J. Pharm. Pharmacol. 1996, 48:228–232
Received April 21, 1995
Accepted April 28, 1995

A Dapsone-induced Blood Dyscrasia in the Mouse: Evidence for the Role of an Active Metabolite

M. AHMADI, L. F. KHALAF, H. J. SMITH AND P. J. NICHOLLS

Welsh School of Pharmacy, UWCC, Cardiff CF1 3XF, UK

Abstract

In the female mouse, dapsone (50–500 mg kg^{-1}, p.o.) caused a dose-related methaemoglobinaemia which peaked at 0·5–1 h with recovery to baseline values occurring by 4 h. Cimetidine (100 mg kg^{-1}, p.o.), a known inhibitor of several hepatic P450 isozymes administered 1 h before dapsone, prevented the methaemoglobinaemia. In-vitro, dapsone required activation by mouse hepatic microsomes to cause methaemoglobin formation in mouse erythrocytes and cytotoxicity to human mononuclear leucocytes. In both instances, the toxic effects were markedly reduced by cimetidine. Daily dosing of mice with dapsone (50 mg kg^{-1}, p.o.) for 3 weeks induced a blood dyscrasia, characterized by a fall of platelet and white blood cell counts, which was inhibited by cimetidine (100 mg kg^{-1}, p.o. daily).

It is concluded that an active metabolite of dapsone arising from a P450-dependent pathway is involved in the genesis not only of the methaemoglobinaemia but also the blood dyscrasia arising from repeated administration of the drug in this species.

Though introduced in the 1940s, dapsone (Fig. 1) remains important in the treatment of leprosy and has more recently been employed in the treatment of *Pneumocystis carinii* pneumonia in AIDS patients (Lee et al 1989). Use of the drug is associated with a number of side effects of which agranulocytosis (Friman et al 1983) is the most serious and methaemoglobinaemia and haemolysis (Cucinell et al 1972) are the more common. These adverse reactions have been attributed to a hydroxylamine metabolite (dapsone-NHOH) of the drug and Coleman & Tingle (1992) have clearly demonstrated this for dapsone-induced methaemoglobin formation and haemolysis in the rat. However such an association for dapsone-NHOH and agranulocytosis has yet to be established.

There are several metabolic pathways for dapsone including the well-studied polymorphic acetylation. Of these, oxidation is responsible for approximately 50% of the metabolism of the administered dose in man (Coleman & Tingle 1992).

The aromatase inhibitor, aminoglutethimide (Fig. 1), may induce bone-marrow failure with leucopenia, thrombocytopenia and pancytope in some women (Messeih et al 1985). As the female mouse is a good model for this blood dyscrasia (Ali & Nicholls 1986) and as aminoglutethimide, like dapsone, is an aromatic amine metabolized partly by *N*-hydroxylation (Dalrymple & Nicholls 1988), dapsone was examined in this model in the present study.

Materials and Methods

Chemicals

Dapsone was obtained from Sigma (Poole, Dorset, UK). Cimetidine and SKF525A were generous gifts from SmithKlineBeecham (Welwyn, Herts, UK). All other chemicals were the highest grade available from BDH (Poole, Dorset, UK).

Correspondence: P. J. Nicholls, Welsh School of Pharmacy, UWCC, Cardiff CF1 3XF, UK.

Treatment of animals

Female albino mice (ICI-derived strain 25–35 g body weight) were housed on softwood sawdust bedding in plastic cages. The animals were kept in an air-conditioned room (21 ± 1°C) on a 12 h light-12 dark cycle with free access to food (Grain Harvesters Ltd, Canterbury) and water. When a dose of an orally administered drug was given on one occasion only, food but not water was withheld for 12 h prior to dosing. Otherwise there were no restrictions regarding food intake. Orally administered drugs were formulated as suspensions in 0·1% (w/v) sodium carboxymethylcellulose. Control animals received the dosing vehicles in place of the drugs. Dosing volumes were 0·1 mL/10 g body weight.

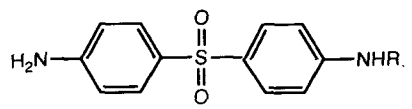
Where blood was collected, animals were anaesthetized (terminally) under ether and blood was withdrawn by cardiac puncture. The blood was immediately transferred to tubes containing either EDTA (for haematological examination) or heparin (for the methaemoglobin studies).

