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Bioactivation of dapsone to a cytotoxic metabolite by human hepatic microsomal enzymes

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1 Using human mononuclear leucocytes as target cells, we have investigated the bioactivation of dapsone (DDS) to a cytotoxic metabolite in the presence of microsomes from nine human livers. Values for NADPH dependent toxicity ranged from 8.8–27% (15.8 ± 5.9%) and were similar to those for microsomes from control mice, 16–24% (19.0 ± 4.8%).
2 Microsomes prepared from mice induced with either phenobarbitone or beta-naphthoflavone did not produce significantly more NADPH dependent toxicity than microsomes prepared from control mice.
3 Cytotoxicity was abolished not only by ascorbic acid, but also by sub-physiological concentrations of *N*-acetylcysteine and glutathione.
4 DDS was metabolised *in vitro* to a hydroxylamine (metabolic conversion 3.1 ± 1.5%), which was oxidised further to a cytotoxic metabolite which also became irreversibly bound to protein.

Keywords dapsone activation human liver

Introduction

Dapsone (DDS) was introduced in the 1940s and remains the mainstay of the treatment of leprosy (Smith, 1988). It is also effective in a number of other conditions including malarial prophylaxis (with pyrimethamine, Bruce-Chwatt, 1982) and other inflammatory diseases such as rheumatoid arthritis (Swinson *et al.*, 1981) and conditions characterized by polymorphonuclear leucocyte infiltration (Lang, 1979). Recently DDS has been used in the treatment of *Pneumocystis carinii* infection in AIDS patients (Green *et al.*, 1988).

Adverse reactions to DDS can be severe and life threatening. Agranulocytosis associated with DDS has been reported at a prevalence of 1:2000 (Friman *et al.*, 1983). Severe haemolytic anaemia has also been reported frequently in individuals deficient in glucose 6-phosphate dehydrogenase, and has been shown to occur in normal patients at higher doses (Grossman & Jollow, 1988). In man, DDS is acetylated (Gelber *et al.*, 1971) to monoacetyl DDS (MADDS) and *N*-hydroxylated to DDS hydroxylamine (DDS NOH, Israili *et al.*, 1973). The hydroxylamine has been associated with haemoglobin oxidation (Uehleke & Tabarelli 1973; Grossman & Jollow, 1988). DDS NOH has also been reported to inhibit granulopoiesis *in vitro* (Weetman *et al.*, 1980). Furthermore, hydroxylamines have been suggested to be the causative agents in sulphonamide hypersensitivity (Shear & Spielberg, 1985). However, it has been postulated that DDS NOH undergoes further oxidation to the nitroso derivative which may be the ultimate mediator of DDS toxicity (Uetrecht *et al.*, 1988). Hence, using an *in vitro* cytotoxicity assay, in which human mononuclear leucocytes (MNL) are exposed to the metabolites generated by both human (Riley *et al.*, 1988) and murine microsomal systems (Spielberg, 1984) we wished to determine the relationship between the metabolism and cytotoxicity of DDS *in vitro*.

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Methods

Chemicals

4,4′ Diaminodiphenyl sulphone (DDS, dapsone), glutathione, *N*-acetylcysteine and human serum albumin were all purchased from Sigma Chemical Co. Ltd (Poole, Dorset, UK). Ring labelled [^{14}C]-DDS was obtained from Amersham International (Bucks, UK). Specific activity was 6.8 mCi mmol^{-1} and radiochemical purity was determined to be 99% by t.l.c. and h.p.l.c. Ascorbic acid was obtained from the Aldrich Chemical Co. (Gillingham, UK).

NADPH (tetrasodium salt) was obtained from BDH Chemicals Ltd (Poole, Dorset, UK). H.p.l.c. grade solvents were products of Fisons plc (Loughborough, Leics. UK). 4,4′ Diaminodiphenyl sulphone hydroxylamine was synthesised using the method of Uetrecht *et al.* (1988). The product was a yellow solid with a m.p. of 195° C and the yield was 96% dapsone hydroxylamine (as determined by h.p.l.c.). The product was also analysed on a VG Tritech TS-250 mass spectrometer. The EI mass spectrum (direct probe, 70 eV) contained identified ions at m/z 264 ([M]$^{+\cdot}$), and 248 ([M-O]$^{+\cdot}$).

