Comparison of the Metabolism and Toxicity of Dapsone in Rat, Mouse and Man1

M. D. TINGLE,2 R. MAHMUD,3 J. L. MAGGS, M. PIRMOHAMED and B. K. PARK

Department of Pharmacology and Therapeutics, The University of Liverpool, Ashton Street Medical School, Liverpool, L69 3GE, United Kingdom

Accepted for publication July 24, 1997

ABSTRACT

The metabolism and toxicity of dapsone was compared in vitro and in vivo in rat, mouse and man. Metabolism was assessed by high-pressure liquid chromatography-mass spectrometry and methemoglobin formation has been used as a toxic endpoint. The greatest toxicity in vitro was seen in microsomes prepared from male Wistar rats (36.6 ± 1.5% methemoglobin), although toxicity was also seen in microsomes from the female rat (8.2 ± 1.3%), male CD1 (4.2 ± 1.6%) and human (10.9 ± 1.1%). The rank order of toxicity agreed with the formation of the hydroxylamine metabolite in vitro. All microsomes were also capable of catalyzing the reverse reaction, i.e., reduction of the hydroxylamine to dapsone. However, in vivo administration of dapsone resulted in significant (P < 0.05) methemoglobinemia only in male rats and humans. This species difference in the susceptibility to dapsone toxicity could not be attributed solely to the sensitivity of the target erythrocytes, because the order of sensitivity to dapsone hydroxylamine was human > mouse > rat. Analysis of bile and urine revealed the formation of dapsone hydroxylamine and its glucuronide in male rats and humans, but not in female rats or mice. This species difference in the metabolism and toxicity of dapsone has important implications in the safety evaluation of related compounds for man.

Dapsone has long been used in the treatment of a wide range of diseases such as leprosy (Vadher and Lalljee, 1992), malaria (Shanks et al., 1992) and dermatitis herpetiformis (Prussick et al., 1992). Dapsone has been evaluated in the prevention and treatment of Pneumocystis carinii and Toxoplasma gondii infections in HIV-positive patients (Jorde et al., 1993; Girard et al., 1993). The mechanism of the antibacterial action for dapsone is similar to that of the sulfonamides, in that it is an antagonist of para-aminobenzoic acid in folate synthesis. Its action as an anti-inflammatory agent and in a variety of skin disorders is because of inhibition of neutrophil adherence and function (Booth et al., 1992; Bozeman et al., 1992; Thuong-Nguyen et al., 1993).

The effective clinical use of dapsone is limited because of dose-dependent adverse hematological reactions, even at the low daily dosages of 100 mg in the chemotherapy of leprosy and dermatological conditions (Coleman and Tingle, 1992). In addition, patients with a genetic deficiency of certain enzymes involved in the process of toxicity are more susceptible to the hematological effects even at therapeutic dosage. The most common adverse reaction is methemoglobinemia, which is significant at a daily dosage of 100 mg in phenotypically normal patients and is severe in patients with a deficiency of NADH-dependent methemoglobin reductase (Ganer et al., 1981). Heinz-body formation and reduced life-span of the red blood cells, which occur in patients receiving chronic dapsone therapy, and in particular those with deficiencies in either glucose-6-phosphate dehydrogenase or glutathione reductase activity, are also important (De Gowin et al., 1966).

The hemotoxicity of dapsone has been shown to be a consequence of N-hydroxylation to yield the hydroxylamine (Hjelm and DeVerdier, 1965; Glader and Conrad, 1973; Grossman and Jollow, 1988). This biotransformation is catalyzed either by hepatic enzymes such as cytochrome P450 (Uehleke and Tabarelli, 1973), flavin monooxygenase and prostaglandin H synthetase in liver, or by myeloperoxidase found in peripheral polymorphonuclear leukocytes (Uetrecht et al., 1988).

To overcome the problem of hemotoxicity, several groups have attempted to synthesize structural analogs of dapsone that have greater pharmacological activity than dapsone. The activity of dapsone analogs has been assessed in vivo against Plasmodium berghei in the mouse (Popoff et al.,...
1971a, b), in cell culture (Colwell et al., 1974; de Benedetti et al., 1987) and in cell-free systems (Wiese et al., 1987). However, toxicity data for any of these analogs is limited (Coleman et al., 1996). The \textit{in vivo} toxicity of dapsone has generally been studied in the rat (Coleman et al., 1990a,c), whereas liver preparations from several species have been investigated for their ability to N-hydroxylate dapsone (Hjelm and DeVerdier, 1965; Uheleke and Tabarelli, 1973; Tingle and Park, 1993).