Haematological examination

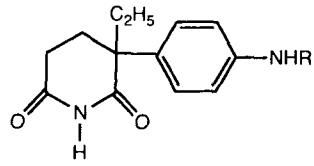
Standard manual determinations (Archer 1965) were carried out for erythrocytes (RBC), platelets (PL) and white blood cells (WBC) as well as haemoglobin (HB) and haematocrit (HC).

Isolation of mononuclear leucocytes from human blood

Human mononuclear leucocytes (MNLs) were isolated from blood freshly drawn from a single healthy male volunteer using a method described by Riley et al (1988). On isolation, the viability of these cells, as determined by trypan blue exclusion was >93%. Isolated cells were kept at room temperature and incubated within 3 h.



Dapsone R = H
N-Hydroxyldapsone R = OH



Aminoglutethimide R = H
N-Hydroxylaminoglutethimide R = OH

FIG. 1. Structures of dapsone and aminoglutethimide.

Preparation of mouse hepatic microsomes

Microsomes were prepared from the pooled livers of 5 phenobarbitone-pretreated (60 mg kg^{-1}, i.p. daily for 3 days) female mice by homogenization (20% w/v) in 0·1 M sodium phosphate buffer pH 7·4. The 10 000-*g* supernatant was centrifuged at 100 000 *g* for 1 h at 4°C to obtain the microsomal pellet which was resuspended in the buffer and stored at −20°C until used (not more than 3 days, over which time no loss of activity occurred). The cytochrome P450 content of the induced mouse microsomes, determined by the procedure described by Omura & Sato (1964), was 1·84 ± 0·14 nmol mg^{-1} microsomal protein (mean ± s.d.) n = 4 batches of pooled microsomes).

Methaemoglobin formation

Methaemoglobin was determined according to the procedure described by Fairbanks (1976). The assay is based on the observation that the absorption spectrum of methaemoglobin at pH 6·6 exhibits a characteristic peak at 630 nm which decreases when methaemoglobin is converted to cyanmethaemoglobin by addition of cyanide.

For the in-vivo formation of methaemoglobin, 0·1 mL aliquots of blood were used. Study of in-vitro formation of methaemoglobin utilized blood from untreated animals. Blood (0·5 mL) was incubated with either 0·06 M phosphate buffer (pH 7·4, 0·1 mL) or a solution of dapsone in the buffer (0·1 mL) in the presence of either the 10 000-*g* supernatant of a 20% (w/v) homogenate of mouse liver in KCl (1·15%, w/v) -Tris (0·1 M) buffer pH 7·4 (0·2 mL) or KCl-Tris buffer (0·2 mL). In each case a solution of NADP (0·2 μmol), glucose-6-phosphate (4 μmol) and $MgCl_2$ (4 μmol) in water (0·2 mL) was added to complete the incubation mixture. At the end of incubation, methaemoglobin was determined as for whole blood.

Table 1. Effect of orally administered dapsone on methaemoglobin levels in the blood of female mice.

Dose (mg kg^{-1})	Methaemoglobin (% total blood pigment)		
	Alone	+ cimetidine (100 mg kg^{-1})	+ SKF525A (200 mg kg^{-1})
0	0·6 ± 0·2	0·6 ± 0·2	0·6 ± 0·2
50	4·5 ± 0·8*	2·3 ± 0·5†	4·7 ± 0·5
200	7·2 ± 1·3*	not determined	not determined
500	9·4 ± 1·1*	1·9 ± 0·5†	9·3 ± 0·8

Values are means ± s.d. (n = 5) from blood taken by cardiac puncture 1 h after an oral dose of dapsone. Cimetidine or SKF525A was administered 1 h before dapsone. *$P < 0·05$ compared with zero dose; †$P < 0·05$ compared with values in absence of cimetidine.

