Direct and metabolism-dependent toxicity of sulphasalazine and its principal metabolites towards human erythrocytes and leucocytes

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- 1 The role of metabolites in sulphasalazine-mediated toxicity has been investigated *in vitro* by the use of human red blood cells and mononuclear leucocytes as target cells, with methaemoglobin formation and cytotoxicity respectively, being the defined toxic end-points.
- 2 Of the metabolites of sulphasalazine investigated, only sulphapyridine was bioactivated by human liver microsomes in the presence of NADPH to a metabolite which caused marked methaemoglobinaemia and a small, but statistically significant degree of mononuclear leucocyte cell death.
- 3 Methaemoglobinaemia was inhibited by ketoconazole but not by ascorbic acid (100 μM), glutathione (500 μM) and *N*-acetylcysteine (50 μM). In contrast, ascorbic acid and the thiols afforded complete protection for mononuclear leucocytes.
- 4 Sulphapyridine (100 μM) was converted *in vitro* to a metabolite (metabolite conversion $6.8 \pm 0.3\%$), the retention time of which on h.p.l.c. corresponded to synthetic sulphapyridine hydroxylamine. The half-life of sulphapyridine hydroxylamine in phosphate buffer (pH 7.4) was found to be 8.1 min.
- 5 In the absence of microsomes and NADPH, sulphapyridine hydroxylamine caused a concentration-dependent (10–500 μM) increase in methaemoglobinaemia (2.9%–24.4%) and cytotoxicity (5.4%–51.4%), whereas sulphasalazine, sulphapyridine, 5-hydroxy sulphapyridine and 5-aminosalicylic acid had no effect.

Keywords sulphasalazine metabolism cytotoxicity methaemoglobinaemia sulphapyridine toxicity

Introduction

Sulphasalazine (SPS) is used in the treatment of rheumatoid arthritis (Pullar, 1989) where it is of comparable efficacy to other disease-modifying agents, such as sodium aurothiomalate (Pullar *et al.*, 1983) and D-penicillamine (Neumann *et al.*, 1983). It is also used for the treatment of inflammatory bowel disease (Riley & Turnberg, 1990) and is currently being investigated for its possible therapeutic potential in multiple sclerosis (Prosiegel *et al.*, 1989).

Sulphasalazine consists of two moieties: sulphapyridine (SPy) and 5-amino salicylic acid (5-ASA), which are linked by an azo bond. SPS reaches the colon unchanged, where bacteria cleave the azo link, liberating SPy which is absorbed and 5-ASA which is excreted in the faeces with minimal systemic absorption (Bondesen *et al.*, 1988; Das & Dubin, 1976; Riley & Turnberg,

1990). Subsequently, SPy undergoes metabolism through hydroxylation and acetylation, to 5-hydroxy sulphapyridine (5-OH SPy) and *N*⁴-acetyl sulphapyridine, respectively. These are predominantly excreted in the urine as their glucuronic acid conjugates (Das & Dubin, 1976).

A wide range of toxic effects has been reported with SPS including leucopenia (Amos & Bax, 1988), hepatotoxicity (Farr *et al.*, 1985), neurotoxicity (Wallace, 1970) and lung disorders (Thomas *et al.*, 1974). In addition, SPS can cause intravascular haemolysis (Das *et al.*, 1973; Mechanick, 1985) particularly in glucose-6-phosphate dehydrogenase deficient patients (Pounder *et al.*, 1975), and methaemoglobinaemia in up to 40% of patients (Pounder *et al.*, 1975).

The moiety responsible for this toxicity is thought to

be SPy as the effects associated with SPS are very similar to those caused by sulphonamides (Das *et al.*, 1973), and minimal amounts of 5-ASA are absorbed and metabolised (Bondesen *et al.*, 1988). However, it is not known whether it is SPy itself or one of its metabolites which is responsible.

Recent evidence suggests that the toxicity of sulphonamides is mediated by the *N*-hydroxy metabolite (Shear & Spielberg, 1985). Moreover, dapsone which is structurally very similar to SPy, and has a similar side-effect profile, also causes toxicity via its hydroxylamine metabolite (Coleman *et al.*, 1989; Uetrecht *et al.*, 1988).