This study investigated the role of metabolism of dapsone in the pharmacokinetics and toxicity of the compound \textit{in vivo} with use of the rat, mouse and man. Furthermore, we used \textit{in vitro} techniques to compare the effects of metabolism by rat and mouse liver preparations with human liver enzymes to assess the relevance of the \textit{in vivo} findings in animals to the situation in man.

Methods

Materials. Dapsone, 3,3′-diaminodiphenyl sulfone, potassium ferricyanide and reduced NADPH were from Sigma Chemical Co. Ltd. (Poole, UK). Potassium cyanide was obtained from BDH Chemicals Ltd. (Poole, UK). HPLC solvents and other reagents were purchased from Fisher Scientific Ltd. (Loughborough, UK). 4-Amino-4′-nitrodiphenylsulfone was prepared by the method of Raizis et al. (1939) and reduced with a palladium-carbon catalyst (Entwistle et al., 1994). Ferricyanide and reduced NADPH were from Sigma Chemical Co.

Confirmation of dapsone hydroxylamine, urine (200–230 g, n = 5) was spiked with an internal standard (3,3′-diaminodiphenyl sulfone; 1 μg) and then extracted with ethyl acetate (2 × 1 ml). The organic layers were combined and treated in the same way as for plasma samples described above. To determine the total excretion of both dapsone and the hydroxylamine metabolite, an aliquot (200 μl) of urine was incubated for 6 h with Glucurase (500 U) in the presence of 500 μM ascorbate. Protein was precipitated with methanol (300 μl) overnight (−20°C) and centrifuged; an aliquot (50 μl) of the supernatant was injected onto the HPLC column and the compounds were eluted as described above.

Metabolism and toxicity of dapsone and dapsone hydroxylamine \textit{in vitro}. Micromolar fractions were prepared from the pooled livers of male and female rats (n = 6), male mice (n = 6) or from six individual human livers, as described previously (Gill et al., 1995). Washed erythrocytes were prepared from the blood of male and female rats, male mice and humans (Tingle and Park, 1993). Dapsone (100 μM) was incubated with microsomes (1 mg of protein) and 1 mM NADPH (omitted from controls) in the presence of washed human erythrocytes (500 μl of a 50% suspension) in HEPESS-buffered saline (1 ml) at 37°C and the methemoglobin content assessed after 1 h. For the metabolism studies, either dapsone or dapsone hydroxylamine (100 μM) were incubated with microsomes (1 mg of protein) and 1 mM NADPH in the presence of 500 μM ascorbate. After 1 h, the reaction was terminated by addition of methanol (2 ml), and protein precipitated overnight at −20°C. Protein was sedimented (750 × g, 30 min), and an aliquot (100 μl) of the supernatant was injected on a Spherisorb HPLC column and eluted as described above. Species-dependent sensitivity toward dapsone hydroxylamine was assayed after incubation of the cells with the compound (0–100 μM) for 1 h as described above.

Data analysis. Data shown in the \textit{in vivo} experiments is mean ± S.D. and values were compared by use of the Student’s \textit{t}-test for nonpaired data after tests to indicate whether the data were distributed normally. Where necessary, data were analyzed by analysis of variance with Bonferroni’s correction. A difference was deemed significant when \( P < 0.05 \). Half-lives and area under the curves (AUC\(_{0-24}\)) were calculated by

