J. Pharm. Pharmacol. 1990, 42: 698–703
Received March 6, 1990

Gonadal Influence on the Metabolism and Haematological Toxicity of Dapsone in the Rat

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Abstract—Administration of dapsone (33 mg kg^{-1}) to intact rats resulted in a marked elevation of methaemoglobin levels in male (435·0 ± 105·2% met Hb h) compared with female rats (59·0 ± 17·2% met Hb h). However, the clearance of dapsone was significantly faster in males compared with females. Female rats showed very low levels of methaemoglobin which were accompanied by significantly higher blood concentrations of parent drug. Clearance of dapsone in castrated animals was less than one-third of that of the intact sham-operated males (252·2 ± 67·2 vs 81·4 ± 33·0 mL h^{-1}). Likewise, clearance of dapsone in ovarectomized rats was approximately half that of intact females. There were no significant differences in the disposition of dapsone between the ovarectomized (AUC, 431·0 ± 31·7 μg h mL^{-1}; $t_{\frac{1}{2}}$, 15·62 ± 1·8 h) and castrated (AUC, 450·6 ± 150·9 μg h mL^{-1}; $t_{\frac{1}{2}}$, 17·6 ± 7·9 h) animals. However, methaemoglobin levels in castrated males, although less than a third of those of intact males, significantly exceeded those of ovarectomized animals. There was no significant difference between the four groups of animals with respect to red cell sensitivity to the methaemoglobin-forming capacity of the toxic metabolite of dapsone, the hydroxylamine. Metabolic conversion of dapsone to the hydroxylamine in the presence of NADPH was 7·6 ± 1·5% for liver microsomes from intact males and was significantly greater ($P < 0·05$) than the corresponding values for liver microsomes from castrated rats (5·3 ± 0·59%). Conversion of dapsone to dapsone-NOH by liver microsomes from intact females and ovarectomized animals was below 1% in both cases. This study illustrates the androgenic control of *N*-hydroxylation in the rat.

Dapsone is a component of multidrug regimens for leprosy (Shepard 1982), malarial prophylaxis (Bruce-Chwatt 1982), inflammatory disease (Lang 1979; Grindulis & McConkey 1984) and more recently for the therapy against *Pneumocystis carinii* in AIDS patients (Green et al 1988). Haematological side effects, such as methaemoglobinaemia, which occur during dapsone therapy (Cucinell et al 1972) have been attributed to the hydroxylamine metabolite (Israili et al 1973). Reduced red cell lifespan due to haemolysis is especially marked in individuals with glucose-6-phosphate dehydrogenase deficiency or diminished glutathione reductase activity (Zuidema et al 1986). The hydroxylamine is unstable in the presence of oxygen (Coleman et al 1989) and is concentrated to a high degree by erythrocytes (Israili et al 1973). Hence this metabolite is difficult to assay directly in-vivo. A previous method of analysis involved oxidation to the nitro derivative, hence measuring the sum of the hydroxylamine and nitroso intermediate species (Uetrecht et al 1988). However, direct measurement of the hydroxylamine in the rat isolated perfused liver indicated that methaemoglobin formation was an effective functional assay for hydroxylamine formation (Coleman et al 1990b). The other major route of metabolism of dapsone is *N*-acetylation, which has not been associated with toxicity (Coleman et al 1990b) unless monoacetyl dapsone is also *N*-hydroxylated (Grossman & Jollow 1988).

Previous studies in our laboratory have indicated that male rats possessed a far greater susceptibility to the haematological toxicity of dapsone compared with female rats. This was accounted for by the greater capacity for the *N*-hydroxylation of dapsone shown in male rats (Coleman et al 1990a). Sex-differences in haemotoxicity due to *N*-hydroxylation have also been reported in beagles after the administration of 4-aminopropiophenone (Bright et al 1987). Gonadectomy has been used to investigate the androgenic and oestrogenic modulation of the biotransformation of other compounds which exhibit sex differences in metabolism, such as imipramine and lignocaine (Skett et al 1980) and dimethylated chlorocyclodiene epoxide (DME, Finnen & Hassall 1980). In the present study, we have investigated the influence of gonadectomy on dapsone toxicity and hence the balance between the *N*-acetylation and *N*-hydroxylation pathways of dapsone metabolism in the rat.