Isolation of human mononuclear leucocytes

Mononuclear leucocytes (MNL) were isolated (*circa* 1 × 10^6 ml^{-1} whole blood) from freshly drawn heparinized (lithium heparin) blood from three healthy male volunteers (21–25 years) using the method of Boyum (1976). The blood (30–40 ml) was diluted (1:1, v/v) with phosphate-buffered saline (PBS; NaCl, 8 g l^{-1}; KCl, 0.2 g l^{-1}; Na_2HPO_4, 1.15 g l^{-1}; KH_2PO_4, 0.2 g l^{-1}; pH 7.2). Aliquots (12 ml) were layered onto Lymphoprep (Nycomed UK Ltd, Birmingham; 9 ml aliquots), and centrifuged at 1900 rev min^{-1} (*circa* 650 *g*) for 13 min in a Centaur 2 centrifuge (MSE, Crawley, Sussex, UK). The interface layer was removed, the suspension of cells was diluted with PBS (1:1, v/v) and the cells were sedimented by centrifugation. They were resuspended gently in PBS and sedimented at a low speed (800 rev min^{-1}, *circa* 115 *g*, for 10 min) to reduce contamination by platelets; this procedure was repeated once. Contaminating erythrocytes were removed by hypotonic lysis. Finally, the MNL were resuspended in 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES)-buffered balanced salt medium (Spielberg, 1980) to obtain *circa* 2 × 10^6 cells ml^{-1}. The isolated cells were left at room temperature and incubated within 3 h. Their viability upon isolation, as determined by trypan blue exclusion, was ≥ 98%. There were no significant differences between the respective sensitivities of the MNL obtained from different donors to drug related toxicity.

Preparation of hepatic microsomes

Histologically normal livers were obtained from nine kidney transplant donors (aged 4–60 years). Ethical approval was granted and consent to removal of the liver samples was obtained from the donors' relatives, as previously indicated (Riley *et al.*, 1988). Ethical approval was also obtained for removal of blood from volunteers.

Washed human liver microsomes were prepared as reported previously (Purba *et al.*, 1987). Microsomes were isolated from the livers of four phenobarbitone pretreated (60 mg kg^{-1} body wt day^{-1}, i.p. in 0.9% w/v saline for 3 days), four beta-naphthoflavone (BNF) pretreated (75 mg kg^{-1} body wt day^{-1}, i.p. in corn oil for 3 days) and four control male CBA mice by the centrifugation procedure used to obtain washed human liver microsomes. Their cytochrome P-450 contents were measured according to the method of Omura & Sato (1964) and found to be 0.92 ± 0.15 and 0.98 ± 0.10 nmol mg^{-1} protein (PB and BNF pre-treated mice) and 0.60 ± 0.17 nmol mg^{-1} protein (control mice). Concentrated suspensions of the microsomes (10–15 mg microsomal protein ml^{-1}) in 0.1 M sodium phosphate buffer (pH 7.4) were prepared for incubation with MNL. The protein content of the liver microsomes was measured by the method of Lowry *et al.* (1951). Aliquots were stored at −80° C; the cytochrome P-450 dependent activities of human liver microsomes stored in phosphate buffers were stable over long periods.

Measurement of MNL viability by trypan blue dye exclusion

Cell viability (% excluding dye) was assessed using 100 µl aliquots of suspensions: they were mixed with 25 µl of a 0.2% (w/v) solution of trypan blue (dye content ≥ 50%; Aldrich Chemical Co., Gillingham, Dorset, UK) in PBS. The cells (≥ 200) were examined on a Neubauer haemocytometer under a Model Wilovert II microscope (Will Wetzlar GmbH, Nauborn, FRG). Cell death could then be expressed as % cells not excluding dye. Samples were examined in a randomised order to avoid bias.

Determination of cytotoxicity

For determination of direct cytotoxicity MNL (1 × 10^6 cells in 1 ml HEPES-buffered medium) were incubated with DDS and DDS NOH added

in h.p.l.c. grade methanol and acetone, respectively (10 μl), in 10 ml polystyrene conical based tubes (Sterilin, Feltham, UK). The tubes, open to the air, were shaken in a water bath at 37° C for 2 h. Control incubations with methanol or acetone alone showed that neither solvent was toxic at 1% (v/v) solution. The initial viabilities of cells incubated without additives and in the presence of methanol or acetone were 98 ± 1.5% ($n = 3$), 98 ± 1% ($n = 3$) and 97.8 ± 1% ($n = 3$). After 2 h, viability was determined by trypan blue exclusion. The cells were sedimented and resuspended in a drug-free medium (HEPES-buffered medium containing 5 mg ml^{-1} human serum albumin). Incubations were continued in loosely capped tubes, without agitation in a humidified incubator at 37° C. After 15 h had elapsed, aliquots were removed for dye exclusion assay.

