where C_{24} was the blood concentration of dapsone at time 24 hr. The area under the curve from zero to infinity (AUC) for dapsone was obtained from the sum of the two areas. The terminal phase elimination rate constant (β) was determined by least-squares regression analysis of the post-distributive blood dapsone concentration–time data and the terminal phase half-life (T_1) from the ratio $0.693/\beta$.

Statistical comparisons between groups were made by the use of Mann-Whitney U-test. Data are tabulated as mean ± SD and presented graphically as mean ± SE. Statistical significance was set at the $P < 0.05$ level.

Results

Within 1 hr of administration of dapsone rapid and acute methaemoglobinaemia resulted (32.6 ± 6.4%) compared with background values of 4.5 ± 1.1%. Twenty-four hours post-dose, levels of methaemoglobin had fallen to less than 8% (7.5 ± 2.1%) in animals to which DDS was given alone. Pretreatment with cimetidine at 100 and 50 mg/kg prior to DDS administration significantly reduced haemoglobin oxidation (Fig. 1). However, pretreatment with cimetidine at the lowest dose (10 mg/kg) did not affect methaemoglobin formation, which was not significantly different from animals which received DDS alone (Table 1). Piperonyl butoxide significantly reduced methaemoglobin formation (Table 1). By contrast, 1 hr pretreatment with methimazole did not reduce DDS mediated haemoglobin oxidation (Table 1). However, 24 hr pretreatment with methimazole significantly ($P < 0.05$) reduced methaemoglobin levels (Table 1). Administration of ketoconazole (50 mg/kg) 1 hr before DDS (33 mg/kg) did not reduce haemoglobin oxidation (Table 1). Moreover, when the experiment was repeated with a 10-fold lower dose of DDS, methaemoglobin formation was significantly ($P < 0.01$) reduced (Table 1).

Both piperonyl butoxide and cimetidine were associated with marked changes in the pharmacokinetics of DDS. The AUC for dapsone after pretreatment with both cimetidine at 100 mg/kg (133.8 ± 12.5 μg/hr/mL) and 50 mg/kg

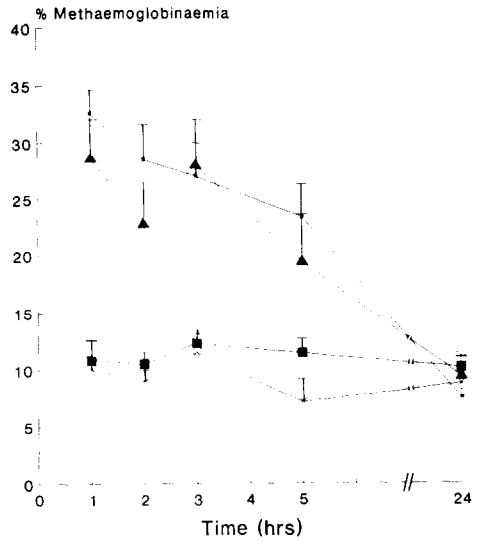

Fig. 1. Percentage methaemoglobinaemia against time after administration of DDS (33 mg/kg) alone (N = 10) (■–■); DDS (33 mg/kg) in the presence of cimetidine (100 mg/kg, N = 5) (×—×); DDS (33 mg/kg) in the presence of cimetidine (50 mg/kg N = 5) (■■). DDS (33 mg/kg) in the presence of cimetidine (▲▲) (10 mg/kg, N = 5). Values are mean ± SE.

(88.7 ± 27.2 μg hr/mL) was significantly greater than the control (59.4 ± 10.3 μg hr/mL). In addition, cimetidine pretreatment at 100 mg/kg resulted in a significantly greater increase ($P < 0.05$) in dapsone AUC in comparison with pretreatment at 50 mg/kg (133.8 ± 12.5 μg hr/mL vs 88.7 ± 27.2 μg hr/mL). However, there was no significant difference between the AUC values for dapsone in the presence of cimetidine at 10 mg/kg (72.6 ± 20.4 μg hr/mL) compared with control (59.4 ± 10.3 μg hr/mL). The AUC for dapsone in the presence of piperonyl butoxide (93.2 ± 32.0 μg hr/mL) was significantly greater than when dapsone was given alone (59.4 ± 10.3 μg hr/mL). Pretreatment with cimetidine (50 and 100 mg/kg) or piperonyl butoxide delayed the onset of the rapid initial drug clearance which was associated with the acute methaemoglobinaemia seen in the control animals (Figs 1 and 2, Table 1).