Materials and Methods

Chemicals

4,4-Diaminodiphenyl sulphone (dapsone) was supplied by the Sigma Chemical Co. (Poole, Dorset). [^{14}C]Ring labelled dapsone was supplied by Amersham Radiochemicals (Bucks, UK) and was radiochemically 99% pure by HPLC analysis. Monoacetyl dapsone was a gift from Dr S. A. Ward, Department of Parasitology, Liverpool School of Tropical Medicine. All other reagents were of HPLC grade and were obtained from BDH Chemicals Ltd (Poole, Dorset). Dapsone hydroxylamine (96% pure, determined by HPLC) was synthesized as previously described (Uetrecht et al 1988).

Animals

Male Wistar rats (n = 24, Department of Pharmacology breeding colony), 4 weeks old were anaesthetized (Hypnorm; 0·3 mg kg^{-1} fentanyl citrate, 10 mg kg^{-1} fluanisone i.m.) and

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were either castrated (n = 12) or underwent sham operation (n = 12). Female Wistar rats (n = 24), 4 weeks old were also anaesthetized and were either ovarectomized (n = 12) or underwent sham operation (n = 12). After recovery, the animals were left for six weeks before further experimentation. Previous studies have shown this time to be adequate for the complete effect of the operations to be seen (Winn & Park 1987; Skett et al 1980).

Protocol

All four groups of animals were dosed intraperitoneally with dapsone (33 mg kg^{-1}) dissolved at a concentration of 49·5 μg mL^{-1} in dimethyl sulphoxide (0·67 mL kg^{-1}). Blood samples (200 μL) were withdrawn from the tail veins of the rats under light diethyl ether anaesthesia. Samples were removed predose, then at 1, 2, 3, 5 and 24 h post dose. In addition, three animals from each of the four groups (untreated) were anaesthetized with diethyl ether and exsanguinated by cardiac puncture. Pooled blood from each group (1 mL per incubation, n = 4 per concentration) were separately exposed to dapsone-hydroxylamine at 1, 3, 10 and 30 μM for 1 h, with the metabolite added as a concentrated solution in methanol (10 μL). Control incubations with methanol (10 μL) added alone to the pooled blood did not result in the formation of methaemoglobin. A sample of blood was removed at 1 h and assayed for methaemoglobin formation.

Preparation of hepatic microsomes

Microsomes were isolated from the pooled livers of three untreated female, three untreated male, three untreated castrated males and three untreated ovarectomized female rats by the method of Purba et al (1987). Mean cytochrome P450 contents were measured according to the technique of Omura & Sato (1964) and found to be 0·54 nmol mg^{-1} microsomal protein (male) 0·68 nmol mg^{-1} protein (females), 0·4 nmol mg^{-1} (castrated males) and 0·5 nmol mg^{-1} (ovarectomized females). Concentrated suspensions of the microsomes (10–15 mg microsomal protein mL^{-1}) were stored in 0·1 M phosphate buffer, pH 7·4. Microsomal protein determination was by the method of Lowry et al (1951). Samples of suspensions were stored at −70 C.

Metabolism of dapsone by rat liver microsomes

[^{14}C]Dapsone (100 μM, 0·1 μCi) was incubated with male or female rat liver microsomes (1·0 mg protein) in phosphate buffer, pH 7·4 (final volume 1 mL). Reactions, performed in quadruplicate, were started by the addition of NADPH (1 mM), and were then incubated in a shaking water bath at 37°C for 1 h. NADPH was omitted from control incubations. Before termination of the reactions by the addition of methanol (1 mL), 50 mM ascorbate (50 μL) was added to each incubation to preserve any dapsone hydroxylamine formed, as previous studies have underlined the instability of this compound (Israili et al 1973; Coleman et al 1989). The tubes were left at −20 C overnight to precipitate all protein.

Analytical procedures

Samples from all four groups plus samples obtained from the in-vitro incubation of rat blood with dapsone hydroxylamine were assayed for methaemoglobin relative to haemoglobin levels using the spectrophotometric technique of Harrison & Jollow (1986). Samples from all four groups were assayed for dapsone using the HPLC method of Grossman & Jollow (1988). Chromatography was performed on a Spectra-Physics 8700 chromatograph. Separation was achieved using a μBondapak C18 column (30 × 0·39 cm i.d. 10 μm, Waters Assoc., Hartford, Cheshire). The mobile phase consisted of 0·1 M ammonium acetate–acetonitrile–methanol (66:12:22 v/v/v, 1·5 mL min^{-1}).