Measurements of metabolism (NADPH)-dependent cytotoxicity were performed in the same manner as above except that the 2 h incubations included murine or human hepatic microsomes (0.5 mg protein) together with NADPH (1 mM). Ascorbic acid, *N*-acetylcysteine and glutathione were incubated with the cells for 10 min prior to addition of drug, microsomes and NADPH (Spielberg *et al.*, 1981). The results shown represent cytotoxicity (% cells not excluding trypan blue) observed after 16 h incubation, as cell death after 2 h was unusual.

Metabolism of DDS by human liver microsomes

[^{14}C]-DDS (100 μM, 0.5 μCi) was incubated with human liver microsomes (0.5 mg protein) and 1×10^6 MNL in 15 mM HEPES-buffered medium (pH 7.4) final volume (1 ml). Reactions were performed in quadruplicate and started by the addition of NADPH (1 mM) in 10 ml polystyrene tubes followed by incubation in a shaking water bath at 37° C for 2 h. NADPH was omitted from control incubations ($n = 4$). In selected incubations, ascorbic acid (2 mM) was added to preserve any DDS NOH formed from autooxidation to nitroso DDS. The reactions were terminated by the addition of 1 ml of methanol to each tube. The tubes were left at −20° C overnight to precipitate all protein.

Analytical procedures

After further precipitation of protein by centrifugation (650 *g* for 20 min), DDS and DDS NOH were analysed by radiometric h.p.l.c. They were identified chromatographically by comparing their reaction times with those of co-injected authentic unlabelled compounds. Aliquots of the supernatant containing approximately 80,000 d min^{-1} were injected onto the column for measurement of approximately 1800–2500 d min^{-1} associated with DDS NOH. Chromatography was performed on an LKB chromatograph using the method of Uetrecht *et al.* (1988). Separation was achieved using a μ Bondapak C_{18} column (30 cm × 0.39 cm i.d., 10 μM, Waters Assoc., Hartford, Cheshire, UK).

The solvent mobile phase consisted of water-acetonitrile-acetic acid-triethylamine (80:20:1: 0.05 v/v) flowing at 1.3 ml min^{-1}. Under these conditions the retention times of DDS NOH and DDS were 8.5 and 10.5 min.

Eluate was monitored at 254 nm and collected in 30 s fractions which were then mixed with 4 ml of scintillant for measurement of radioactivity.

Statistical analysis was accomplished by the use of the Mann-Whitney 'U' test accepting $P \leq 0.05$ as significant. Data are presented as the mean ± s.d.

Irreversible binding of radiolabelled material

Radiolabelled material irreversibly bound to the precipitated protein was measured by extensive solvent extraction. The protein was successively extracted with methanol (1 ml × 2) and 70% (v/v) methanol (1 ml × 2) by vigorous vortex mixing. Extracts were assayed for radioactivity to ensure that removal of the reversibly bound material was complete. The protein was solubilized in 1 ml of 0.5 M sodium hydroxide and heated at 60° C. Aliquots were taken for protein measurement (Lowry *et al.*, 1951) and liquid scintillation counting.

Results

Activation of DDS to a cytotoxic metabolite

Incubation of DDS alone with MNL showed no significant cytotoxicity above background levels (2.0 ± 0.7%) up to 1 mM (100 μM, 2.4 ± 0.5%; 500 μM, 2.8 ± 0.7%; 1 mM, 3.2 ± 0.9%). However, in all nine human livers tested DDS was activated in the presence of microsomes and NADPH to a cytotoxic metabolite at 100 μM concentrations (Table 1, Figure 1). However, variation in the degree of activation was considerable, with a mean NADPH dependent toxicity value of 15.8 ± 5.9% ($n = 9$). Values for NADPH dependent toxicity of DDS in murine microsomal systems were in a similar range to those for human microsomes. There was no significant difference in the toxicity of DDS with microsomes prepared from phenobarbitone-induced mice