Analysis of bile and urine by reverse-phase HPLC linked to mass spectrometry. Aliquots of bile or urine (10 ml) were eluted from a \( \mu \) Bondapak C\(_{18}\) column (30 × 0.46 cm i.d.) with acetonitrile/20 mM ammonium formate, pH 3.5. The mobile phase consisted of 7.5% acetonitrile for 10 min, followed by a linear increase up to 25% over 10 min. The flow rate was 1.2 ml/min. Two Jasco PU980 pumps were linked to an HG-980–30 mixing module. Eluate passed through a Jasco UV-975 absorbance detector (254 nm), and thence, via a stream splitter, to either a radiometric detector (A200, Canberra Packard, Reading, UK) or to the electrospray probe and interface of a Quattro II mass spectrometer (Fisons Biotech MS, Manchester, UK). The splitter and probe were connected by 1.5 m of 75-nm fused silica capillary. Nebulizing and drying gas (nitrogen) were delivered at 13 liters/h and 300 liters/h, respectively. The interface temperature was 60°C; the capillary voltage was 6 × 10\(^3\) V. Compressed centroid spectra were acquired between m/z 100–650 with a scan duration of 4 or 5 s; the photomultiplier voltage was 530 V. Fragmentation of analyte ions was enhanced by increasing the cone voltage.

Toxicity and plasma kinetics of dapsone in humans. The pharmacokinetics and toxicity of a single dose (100 mg) of dapsone were determined in five male volunteers (65–93 kg). The study was approved by the local ethics committee, and all volunteers gave informed consent. After the dose of dapsone, blood samples were taken at 1, 2, 3, 4, 8 and 24 h. Methemoglobin levels were determined immediately and plasma was prepared for centrifugation and stored at −20°C until analyzed by HPLC. Urine was collected over ascorbate (1 g) for 24 h and stored −20°C until analyzed by HPLC. To quantify the amount of free (nonconjugated) dapsone and dapsone hydroxylamine, urine (200 μl) was spiked with internal standard (3,3′-diaminodiphenyl sulfone; 1 μg) and then extracted with ethyl acetate (2 × 1 ml). The organic layers were combined and treated in the same way as for plasma samples described above. To determine the total excretion of both dapsone and the hydroxylamine metabolite, an aliquot (50 μl) of the supernatant was injected onto the HPLC column and the compounds were eluted as described above.
the linear trapezoidal rule with the Topfit program (Schering, Germany).

Results

Toxicity and plasma kinetics of dapsone in the rat and mouse. Administration of dapsone to male Wistar rats resulted in a time-dependent increase in methemoglobinemia (fig. 1A), which reached a maximum of 29.1 ± 9.3% at 1 h. In contrast, there was no significant increase in methemoglobinemia after administration of dapsone to either the female rat or to the male mouse (fig. 1A). The area under the curve (AUC(0–24)) for methemoglobinemia was 348% metHb h for male rats, 74.0% metHb h for female rats and 49.7% metHb h for male mice.

Analysis of plasma revealed that in the male rat, peak plasma concentrations of dapsone (29.2 ± 8.4 μg·ml⁻¹) were reached 2 h after the dose (fig. 1B), with a half-life of 7.7 ± 1.4 h. The area under the curve for dapsone AUC(0–24) was 243.0 ± 105.6 μg·ml⁻¹·h. In the female rat, although there was no significant difference in the peak plasma concentrations (32.0 ± 6.7 μg·ml⁻¹), they were reached 3 h after the dose, and declined with a significantly (P < .05) longer half-life of 14.5 ± 1.0 h. The AUC(0–24) for dapsone was also significantly (P < .05) greater at 496.8 ± 103.3 μg·ml⁻¹·h. In the male mouse, peak plasma concentrations were 23.4 ± 2.6 μg·ml⁻¹ after 2 h, declining with a half-life of 7.6 ± 1.0 h. The AUC(0–24) for dapsone was 233.1 ± 31.3 μg·ml⁻¹·h. No dapsone hydroxylamine could be detected in plasma at any time point (limit of detection, 10 ng·ml⁻¹).

Excretion and metabolism of [14C]dapsone in the rat. The cumulative biliary excretion of radioactivity after administration of [14C]dapsone to male and female Wistar rats is shown in figure 2. Over 300 min 46.3 ± 13.6% of the dose was excreted into the bile of males compared with 20.9 ± 9.2% in females. There was significantly (P < .01) greater excretion of radioactivity into bile for male rats at all time points after 30 min. There was no significant difference in the urinary excretion of radioactivity between males (4.8 ± 2.9%) and females (3.4 ± 3.1%).