Determination of metabolism-dependent cytotoxicity

Metabolism-dependent toxicity was assessed by incubating MNLs (0·5 mL) and drug (in methanol, 10 μL) in the presence of the mouse hepatic microsomal suspension (0·5 mg microsomal protein, NADP 0·6 mM, glucose-6-phosphate 2·4 mM, glucose-6-phosphate dehydrogenase 2 units in 0·1 M phosphate buffer pH 7·2, 0·5 mL) for 2 h at 37°C in air. After incubation, the cells were sedimented by centrifugation (250 *g*, 10 min at room temperature) and resuspended in 1 mL fresh HEPES-buffered medium containing human serum albumin fraction V (5 mg mL^{-1}). The suspension was incubated without agitation in a humidified air-CO_2 (95 : 5) incubator at 37°C for 16 h. Aliquots were then removed to determine cell viability by trypan blue dye exclusion (0·1% w/v trypan blue applied for 5 min). Failure to exclude the dye was taken as the index of cell death. In excess of 200 cells were counted using a Neubauer haemocytometer.

Results

Dapsone (50 mg kg^{-1}) caused an elevation of methaemoglobin to about 4%, 1 h after dosing and this was significantly greater than the control level (Table 1). Although

Table 2. Formation of methaemoglobin in mouse blood by dapsone in the absence and presence of mouse liver microsomes in-vitro.

Compound (5 μg mL^{-1})	Methaemoglobin (% total blood pigment)			
	Without microsomes	With microsomes	+ cimetidine	+ SKF525A
None	0	0·5 ± 0·2	0·7 ± 0·2	0·5 ± 0·2
Dapsone	0	4·9 ± 0·5*	2·9 ± 0·5†	4·9 ± 0·4*

Values are means ± s.d. (n = 5). Concentrations of cimetidine and SKF525 when present were 10 μg mL^{-1}. *$P < 0·05$ compared with control; †$P < 0·05$ compared with corresponding experiment without cimetidine.

Table 3. Cytotoxicity of dapsone, in the presence of mouse liver microsomes, to human mononuclear leucocytes.

Compound	Cytotoxicity (% mortality)		
	without NADPH	with NADPH	with NADPH + cimetidine
Dapsone (25 μg mL^{-1})	3·0 ± 1·2	17·3 ± 3·4	6·5 ± 0·5*

Values are mean ± s.d. (n = 4). Cimetidine was present at 10 μg mL^{-1}. *$P < 0.05$ compared with the corresponding experiment without cimetidine.

there was evidence of a dose-response relationship, there was only a modest rise of methaemoglobin at 200 and 500 mg kg^{-1}. A possible explanation for this may be a dose-related oral bioavailability. However, the present study did not address this question and there is no evidence that such a phenomenon has been observed previously. At all these doses, the peak level of methaemoglobin ocurred 0·5–1 h after dosing. Recovery was complete by 4 h, values for methaemoglobin (% total blood pigment) being 0·6 ± 0·2, 0·6 ± 0·1, 0·5 ± 0·2, 0·6 ± 0·2% (means ± s.d., n = 5) for control and dapsone 50, 200 and 500 mg kg^{-1} groups, respectively. It may be observed that cimetidine (100 mg kg^{-1}, p.o.) was able to inhibit the formation of methaemoglobin caused by dapsone administered at both 50 and 500 mg kg^{-1}. In contrast, SKF525A (20 mg kg^{-1}, i.p.) was without effect on this action of dapsone.

When dapsone was incubated with mouse blood in the absence of hepatic microsomes, there was no change in methaemoglobin level (Table 2). However, when blood was incubated with dapsone in the presence of mouse liver microsomes, a significant formation of methaemoglobin above background occurred. It may be observed that this action was blocked by cimetidine but not by SKF525A.

Dapsone caused an NADPH-dependent cytotoxicity and this was almost abolished by the presence of cimetidine (Table 3).

The results presented in Table 4 show that dapsone (50 mg kg^{-1}) daily for 3 weeks significantly lowered platelet and white blood cell counts. However, the drug was unable to influence the erythrocyte count, the haemoglobin concentration or the haematocrit. Cimetidine (100 mg kg^{-1}, p.o.) administered daily to mice for 3 weeks had no influence on the blood picture. However, this regimen of the drug was able to prevent the effects of dapsone on platelet and white cell counts.

In a further experiment, the effect of daily dosing with SKF525A (20 mg kg^{-1} ip) concurrently with dapsone (50 mg kg^{-1}) was examined. The dapsone-induced lowering of platelet and white cell counts (851 ± 70 and 4·8 ± 0·6, × 10^3 mm^{-3}, respectively) was not significantly altered by SKF525A (840 ± 62 and 4·3 ± 0·5, × 10^3 mm^{-3}, respectively).