Table 1 Cytotoxicity at 16 h after incubation of MNL with NADPH, DDS (100 μM) and various microsomal preparations over 2 h at 37° C

Incubation microsomes	n	*Protectant*	*NADPH dependent toxicity (%)*
Human	9	—	15.8 ± 5.9
Control mouse	4	—	19.0 ± 4.8
PB+	4	—	19.7 ± 3.9
BNF*	4	—	13.8 ± 7.1
Control mouse	4	ASC (100 μM)	1.0 ± 0.2
Control mouse	4	NAc (50 μM)	1.2 ± 1.0
Control mouse	4	GSH (500 μM)	1.1 ± 0.2

+ Microsomes isolated from phenobarbitone induced mice.
* Microsomes isolated from BNF induced mice.
ASC: Ascorbate.
NAC: *N*-acetylcysteine.
GSH: Glutathione.

(19.8 ± 3.9%), BNF-induced mice (13.8 ± 7.1%) and control mice (19.0 ± 4.8%) (Table 1). The minimum protective effects of *N*-acetylcysteine, ascorbate and glutathione were determined by incubation of increasing concentrations (5–550 μM) of these compounds with MNL in the presence of DDS (100 μM), control microsomes and NADPH. *N*-Acetylcysteine and ascorbate abolished toxicity at 50 μM and 100 μM while glutathione significantly protected the cells only at 500 μM.

Toxicity of DDS NOH

DDS NOH was incubated with MNL alone across an identical concentration range to DDS (100 μM–1 mM). At 100 μM, DDS NOH was shown to cause a four fold increase in cytotoxicity with respect to control (8.2 ± 3.3 *vs* 2.0 ± 0.7%). At 1 mM, DDS NOH cytotoxicity exceeded 67% (Figure 2); while DDS toxicity was not significantly different from background (3.1 ± 1.2 *vs* 2.0 ± 0.7%). Incubation of DDS NOH with human liver microsomes (Liver 3) and MNL showed no significant difference in cytotoxicity with respect to DDS NOH with MNL alone (8.2 ± 3.3 *vs* 7.0 ± 3.2%). However, the addition of NADPH (1 mM) caused a significant decrease (≥ 50%) in cytotoxicity (2.2 ± 0.8 *vs* 7.0 ± 3.2%).

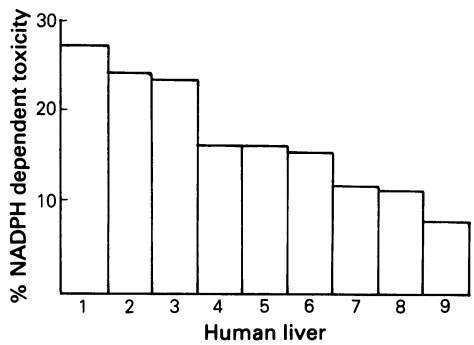

Figure 1 MNL cytotoxicity after incubation of human liver microsomes with MNL and NADPH ($n = 9$).

Incubation of DDS NOH with MNL across the concentration range 100 μM–1 mM over 2 h in the presence of ascorbate (500 μM) revealed a marked fall in overall cytotoxicity (Figure 2). Cytotoxicity of the DDS NOH at 1 mM was reduced by almost half (63.6 ± 9.3 *vs* 33.4 ± 13%) in the presence of ascorbate (500 μM). H.p.l.c. analysis of DDS NOH in the incubations in the presence of ascorbate revealed the preservation of DDS NOH with respect to incubations

Table 2 Total DDS NOH determined by h.p.l.c. in the presence of ascorbate and irreversible binding in the absence of ascorbate after incubation of [^{14}C]-DDS (100 mM) with NADPH and human liver microsomes over 2 h at 37° C : $n = 4$ per incubation

	Total stable metabolite DDS NOH (% radioactivity)*		*Irreversible binding+ (nmol mg^{-1} protein)*	
Liver	*−NADPH*	*+NADPH*	*−NADPH*	*+NADPH*
3	2.5 ± 0.6	5.1 ± 0.8	0.56 ± 0.2	1.12 ± 0.13
4	0.4 ± 0.07	2.75 ± 0.3	0.33 ± 0.09	0.86 ± 0.17
7	0.14 ± 0.04	2.5 ± 1.1	0.32 ± 0.07	0.61 ± 0.1
5	0.1 ± 0.03	1.8 ± 0.15	0.33 ± 0.17	0.81 ± 0.17