Analysis of bile from male rats by reverse-phase HPLC with radiometric detection linked to LCMS, revealed that the major metabolite (59.7 ± 17.8% of radioactivity) was a glucuronide of dapsone with the presence of a dapsone hydroxylamine glucuronide (27.8 ± 19.3%) and unchanged dapsone (11.6 ± 3.6%). In the urine it was found that the major metabolite was the glucuronide of dapsone (59.7 ± 6.7%), whereas dapsone hydroxylamine glucuronide (8.4 ± 4.2%) and unchanged dapsone (31.8 ± 6.7%) were also detected. Analysis of bile from female rats revealed the presence of dapsone glucuronide (77.1 ± 5.0%) and dapsone (22.9 ± 5.0%), with no detectable levels of dapsone hydroxylamine glucuronide. In the urine, dapsone glucuronide (42.3 ± 15.3%) and dapsone (57.7 ± 15.3%) were present. Trace amounts (<1% of radioactivity) of N-acetyl dapsone and N-acetyl dapsone glucuronide were detected by mass spectrom-

Fig. 1. Methemoglobin levels (A) and plasma levels of dapsone (B) after administration of dapsone (100 μmol·kg⁻¹ i.p.) to male (○) and female (●) Wistar rats plus male CD1 mice (□). Values shown are mean ± S.E.M. (n = 4). ***P < 0.001 compared with 0 h. # P < 0.05; ###P < 0.001 compared with male rats.

Fig. 2. Excretion of radioactivity into bile after administration of dapsone (100 μmol·kg⁻¹, 3 μCi i.v.) to male (○) and female (●) Wistar rats plus male CD1 mice (□). Values shown are mean ± S.E.M. (n = 4). **P < 0.01; ***P < 0.001 compared with male rats.
etrical, but not radiometric detection, in the urine and bile of both male and female rats.

**Excretion and metabolism of dapsone in the mouse.** After administration of [14C]dapsone to male mice, 4.1 ± 2.3% of the dose was excreted into the bile and 3.0 ± 2.2% into urine over 180 min. Analysis of bile and urine by reversed-phase HPLC/MS revealed the presence of dapsone alone, with no glucuronides of either dapsone or dapsone hydroxylamine.

**Toxicity and plasma kinetics of dapsone in humans.** After administration of dapsone (100 mg), it was possible to detect a significant (P < 0.05) increase in methemoglobin levels in four of the five volunteers. No significant elevation of methemoglobin levels were detected in one subject at any time point. The AUC(0–24) for methemoglobin was 37.3 ± 11.0% metHb·h⁻¹. The mean plasma levels of dapsone are shown in figure 3. The AUC(0–24) for dapsone was 12.3 ± 1.3 μg·ml⁻¹·h⁻¹. Three control subjects were classified as rapid acetylators and two as slow acetylators based on the ratio of monoacetyl dapsone to dapsone in plasma 3 h postdose (Gelber et al., 1971). There was no correlation between acetylator status and methemoglobinemia (r = 0.3).

Initial analysis of human urine by LCMS revealed the presence of dapsone, dapsone hydroxylamine and their respective glucuronides, plus some monoacetyl dapsone. During 24 h postdose, 5.9 ± 2.6% of the dose was excreted as free (nonconjugated) dapsone, 0.5 ± 0.3% as free hydroxylamine and 0.6 ± 0.2% as monoacetyl dapsone. After hydrolysis with β-glucuronidase, 15.3 ± 2.4% of the dose was quantified as dapsone and 13.1 ± 2.6% as dapsone hydroxylamine.

**Metabolism and toxicity of dapsone and dapsone hydroxylamine in vitro.** Metabolism (NADPH)-dependent methemoglobin formation in human erythrocytes was observed when dapsone was incubated with liver microsomes prepared from all three species (table 1). However, dapsone hydroxylamine could only be detected by HPLC after incubation with rat and human liver enzymes. Microsomes prepared from the livers of all three species catalyzed the reverse reaction, i.e., reduction of dapsone hydroxylamine to dapsone (table 1).