The eluate was monitored at 254 nm with a Pye Unicam UV detector. The retention times of dapsone, monoacetyl dapsone and internal standard were 4·6, 6·5 and 7·5 min, respectively.

Analysis of dapsone hydroxylamine was by radiometric HPLC using the method of Uetrecht et al (1988). Dapsone and its hydroxylamine metabolite were identified chromatographically by comparison of their retention times with those of co-injected authentic unlabelled compounds (dapsone hydroxylamine 8·5 min; dapsone 10·5 min). After further precipitation of protein by centrifugation (650 g, 20 min), portions (100 μL) of the supernatant containing approximately 130 000 d min^{-1} were injected onto the column for measurement of 8–10 000 d min^{-1} associated with dapsone hydroxylamine. Separation was achieved using the C18 μBondapak column. The mobile phase consisted of water–acetonitrile–acetic acid–triethylamine (80:20:1:0·05 v/v/v/v), flowing at 1·2 mL min^{-1}. Eluate was again monitored at 254 nm and collected in 30 s fractions which were then mixed with 4 mL of scintillant fluid for measurement of radioactivity.

Calculations

AUC values from 0–24 h for blood methaemoglobin and dapsone were estimated by the trapezoidal rule (Gibaldi & Perrier 1982). The AUC from 24 h to infinity for blood dapsone was calculated by the ratio C_{24}/β where C_{24} was the blood level of dapsone at 24 h. 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 half-life ($t_{\frac{1}{2}}$) from the ratio $0{\cdot}693/\beta$. Clearance was calculated by the formula:

$$CL = \frac{\text{Dose}}{AUC_{\infty}}$$

volume of distribution was calculated from:

$$V_d = \frac{CL \times t_{\frac{1}{2}}}{0{\cdot}693}$$

Analysis was by the Wilcoxon Signed Rank test accepting $P < 0{\cdot}05$ as significant. Data are presented in the text and tabulated as mean ± s.d. and presented graphically as mean ± s.e.m.

Results

Administration of dapsone (33 mg kg^{-1}) to intact rats resulted in a marked elevation of methaemoglobin AUC in male (435·0 ± 105·2% met Hb h) compared with female rats (59·0 ± 17·2% met Hb h; Fig. 1). However, the clearance of dapsone was significantly faster in males compared with females (Table 1). Female rats showed very low levels of methaemoglobin which were accompanied by significantly

Table 1. Pharmacokinetic parameters of dapsone and monoacetyl dapsone after the administration of dapsone (33 mg kg^{-1}) to normal male, normal female, castrated and ovarectomized rats.

	Male (castrated)	Female (ovarectomized)	*P*
Met Hb AUC_{0-24} (%h)	141·7 ± 50·2	57·0 ± 11·7	< 0·05
Dapsone AUC (μg h mL^{-1})	450·6 ± 150·9	431·0 ± 31·7	NS
Monoacetyl dapsone AUC (μg h mL^{-1})	98·3 ± 25·5	116·4 ± 20·4	NS
CL (mL min^{-1})	81·4 ± 33	76·9 ± 6·3	NS
V_d (mL)	1855·0 ± 384	1726·0 ± 166·0	NS
$t_{\frac{1}{2}}$ (h)	17·6 ± 7·9	15·62 ± 1·8	NS
	Male (intact)	Female (intact)	*P*
Met Hb AUC_{0-24} (%h)	435·0 ± 105·2	59·1 ± 17·2	< 0·005
Dapsone AUC (μg h mL^{-1})	138·2 ± 36	222·0 ± 22·1	< 0·005
Monoacetyl dapsone AUC (μg h mL^{-1})	14·52 ± 8·12	84·2 ± 16·2	< 0·005
CL (mL min^{-1})	252·2 ± 67·2	149·1 ± 13·9	< 0·005
V_d (mL)	2781·1 ± 287	1879·2 ± 213·6	< 0·05
$t_{\frac{1}{2}}$ (h)	8·0 ± 1·07	8·8 ± 1·94	NS

NS = not significant.

higher blood concentrations of parent drug (Fig. 2, Table 1). The mean monoacetyl dapsone AUC in the female rats was five-fold greater than that of the males (Fig. 3). Hence, although the respective half-lives of dapsone within the two groups of intact animals did not differ, rapid removal of dapsone from blood was associated with toxicity in the form of haemoglobin oxidation.