* Presence of 2 mM ascorbate.
+ Absence of ascorbate

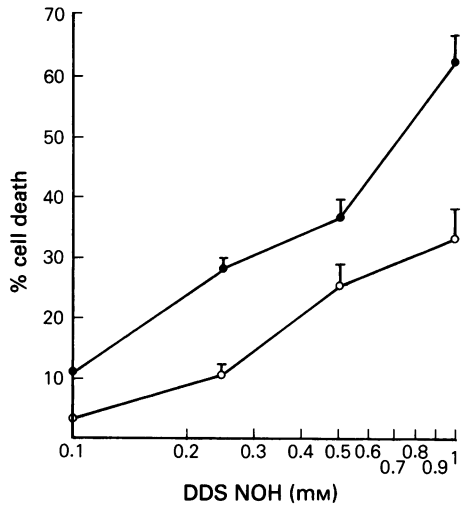

Figure 2 Incubation of DDS NOH with MNL over 2 h (37° C) with (o——o) and without (•——•) ascorbate (500 μM) (n = 5 per point).

containing DDS NOH alone. At 500 μM, DDS NOH concentrations were maintained in the presence of ascorbate at 26.5 ± 5.3% of the starting levels compared with 7.8 ± 2.4% remaining in the control incubations. At 1 mM preservation of DDS NOH was even more marked, with 57.3 ± 0.57% of the compound remaining intact in the presence of ascorbate compared with only 26.6 ± 5.3% remaining in the absence of ascorbate.

Metabolism and covalent binding of DDS NOH

Radiometric analysis of incubations containing DDS, NADPH, microsomes from four human livers (1–4) and 2 mM ascorbic acid revealed a peak corresponding to the retention time of DDS NOH (Figure 3). No other oxidative metabolites of DDS could be detected. Conversion of radiolabelled DDS to DDS NOH ranged from 1.8–5.1% (3.0 ± 1.4%; mean ± s.d.; n = 4). Incubation of DDS, human liver microsomes and NADPH in the absence of ascorbic acid revealed considerable irreversible binding of DDS related material. Binding in the absence of NADPH ranged from 0.3–0.56 nmol mg^{-1} protein. However, these values more than doubled in the presence of NADPH (0.86–1.12 nmol mg^{-1} protein).

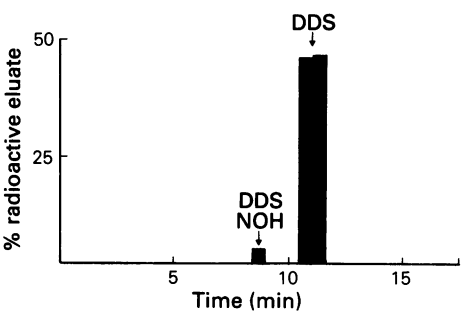

Figure 3 High performance liquid chromatogram of an extract of an incubation containing human liver microsomes (0.5 mg protein), NADPH and 2 mM ascorbate performed at 37° C for 2 h. The peaks eluted at 8.5 min and 10.5 min were DDS NOH and DDS respectively.

Discussion

DDS is associated with a number of adverse reactions, many of which are dose related (Smith, 1988; Weetman *et al.*, 1980). A number of studies have also noted the marked variation in DDS levels in individuals taking the same drug dose daily (DeGowin *et al.*, 1966; Alexander *et al.*, 1970). The major route of elimination of DDS is *N*-acetylation, which exhibits genetic polymorphism (Gelber *et al.*, 1971). However, DDS NOH has been found in the urine of patients receiving DDS and approximately one third to one half of the dose may be eliminated in man as *N*-hydroxylated products (Israili *et al.*, 1973; Uehleke & Tabarelli, 1973). However, the instability of DDS NOH in solutions containing oxygen, casts doubt on this estimate. The *N*-hydroxylation of DDS may be of toxicological significance, since it has been suggested that either DDS NOH (Weetman *et al.*, 1980; Grossman & Jollow, 1988) or its further oxidative product, 4,4 nitrosoaminodiphenyl sulphone (Uetrecht *et al.*, 1988) is responsible for the toxicity of DDS in man.