The dapsone hydroxylamine-dependent methemoglobinemia in erythrocytes, isolated from male and female Wistar rats, CD1 mice or human blood is shown in figure 4. Of the three species investigated, human blood appeared to be the most sensitive, with 63.6 ± 3.1% methemoglobin at 100 μM hydroxylamine, compared with 28.8 ± 3.9 for the male rat, 31.3 ± 3.5 for the female rat and 45.3 ± 1.4 for the mouse.

**Discussion**

Administration of dapsone to male Wistar rats resulted in methemotoxicity, in the form of methemoglobinemia, in agreement with previous studies (Coleman et al., 1990a,c). Methemoglobin formation is a consequence of the N-hydroxylation of dapsone to a hydroxylamine metabolite, which is well established as being toxic toward erythrocytes (Scott and Raskind, 1973; Glader and Conrad, 1973). In this study, we have confirmed that dapsone is N-hydroxylated by male rat liver enzymes in vitro, and that in vivo there is formation of the further metabolite, dapsone hydroxylamine glucuronide, which is excreted into bile and urine. The levels of N-oxidation products found in bile from male rats in this study (14.2 ± 9.9% of the dose over 5 h) may be higher than reported previously (0.1–3.2% of the dose) (Isaïli et al., 1973), because we have determined them directly by radiometric HPLC/MS, rather than after a complex chemical work-up involving hydrolysis and azoxy product formation.

Dapsone did not cause any methemoglobinemia in the female rat, in keeping with a previous study (Coleman et al., 1990c), nor was any hydroxylamine or hydroxylamine glucuronide detected. Although some N-hydroxylation was detected in vitro with microsomes prepared from the livers of female rats, the rate of reduction for the hydroxylamine to the amine was greater. This sex difference in the bioactivation of dapsone in the rat has been ascribed to the sex-dependent expression of cytochrome P450 enzymes, and in particular to CYP2C11 and CYP3A1 (Coleman et al., 1990c; Vage and Svensson, 1994), which are expressed only in male rats (Guengerich et al., 1986). Clearly, there may also be sex-dependent expression of the cytochrome P450 enzyme(s) involved in the reduction of dapsone hydroxylamine. Sulfa-methoxazole is N-hydroxylated by CYP2C9 in man and by CYP2C6 in rat, but the hydroxylamine is reduced by CYP3A in both species (Cribb et al., 1995).

In both sexes, only trace amounts of N-acetylated metabolites of dapsone were detected in the bile and urine, whereas no monoacetyl dapsone was detected in plasma, which suggests that the Wistar rat is a “slow” acetylator of dapsone. No products of sulfation or glutathione adducts were detected by either radiometric HPLC or LCMS.

Administration of dapsone to male CD1 mice did not result in any significant methemoglobinemia, even at doses of 1 mmol·kg⁻¹ which result in neurotoxicity (M. D. Tingle, unpublished observations). This lack of hemotoxicity is not caused by insensitivity of the target cells, because erythrocytes prepared from mouse blood were more sensitive than rat cells to the methemoglobin-forming capacity of dapsone hydroxylamine. However, the lack of toxicity can be rationalized by the fact that no N-hydroxylation could be measured in vivo, with no hydroxylamine or further metabolites detected. Although there was some metabolism-dependent methemoglobin formation in vitro with microsomes prepared from mouse livers, no hydroxylamine could be detected in vitro. This apparent discrepancy may be caused by the accumula-
and biliary excretion in male plasma clearance is borne out in both the plasma kinetics - (1990). This relationship between N rate of N the mouse model (Popoff et al., 1997). Species Differences in Dapsone Metabolism 821
either rats, which do not N -dependent methemoglobin formation in vitro (46x58). female rat (●) or male mouse (□) blood, to dapsone hydroxylamine-dependent methemoglobin formation in vitro. Values shown are mean ± S.E.M. (n = 4). ***P < .001 compared with human.

Despite the lack of metabolism plus low urinary and biliary excretion (fig. 2), the clearance of dapsone from plasma in the mouse was not significantly different from that observed in the male rat. This may suggest that there is tissue accumulation of dapsone in the mouse, perhaps into the skin (Chatterjee and Poddar, 1957) and brain, which may account for the different toxicity observed. The absence of N-hydroxylation in the mouse would suggest that this metabolic pathway is not important in the mechanism of antiparasitic activity for dapsone, because dapsone is active against P. berghei in the mouse model (Popoff et al., 1971a).