Administration of dapsone to castrated and ovarectomized animals resulted in diminished clearance of the drug compared with control (sham-operated) rats. Clearance of dapsone in castrated animals was less than one-third that of the intact males (Table 1, Fig. 2). Likewise, clearance of dapsone in ovarectomized rats was approximately half that of intact females. There were no differences in the disposition of dapsone between the ovarectomized and castrated animals (Table 1). However, methaemoglobin levels in castrated males, although less than a third of those of intact males, significantly exceeded those of ovarectomized animals (Fig. 1). There was no significant difference between the four groups of animals in respect of red cell sensitivity to the methaemoglobin-forming capacity of the toxic metabolite of dapsone, dapsone-hydroxylamine (Table 2). There was no significant difference between the monoacetyl dapsone plasma concentrations of the castrated and ovarectomized groups. However, monoacetyl dapsone formation was significantly greater ($P < 0·05$) in ovarectomized compared with intact females (Table 1, Fig. 3).

Radiometric analysis of incubations containing dapsone

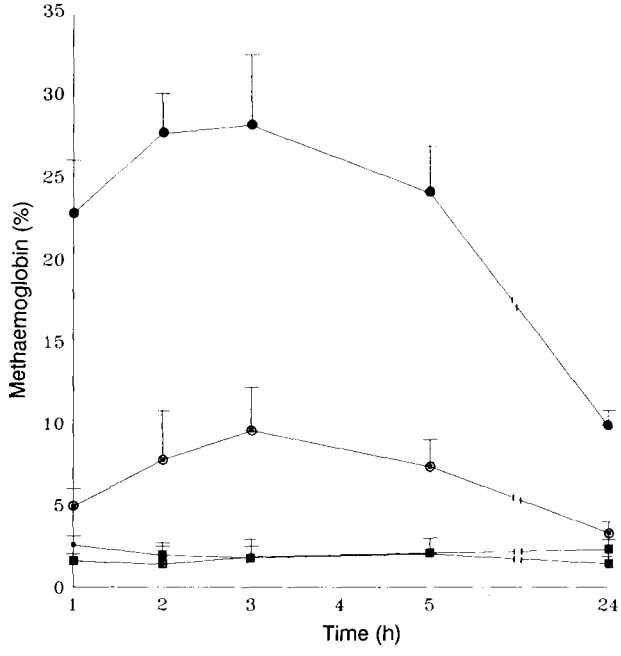

FIG. 1. Percentage of methaemoglobinaemia against time after the administration of dapsone (33 mg kg^{-1}) to male (●), female (■), castrated (⊙) and ovarectomized (⊡) rats. n = 6/group values are means ± s.e.m.

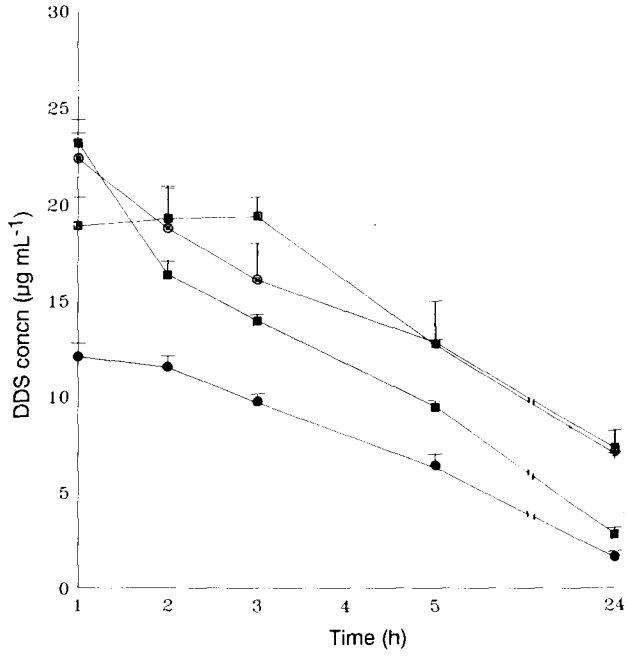

FIG. 2. Concentrations of dapsone (DDS, μg mL^{-1}) against time after the administration of dapsone (33 mg kg^{-1}) to male (●), female (■), castrated (⊙) and ovarectomized (⊡) rats. n = 6/group; mean ± s.e.m.