Discussion

Dapsone is an aromatic amine that undergoes *N*-hydroxylation and this process is believed to be an important step in the mechanism of its toxicity (Coleman & Tingle 1992). In subjects receiving the drug, the commonest haematological reactions are methaemoglobinaemia and haemolytic anaemia (Zuidema et al 1986), haemolysis being the less common. Dapsone-NHOH is the main oxidative metabolite of dapsone which can enter the circulating erythrocyte. Here it is co-oxidized with haemoglobin to give rise to the nitroso derivative of dapsone and methaemoglobin (Kramer et al 1972). Although the mouse has a much higher methaemoglobin reductase activity than that of man, the former species has been successfully used to study chemically-induced methaemoglobinaemias (Smith et al 1967). Thus it was anticipated that dapsone would cause a methaemoglobinaemia in the mouse. The peak levels achieved and the time-course (baseline levels returning within 4 h after dosing) were similar to those previously recorded for the female rat (Coleman et al 1990b).

Studies with human liver microsomes have shown that cytochrome CYP1A2 is responsible for the activation, through *N*-oxidation, of many arylamine procarcinogens (Butler et al 1989). Recently it has been shown that cytochrome CYP3A4 is chiefly responsible for the *N*-oxidation of dapsone in man (Fleming et al 1992). However, it is considered possible (Coleman & Tingle 1992) that a number of P450 isozymes with varying substrate affinities are capable of oxidising dapsone, as is the case for the *N*-oxidation of 3-methoxy-4-aminoazobenzene (Yamasaki et al 1991).

Cimetidine binds to various P450 isozymes and inhibits the metabolism of a variety of xenobiotics (Knodell et al 1991). In the rat, it inhibits both dapsone-induced

Table 4. Haemotoxicity of oral dapsone (50 mg kg^{-1}) administered daily for three weeks to female mice.

Group	Platelets (10^3 mm^{-3})	White blood cells (10^3 mm^{-3})	Red blood cells (10^3 mm^{-3})	Haemoglobin (g dL^{-1})	Haematocrit (%)
Vehicle	1630 ± 60	8·2 ± 0·1	11·4 ± 0·8	15·0 ± 0·3	42 ± 1
Cimetidine	1535 ± 65	8·1 ± 0·2	11·7 ± 0·1	14·6 ± 0·3	43 ± 1
Dapsone	920 ± 85*	6·7 ± 0·5*	9·6 ± 0·9	14·8 ± 0·5	39 ± 2
+ cimetidine	1440 ± 80	7·9 ± 0·3	10·9 ± 0·9	15·0 ± 0·2	40 ± 2

Values are mean ± s.d. (n = 10). Blood was collected by cardiac puncture 24 h after the final dose od dapsone. Cimetidine was given at an oral dose of 100 mg kg^{-1}, daily. *$p < 0.05$ compared with vehicle.

methaemoglobinaemia (Coleman et al 1990b) and the formation of dapsone-NHOH (Coleman et al 1990a). From the present results it is clear that cimetidine also abolishes dapsone-induced methaemoglobiaemia in the mouse. It was equally effective in the in-vitro system measuring methaemoglobin formation in erythrocytes in the presence of liver microsomes and in preventing the cytotoxicity of metabolically-activated dapsone to human leucocytes. In addition, cimetidine alone administered daily for 3 weeks was without effect on the blood picture of mice. However, this regime effectively prevented the dapsone-induced reductions in blood platelet and erythrocyte counts.

The mouse, but not the rat, has been found to be a good model in which to reproduce the clinically important blood dyscrasia caused by aminoglutethimide (Ali & Nicholls 1986). Although the mechanism for this toxicity has not been fully elucidated, there is sufficient evidence to implicate the metabolic activation of aminoglutethimide to aminoglutethimide-NHOH (Ali et al 1990). It is probable that a similar consideration also applies to the dapsone-induced blood dyscrasia described here in the mouse and this is supported by the inhibitory effects of cimetidine.