In the present study we found that DDS underwent bioactivation to a cytotoxic metabolite, in an NADPH dependent reaction catalysed by microsomes prepared from nine individual human livers (Figure 1). There was considerable variation in cytotoxicity between livers. Cytotoxicity was effectively abolished by addition of glutathione, *N*-acetylcysteine or ascorbic acid, in concentrations comparable to those found *in vivo* (Ecobichon, 1984; Malloy *et al.*, 1981; Woodruff, 1964). However, it should be noted that glutathione added exogenously will not penetrate the cells, whereas *N*-acetylcysteine may diffuse into the cells and serve as a precursor for intracellular glutathione. Chromatographic analysis of incubations which contained ascorbic acid revealed the presence of a single detectable metabolite which co-chromatographed (retention

time 8.5 min) with synthesised DDS NOH, and that metabolic conversion was less than 5%. The radioactivity in fractions associated with DDS NOH was at least 20 times greater than background. No other product was detected. In further experiments it was found that ascorbate significantly reduced the cytotoxicity of DDS NOH and prevented autoxidative decomposition. Hence in the absence of reducing agents such as ascorbate and glutathione it is likely that DDS NOH undergoes further oxidation to a reactive nitroso derivative. Ascorbic acid and NADPH are known to reduce nitroso derivatives but do not react with the corresponding hydroxylamine (Becker & Sternson, 1980). Thus it is also possible that in the present study the cytotoxicity induced by activation of DDS may have been slightly reduced by the antioxidant effect of the NADPH present in the incubations.

Studies with procainamide hydroxylamine have indicated that the metabolite undergoes spontaneous oxidation to a nitroso derivative, which is thought to be responsible for the agranulocytosis, hepatotoxicity and lupus like syndrome associated with procainamide therapy (Rubin *et al.*, 1987). In addition, the nitroso derivative of procainamide also undergoes considerable irreversible binding *in vitro* (Uetrecht, 1984). In the present study, NADPH dependent irreversible binding was comparable with that of the more toxic drug mianserin and greater than that of phenytoin observed under similar conditions (Riley *et al.*, 1988). Compounds such as procainamide, DDS and sulphonamides contain an aromatic amine group which is readily acetylated by cytosolic hepatic acetyltransferases. So called 'fast acetylators' are at lower risk from procainamide induced adverse reactions (Woosley *et al.*, 1978), as a lower proportion of the drug is available for *N*-hydroxylation. However, a hydroxylated derivative of MADDS has been found in human urine (Israili *et al.*, 1973), and has been shown to be equipotent with DDS NOH as a haemolytic agent in rats (Grossman & Jollow, 1988). Therefore, acetylator status may have less bearing on the occurrence of toxic reactions to DDS in comparison with procainamide.

The adverse reactions to DDS are less frequent than might be anticipated considering that most human livers activate the drug to the hydroxylamine. Although the liver is thought to be the major site of production for the metabolite (Uehleke & Tabarelli, 1973), hepatotoxicity has only been reported in instances where doses exceeded 300 mg day^{-1} (Barnes & Barnes, 1951). In fact, hepatic concentrations of glutathione (in the rat, $\geqslant 6$ mM Ecobichon, 1984) would prevent further oxidation of DDS NOH to the nitroso derivative until it escapes the liver. Likewise, plasma cysteine levels (Malloy *et al.*, 1980) would also preserve the hydroxylamine. However, although red cells contain relatively high levels of glutathione (Beutler *et al.*, 1963), intracellular DDS NOH oxidation of haemoglobin outstrips its reduction by NADH-dependent methaemoglobin reductase (Modderman *et al.*, 1983). Impairment of this enzyme function may cause severe methaemoglobinaemia (Ganer *et al.*, 1981). In addition, due to haemolysis, the lifespan of red cells is reduced in all subjects taking DDS. This is especially marked in individuals with glucose-6-phosphate dehydrogenase deficiency who have diminished activity of glutathione reductase (Zuidema *et al.*, 1986). Further oxidation of DDS NOH may also occur in target cells such as white cells (Uetrecht *et al.*, 1988) and bone marrow (Weetman *et al.*, 1980). Thus, serious adverse reactions to dapsone may arise as a consequence of inter-individual variations in the acetylation and oxidation of dapsone, and also variation in detoxication of dapsone metabolites in particular cells.

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