Discussion

Although a major metabolite of dapsone in man is monoacetyldapsone [8], it has been estimated that almost half a given dose undergoes cytochrome P-450 mediated N-hydroxylation [6]. However, the hydroxylamine of dapsone is unstable in oxygen and its measurement in urine may underestimate the true levels excreted. Hydroxylamine metabolites of dapsone have been shown to cause toxicity in bone marrow [14], and red cells [22]. Dapsone itself is not toxic to red cells [6] and was shown not to be cytotoxic even at concentrations up to 1 mM when incubated with human mononuclear leucocytes *in vitro*. However, incubation of the drug at only 0.1 mM with NADPH and human hepatic microsomes caused considerable cytotoxicity [7]. Metabolic activation by cytochrome P-450 through N-hydroxylation appears to be responsible for toxicity associated with dapsone therapy. Hence, by inhibition of cytochrome P-450, we wished to selectively inhibit N-hydroxylation of dapsone, without affecting cytosolic acetylation of the drug. Therefore, drug elimination would still occur without leading to toxicity.

In support of this hypothesis, we have shown that piperonyl butoxide, the irreversible inhibitor of cytochrome P-450 [23], successfully prevented the formation of methaemoglobin for over 24 hr post administration of dapsone. Cimetidine produced a dose dependent inhibition of methaemoglobin formation. This was accompanied by a significant increase in the AUC of dapsone on increasing cimetidine dose. At the lowest cimetidine dose, although blood concentrations of dapsone were slightly higher than control, this did not achieve significance. This was reflected in the failure of this dose of cimetidine to inhibit methaemoglobin formation.

Hence, it is likely that cimetidine, at the higher doses, caused inhibition of cytochrome P-450 mediated N-hydroxylation of dapsone, which resulted in prevention of methaemoglobinaemia. Whilst cimetidine at the higher

Table 1. Area under the methaemoglobin/time course curves (corrected for 4.5% baseline methaemoglobinaemia) expressed as % methaemoglobin/hr after the administration of DDS (33.3 mg/kg) and various metabolic inhibitors

Treatment group	AUC % methaemoglobin/hr	P
DDS	481.4 ± 118.0	
DDS/cimetidine (100 mg/kg)	255.0 ± 52.2	<0.01
DDS/cimetidine (50 mg/kg)	187.1 ± 90.4	<0.01
DDS/cimetidine (10 mg/kg)	380.8 ± 140	NS
DDS	297.0 ± 77.8	<0.01
DDS/piperonyl butoxide	79.2 ± 15.1	
DDS	284.8 ± 27.0	NS
DDS/methimazole*	282.6 ± 63.6	
DDS	482.0 ± 63.2	<0.05
DDS/methimazole†	384.0 ± 55.5	
DDS	595.0 ± 102	NS
DDS/ketoconazole	548.0 ± 88.2	
DDS‡	139.6 ± 35.1	<0.01
DDS/ketoconazole	48.6 ± 21.0	

N = 5 per group, values are means ± SD.
* One hour pretreatment.
† Twenty-four hour pretreatment.
‡ DDS dose 3.3 mg/kg.
NS, not significant.

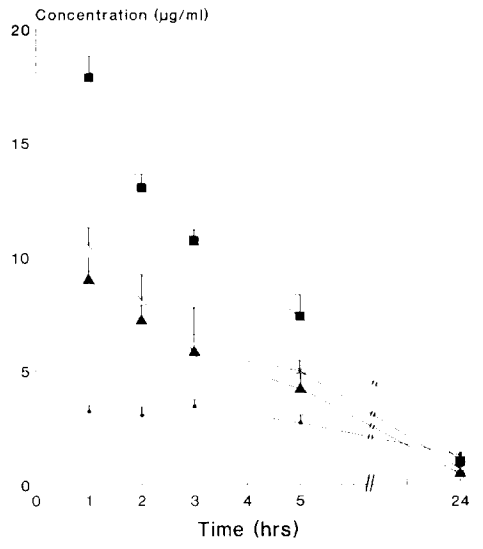

Fig. 2. Concentrations of DDS (μg/mL) alone (■–■) and in the presence of cimetidine at 100 mg/kg (■■), 50 mg/kg (×—×) and 10 mg/kg (▲▲) N = 5 per group. Values are mean ± SE.