It was anticipated that SKF525A would have exhibited a similar inhibitory effect to that of cimetidine on the haematological effects of dapsone as this compound is a well-recognised inhibitor of hepatic mixed-function oxidase (Cooper et al 1954). However, it was without effect in in-vitro and in-vivo tests with dapsone. Undoubtedly, this is a reflection of the fact that the cytochrome P-450 is a family of enzymes rather than a single protein and that inhibitors of this system show a high selectivity in their inhibitory effects on the different isozymes (Knodell et al 1991). Thus 4-aminopropriophenone-induced methaemoglobinaemia in the mouse (Smith et al 1967) and aniline-induced methaemoglobinaemia in the cat (McLean et al 1967) and mouse (Ali 1987) are not affected by SKF525A. However, this agent is able to inhibit the methaemoglobin-forming activity of 3,4-dichloroaniline in the mouse (Singleton & Murphy 1973) and the *N*-oxidation of 4-chloroaniline (Uehleke 1972) and dapsone (Cucinell et al 1972) in the rat. More recently, it has been shown that even the cytochrome P450 inhibitor, ketoconazole and the selective *N*-hydroxylation inhibitor, methimazole are poor inhibitors of dapsone-induced methaemoglobinaemia in the rat (Coleman et al 1989, 1990b).

Oxidative metabolites of dapsone can bind to human tissue in-vitro (Coleman et al 1989) and it is believed that, in man, dapsone-induced agranulocytosis occurs through an immunological mechanism involving the oxidative metabolic activation of the drug. Accordingly, the causes of agranulocytosis may be linked to the binding of hydroxylamine metabolites to granulocytes, leading to haptenation and eventual cell destruction. There is recent evidence (Coleman et al 1994) that erythrocytes may act as a delivery system enabling dapsone-NHOH to reach the bone marrow and cause haptenation.

Whatever the detailed mechanism for the dapsone-induced blood dyscrasia, it is clear from the present results that a cytochrome P450-dependent and cimetidine-sensitive step of activation is involved. Thus in high-risk patients receiving dapsone, it should be possible to prevent or attenuate agranulocytosis by the coadministration of cimetidine. Such a strategy has proved effective in reducing dapsone-dependent methaemoglobinaemia in dermatitis herpetiformis patients (Coleman et al 1992).

References

Ali, H., Nicholls, P. J. (1986) Haematological effects of aminoglutethimide in the mouse. J. Pharm. Pharmacol. 38: 92P

Ali, H., Khalaf, L., Nicholls, P. J., Poole, A. (1990) Comparison of in vitro and in vivo haemotoxic effects of aminoglutethimide and glutethimide. Toxicology In Vitro 4: 381–383

Ali, H. (1987) Ph.D. Thesis. University of Wales

Archer, R. K. (1965) Haematological Techniques of Use on Animals (2nd edn) Blackwell, Oxford

Butler, M. A., Iwasaki, M., Guengerich, F. P., Kadlubar, F. F. (1989) Human cytochrome P-450PA (P-450IA2), the phenacetin O-de-ethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. Proc. Natl. Acad. Sci. USA 86: 7696–7700

Coleman, M. D., Tingle, M. D. (1992) Use of a metabolic inhibitor to reduce dapsone-dependent haematological toxicity. Drug Dev. Res. 15: 1–16

Coleman, M. D., Breckenridge, A. M., Park, B. K. (1989) Bioactivation of dapsone to a cytotoxic metabolite by human hepatic microsomal enzymes. Br. J. Clin. Pharmacol. 28: 389–395

Coleman, M. D., Rhodes, L. A., Scott, A. K., Verbov, J. L., Friedman, P. S., Breckenridge, A. M., Park, B. K. (1992) The use of cimetidine to reduce dapsone-dependent methaemoglobinaemia in dermatitis herpetiformis patients. Br. J. Clin. Pharmacol. 34: 244–249

Coleman, M. D., Scott, A. K., Breckenridge, A. M., Park, B. K. (1990a) The use of cimetidine as a selective inhibitor of dapsone *N*-hydroxylation in man. Br. J. Clin. Pharmacol. 30: 761–767

Coleman, M. D., Simpson, J., Jacobus, D. P. (1994) Reduction of dapsone hydroxylamine to dapsone during methaemoglobin formation in human erythrocytes in vitro, IV: implications for the development of agranulocytosis. Biochem. Pharmacol. 48: 1349–1354

Coleman, M. D., Winn, M. J., Breckenridge, A. M., Park, B. K. (1990b) Sex-dependent sensitivity to dapsone-induced methaemoglobinaemia in the rat. Biochem. Pharmacol. 39: 805–809