Table 2. Methaemoglobin (%) in incubations of whole blood, removed from intact male and female rats, castrated and ovarectomized rats, with increasing concentration of dapsone-hydroxylamine in-vitro (n = 4/incubation, mean ± s.d.).

Dapsone-hydroxylamine (μM)	Methaemoglobin (%)				
	Male (castrated)	Female (ovarectomized)	Male (intact)	Female (intact)	*P*
1	8·14 ± 1·19	9·7 ± 3·8	8·8 ± 1·7	8·2 ± 4·0	NS
3	10·5 ± 4·2	10·6 ± 3·6	14·0 ± 1·2	11·2 ± 46	NS
10	12·1 ± 5·3	14·0 ± 2·1	16·5 ± 0·85	17·0 ± 0·6	NS
30	30·5 ± 7·6	30·0 ± 4	36·0 ± 0·81	33·1 ± 1·2	NS

NS = not significant.

and microsomes prepared from the livers of male intact or castrated rats revealed the presence of dapsone-hydroxylamine. Metabolic conversion of dapsone to the hydroxylamine in the presence of NADPH was 7·6 ± 1·5% for microsomes from intact male rats and was significantly greater ($P < 0·05$) than the corresponding values for microsomes from castrated rats (5·3 ± 0·59%). There was no detectable metabolism of the drug in the absence of NADPH. Conversion of dapsone to dapsone-hydroxylamine by microsomes prepared from female intact and ovarectomized rats was below 1% in both cases.

Discussion

Sex differences in the metabolism of drugs has been well documented (Kato 1974). Indeed, a number of constitutive forms of cytochrome P450 in male rats are not present in females (Kamataki et al 1983). This is underlined by both our present and previous studies (Coleman et al 1990a), where the capacity of male rats to *N*-hydroxylate dapsone is far in excess of that of females. Severe methaemoglobinaemia has been shown to be a consequence of rapid dapsone metabolism to dapsone-hydroxylamine in the male rat (Grossman & Jollow 1988) and in the rat isolated perfused liver (Coleman et al 1990b). The marked fall in oxidative metabolism, and thus the toxicity of dapsone in the castrated rats compared with normal animals, illustrates that continual secretion of gonadal androgens is necessary to maintain the high capacity of *N*-hydroxylation in the male. Sex-related differences in metabolism are thought to be determined primarily by secretion of testicular androgens during the neonatal period, where a latent masculine potential is imprinted (Shimada et al 1987). Hence, neonatal castration results in loss of expression of male specific cytochrome P450 (Kamataki et al 1984; Waxman et al 1985). Finnen & Hassall (1980) reported that neonatal castration abolished 'masculine' metabolism of dimethylated chlorocyclodiene epoxide (DME), whereas castration of adult animals did not result in changes in DME 'masculine' metabolism. In the present study, castration of the rats at 28 days i.e. as young adults, may have allowed the neonatal imprinting of male specific high capacity *N*-hydroxylation, since although the methaemoglobinaemia mediated by dapsone-hydroxylamine is markedly reduced in the castrated rats compared with normals, it is not abolished entirely. Similarly, hepatic microsomes isolated from the castrated rats partially retained the ability to *N*-hydroxylate dapsone. Of the sex-specific rat cytochrome P450s studied, P450 h (IIC11) is expressed only in adult males, is subject to neonatal imprinting (Gonzalez 1989) and is not inducible (Ryan et al 1984). Previous studies in induced mouse (Coleman et al 1989) and in rat (Tingle et al 1990) have indicated that the *N*-hydroxylation of dapsone in rodents is apparently non-inducible, and it may be that P450 h (IIC11) is responsible for the *N*-hydroxylation of dapsone in the male rat.