A previous study in man has suggested a link between the rate of N-hydroxylation and clearance of dapsone (May et al., 1990). This relationship between N-hydroxylation and plasma clearance is borne out in both the plasma kinetics and biliary excretion in male versus female rats. In the female rats, which do not N-hydroxylate dapsone, the half-life of dapsone in plasma is significantly (P < 0.05) longer after intraperitoneal administration, although peak concentrations do not differ significantly, whereas after intravenous administration, approximately half as much radioactivity (24.3 vs. 51.1%) is excreted into the bile and urine over 5 h in female compared with male rats. However, the similarity of clearance of dapsone from plasma in both the male Wistar rat and the male CD1 mouse, in which no N-hydroxylation of dapsone could be detected, would suggest that factors such as tissue accumulation are also very important.

Although human liver enzymes in vitro had a lower activity with respect to dapsone N-hydroxylation than rat liver microsomes, in vivo there was extensive N-hydroxylation in humans, with 46% of the recovered dose excreted as hydroxylamine or hydroxylamine glucuronide over 24 h, in agreement with previous studies (Coleman et al., 1990b; Israili et al., 1973). The apparently lower rate of N-hydroxylation in vitro by human microsomes compared with rat may be a function of the greater capacity for human enzymes to catalyze the reverse reaction, i.e., reduction of the hydroxylamine back to the amine in vivo. Dapsone N-hydroxylation is known to be catalyzed by CYP2C9, CYP2E1 and CYP3A4 in man (Gill et al., 1995; Mitra et al., 1995) and by CYP2C11 and CYP3A1 in rat (Coleman et al., 1990c; Vage and Svensson, 1994). However, it is not known which isof orm(s) of cytochrome P450 catalyze the reduction pathway.

The hemotoxicity of dapsone may also be greater in man than the rat because human erythrocytes are significantly more susceptible than rodent erythrocytes to the toxic effects of the hydroxylamine. There is no significant difference in the levels of the enzyme NADH- and NADPH-methemoglobin reductase between these two species (Agar and Harley,
1972), so differences in sensitivity may reflect species differences in the ability of the cells to reduce nitroso dapsone back to the hydroxylamine in a futile cycle (Kramer et al., 1972). Indeed, a single oral dose of dapsone (100 mg, approximately 5 μmol·kg⁻¹) in man resulted in more than 2% methemoglobin. If this is multiplied up to an equivalent dose administered to the rat (100 μmol·kg⁻¹), then man would have more than 50% methemoglobin, a lethal concentration (Bodansky, 1951).

There was no correlation between the acetylation ratio and methemoglobin formation in the volunteers. The N-acetylation of dapsone is catalyzed by the polymorphic N-acetyltransferase present in the liver (Zuidema et al., 1986), possibly because acetylation and deacetylation occur at a constant equilibrium in the plasma after administration, 1986), possibly because acetylation and deacetylation occur at a constant equilibrium in the plasma after administration of the drug (Zuidema et al., 1971). Although generally considered as a phase II detoxication reaction, N-acetylation of dapsone does not seem to be important in the toxicity associated with the drug (Zuidema et al., 1986), possibly because acetylation and deacetylation occur at a constant equilibrium in the plasma after administration of the drug (Zuidema et al., 1971).

In conclusion, microsomes prepared from rat, mouse and human livers N-hydroxylate dapsone to produce a hydroxylamine which is toxic to erythrocytes from all three species in vitro. Microsomes from all three species also catalyzed the reduction of the hydroxylamine to dapsone. However, dapsone hydroxylamine and its glucuronide, as well as significant methemoglobinemia, were only detected in humans and male rats. The balance between oxidation, reduction and conjugation (fig. 5) is of great importance for the safety evaluation of compounds, and in the case of dapsone, the use of animal models may seriously underestimate the risk of exposure to man. This species variation in metabolism, and hence toxicity, of dapsone is important in the selection of a suitable animal model to investigate the relationship between disposition and toxicity for novel diarylsulfones, and for safety evaluation of aromatic amines in general.

References


Send reprint requests to: M.D. Tingle, Department of Pharmacology, University of Auckland, Private bag 92019, Auckland, New Zealand.