Cooper, J. R., Axelrod, J., Brodie, B. B. (1954) Inhibitory effects of β-diethylaminoethyldiphenylpropylacetate on a variety of drug metabolic pathways in vitro. J. Pharmacol. Exp. Ther. 112: 55–63

Cucinell, S. A., Israeli, Z. H., Dayton, P. G. (1972) Microsomal *N*-oxidation of dapsone as a cause of methaemoglobin formation in human red cells. Am. J. Tropical Med. Hyg. 21: 322–331

Dalrymple, P. D., Nicholls, P. J. (1988) Metabolism profiles and excretion of 14C-aminoglutethimide in several animal species and man. Xenobiotica 18: 75–81

Fairbanks, V. F. (1976) Hemoglobin, hemoglobin derivatives and myoglobin. In: Tietz, N.W. (ed.) Fundamentals of Clinical Chemistry. Saunders, Philadelphia

Fleming, C. M., Branch, R. A., Wilkinson, G. R., Guengerich, F, P. (1992) Human liver microsomal *N*-hydroxylation of dapsone by cytochrome P-4503A4. Mol. Pharmacol. 41: 975–980

Friman, G., Nystrom-Rosander, C., Jonsell, G., Bjorkman, A., Lekas, G., Sendsrup, B. N. (1983) Agranulcytosis associated with malaria prophylaxis with Maloprim. Br. Med. J. 286: 1244–1245

Knodell, R. G., Browne, D. G., Gwozdz, G. P., Brian, W. R., Guengerich, F. P. (1991) Differential inhibition of individual human liver, cytochromes P-450 by cimetidine. Gastroenterology 101: 1680–1691

Kramer, P. A., Glader, B. E., Li, T. K. (1972) Mechanism of methemoglobin formation by diphenylsulfones. Effect of 4-amino-4'-hydroxyaminodiphenylsulfone and other *p*-substituted derivatives. Biochem. Pharmacol. 21: 1265–1274

Lee, B. L., Medina, I., Benowitz, N. L., Jacob, P., Wofsy, C. B., Mills, J. (1989) Dapsone, trimethoprim and sulfamethizole plasma levels during treatment of pneumocystis pneumonia in patients with the acquired immunodeficiency syndrome (AIDS). Ann. Intern. Med. 110: 606–611

McLean, S., Robinson, J., Starmer, G. A., Thomas, J. (1967) The influence of anaesthetic agents on the formation of methaemoglobin induced by aniline in cats. J. Pharm. Pharmacol. 19: 803–809

Messeih, A. A., Lipton, A., Santen, R. J., Harvey, H. A., Boucher, A. E., Murray, R., Ragaz, J., Buzdar, A. U., Nagel, G. A., Henderson, I. C. (1985) Aminoglutethimide-induced haematologic toxicity : world-wide experience. Cancer Treat. Reports 69: 1003–1004

Omura, T., Sato, R. (1964) The carbon monoxide binding pigment of liver microsomes. J. Biol. Chem. 221: 253–264

Riley, R. J., Maggs, J. L., Lambert, C., Kitteringham, N. R., Park, B. K. (1988) An in vitro study of the microsonal metabolism and cellular toxicity of phenytoin, sorbinil and mianserin. Br. J. Clin. Pharmacol. 26: 577–588

Singleton, S. D., Murphy, S. D. (1973) Propanil (3,4-dichloropropionanilide)-induced methaemoglobin formation in mice in relation to acylamidase activity. Toxicol. Appl. Pharmacol. 25: 20–29

Smith, R. P., Alkaitis, A. A., Schafer, P. R. (1967) Chemically induced methemoglobinemias in the mouse. Biochem. Pharmacol. 16: 317–328

Uehleke, H. (1972) In: Bridges J. W., Gorrod, J. W., Parke, D. V. (eds) Biological Oxidation of Nitrogen in Organic Molecules. Taylor and Francis, London

Yamasaki, H., Degawn, M., Funae, Y., Imaoka, S., Inui, T., Guengerich, F. P. (1991) Roles of different cytochrome P450 enzymes in bioactivation of the potent hepatocarcinogen 3-methoxy-4-aminoazobenzene by rat and human liver microsomes. Carcinogenesis 12: 133–139

Zuidema. J., Hilbers-Modderman, E. S. M., Markus, F. W. H. M. (1986) Clinical Pharmacokinetics of dapsone. Clin. Pharmacokin. 11: 299–325