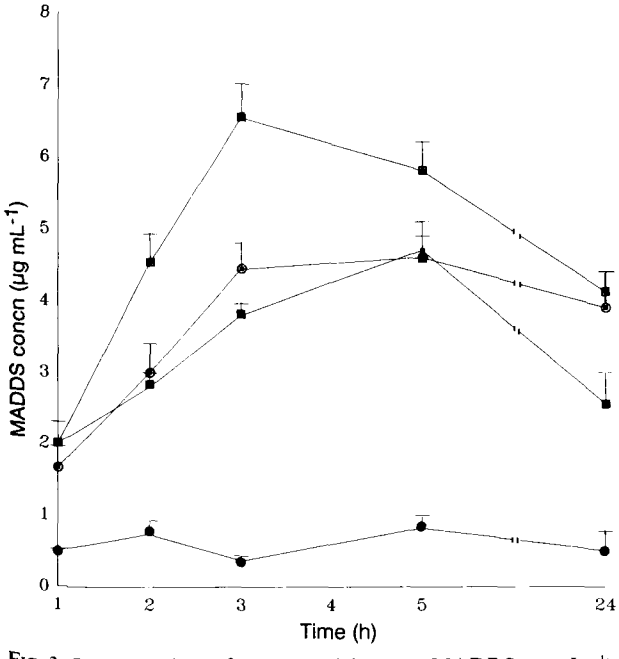

FIG. 3. Concentrations of monoacetyl dapsone (MADDS, μg mL^{-1}) plotted on a semi-logarithmic scale against time after the administration of dapsone (33 mg kg^{-1}) to male (●), female (■), castrated (○) and ovarectomized (□) rats. n = 6/group; mean ± s.e.m.

In the female rat, it has been suggested that the pituitary exerts the primary control of hepatic drug metabolism (Gustafsson et al 1977; Skett et al 1980; Finnen & Hassall 1980). In fact ovarectomy did not affect the hepatic metabolism of either imipramine or lignocaine (Skett et al 1980). However, in the present study, ovarectomy clearly caused a reduction in dapsone clearance. This is unlikely to be due to effects on the oxidative metabolism of dapsone, as not only was methaemoglobinaemia negligible in both intact and ovarectomized females, but *N*-hydroxylation was barely detectable in-vitro. Since it is thought that the only phase I oxidation dapsone undergoes is *N*-hydroxylation (Israili et al 1973; Coleman et al 1989) the decreased drug clearance after ovarectomy cannot be explained in terms of oxidative

metabolism. Therefore, retardation in excretion of dapsone may account for the fall in drug clearance after ovarectomy.

Monoacetyl dapsone is highly protein bound and does not undergo extensive glomerular filtration (Zuidema et al 1986) and consequently it is found in urine in only very low levels (Gelber et al 1971). In addition, it has been suggested that monoacetyl dapsone must be de-acetylated before renal excretion may occur (Zuidema et al 1986). Hence the high concentrations of monoacetyl dapsone seen in the ovarectomized animals may well have retarded the overall excretion of dapsone. Dapsone itself is known to undergo active tubular transport (Goodwin & Sparell 1969), with 5–15% of the dose eliminated unchanged (Ellard 1966; Gelber et al 1971). Oestrogens and androgens have been shown to have potent effects on renal growth and maintenance of function (Petrovic et al 1977; Katayama & Lee 1985; Blantz et al 1988) so it is conceivable that the loss of hormonal influence due to gonadectomy may have reduced the renal excretion of dapsone and contributed to the rise in blood drug levels seen in the ovarectomized, and to a lesser extent, the castrated animals.

In the rat isolated perfused liver, acetylation was significantly increased during inhibition of the oxidative metabolism of dapsone by cimetidine (Coleman et al 1990b). In the present study, a large increase in acetylation compensated for the loss of oxidative metabolism of the drug, which had been reduced by the removal of the influence of androgens. Hence, although cytochrome P450-mediated *N*-hydroxylation is heavily influenced by androgens, it appears that cytosolic *N*-acetyl transferase does not require hormonal maintenance and possesses considerable reserve capacity in the absence of *N*-hydroxylation.

In summary, we have illustrated the potent androgenic control of oxidative dapsone metabolism in the rat. The sex-difference in the acetylation of dapsone in the rat may be a compensatory response to the marked differences in the ability to *N*-hydroxylate the drug.

Acknowledgements

The authors would like to thank Dr Euan Birnie and Mr D. Trafford for assistance with animal operations. MDC, MDT and BKP are supported by the Wellcome Trust. This work received additional support from the World Health Organization and the Wolfson Trust.

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