doses prevented the formation of toxic hydroxylated metabolites, it is apparent from Fig. 2 that dapsone plasma concentrations still declined, despite the presence of the inhibitor. The extent of the renal clearance of unchanged dapsone is poorly documented, but it appears to be relatively low [6]. Therefore it is likely that dapsone was largely cleared by acetylation, as this process does not result in a toxic metabolite and is not inhibited by cimetidine *in vivo* [24]. In the control animals, measured methaemoglobin levels reached a maximum 1 hr post dose. In the presence of the inhibitors, dapsone clearance through acetylation appeared to occur relatively slowly. Hence it is possible that N-hydroxylation, the process indirectly responsible for methaemoglobin formation, occurs at a rate which is probably greater than acetylation in the rat.

Cimetidine is a well established and potent inhibitor of the oxidative metabolism of a number of drugs in both man and animals [25, 26]. Ketoconazole, is also a potent reversible inhibitor of oxidative drug metabolism, both *in vivo* and *in vitro* [27]. However ketoconazole was a far less potent inhibitor in the present study in comparison with cimetidine or piperonyl butoxide. Ketoconazole only successfully inhibited methaemoglobin formation at greater than seven times the molar equivalent dose of dapsone. Previous studies have illustrated ketoconazole mediated inhibition of cytochrome P-450 to be selective. The drug is a potent inhibitor of P-450-mediated steroid hydroxylation [28] and is four-fold more potent than cimetidine in inhibition of aminopyrine metabolism *in vivo* [29]. However studies in man suggest that ketoconazole has no affect on antipyrine clearance [30]. Hence it may be that the affinity of ketoconazole for the variant of cytochrome P-450 which hydroxylates dapsone in the rat may be relatively low. A metabolite of methimazole generated by flavin monooxygenase is thought to be a specific inhibitor of N-hydroxylation, [31]. However, methimazole was again far less effective in preventing methaemoglobin formation compared with cimetidine. The reasons for this lack of effect are unknown.

N-Hydroxylation is widely regarded as an essential step in the bioactivation of aromatic amines to carcinogenic species [32]. In addition, a number of widely used drugs, such as procainamide and the sulphonamides undergo N-hydroxylation to hydroxylamines as well as cytosolic acetylation [33, 34]. Slow acetylators are known to suffer a high incidence of adverse reactions to these compounds [35], as a higher proportion of the drug is metabolized to a hydroxylamine. N-Hydroxylation of these compounds does not appear to contribute to the therapeutic actions of these drugs but is associated with considerable toxicity.

Dapsone is presently administered in combination with rifampicin and clofazamine for leprosy therapy [13]. Clofazamine does not appreciably influence dapsone disposition [36]. Rifampicin, however, through enzyme induction, significantly reduces skin and nerve concentrations of dapsone [37, 38]. It has been speculated that this may promote resistance to dapsone [13]. Co-administration of a reversible metabolic inhibitor, such as cimetidine, with the triple combination may well maintain higher tissue concentrations of dapsone.

In summary, pretreatment with cimetidine or piperonyl butoxide markedly reduced methaemoglobin production by hydroxylated metabolites of dapsone. This was reflected by a significant increase in parent drug levels. Hence co-administration of a reversible metabolic inhibitor with compounds which undergo N-hydroxylation may result in fewer adverse reactions without change in pharmacodynamic activity.

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Department of Pharmacology and Therapeutics
Ashton Street
New Medical School
Liverpool L69 3BX
U.K.

M. D. Coleman*
M. J. Winn
A. M. Breckenridge
B. K. Park

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* To whom correspondence should be addressed.

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Sex-dependent sensitivity to dapsone-induced methaemoglobinaemia in the rat

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Dapsone is a major component of the multidrug regimen for the treatment of leprosy [1]. It is also used in malarial prophylaxis [2], inflammatory disease [3, 4] and more recently in the therapy of *Pneumocystis carinii* in AIDS patients [5]. Dapsone is extensively metabolized in man and experimental animals to hydroxylamine derivatives [6–8]. Haematological side effects which occur during dapsone therapy such as methaemoglobinaemia [9], have been attributed to the hydroxylamine metabolite [10]. Reduced red cell lifespan due to haemolysis is especially marked in individuals with glucose-6-phosphate dehydrogenase deficiency or diminished glutathione reductase activity [11].