In this study, we have used erythrocytes and leucocytes as target cells with methaemoglobin formation and cytotoxicity, respectively as the defined toxic end-points to establish the biochemical basis of SPS-mediated toxicity.

Methods

Chemicals

Sulphasalazine, sulphapyridine, 5-amino salicylic acid, glutathione, *N*-acetylcysteine, ascorbic acid and human serum albumin (HSA, fraction V) were purchased from Sigma Chemical Co. (Poole, Dorset, UK). 5-Hydroxy sulphapyridine was a gift from Pharmacia Ltd (Milton Keynes, 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).

Synthesis of sulphapyridine hydroxylamine (SPy-NOH)

Synthesis was performed using *p*-nitrobenzene sulphonyl chloride and 2-aminopyridine as starting materials (Fel'dman & Mikhailova, 1965). The resulting nitro derivative was reduced to SPy-NOH with hydrogen in the presence of a platinum catalyst (Grossman & Jollow, 1988). The resulting product was purified by flash column chromatography on silica gel (Merck 9385). The final product was a yellow solid of melting point 232–233° C; the yield was 96% SPy-NOH (as determined by h.p.l.c.). It was also fully characterised by mass spectrometry, n.m.r. and i.r. spectrometry.

Preparation of hepatic microsomes

Histologically normal livers were obtained from six kidney transplant donors (aged 8–46 years). Ethical approval was granted and informed consent obtained from donors' relatives, as previously indicated (Riley *et al.*, 1988). Livers from donors were cooled to 0° C, divided into 10–20 g portions and frozen in liquid nitrogen within 1 h of removal and stored at –80° C until required for use. Washed human liver microsomes were prepared as reported previously (Purba *et al.*, 1987). Six male CBA/ca mice (25–50 g) were administered phenobarbitone (PB) once daily for 3 days (60 mg kg⁻¹) by i.p. injections (0.5 ml) in 0.9% saline. Another group of six mice received i.p. injections (0.2 ml) of β -naphthoflavone (BNF) suspended in corn oil at a dose

of 75 mg kg⁻¹ for 3 days. After the final set of injections, the mice were fasted for 24 h and then killed by cervical dislocation. The livers were removed and pooled; the microsomes were prepared by the same centrifugation procedure used to obtain washed human liver microsomes. The protein content of the liver microsomes was measured by the method of Lowry *et al.* (1951). The microsomes were either used immediately or stored for up to 72 h at –80° C as an intact pellet.

Isolation of cells

Peripheral blood mononuclear leucocytes (MNL) were isolated from fresh heparinized venous blood from six healthy male volunteers (aged 22–36 years) as described by Riley *et al.* (1988). Their viability upon isolation as determined by trypan blue dye exclusion, was > 95%. The MNL were suspended in 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES)-buffered balanced salt medium (Riley *et al.*, 1988) to obtain 1×10^6 cells ml⁻¹.

Peripheral blood neutrophils were isolated on a discontinuous density gradient of ficoll-hypaque (Sigma Chemical Co. Ltd, Poole, Dorset, UK; Ringertz *et al.*, 1982). The band containing granulocytes was washed with phosphate-buffered saline (PBS; NaCl 8 g l⁻¹, KCl 0.2 g l⁻¹, Na₂HPO₄ 1.15 g l⁻¹, KH₂PO₄ 0.2 g l⁻¹; pH 7.2) and the contaminating erythrocytes removed by lysis with NH₄Cl (0.85% for 5 min) following a washing phase in RPMI 1640 medium. Finally, the neutrophils were resuspended in HEPES-buffered medium to obtain approximately 1×10^6 cells ml⁻¹. The procedure yielded preparations containing > 95% of neutrophils (viability > 95%).

T- and B-lymphocytes were isolated by a process of negative selection using magnetic monosized polymer microspheres (Dynabeads M-450, Dynal (UK), Ltd, Wirral, UK) coated with monoclonal antibodies specific for the B-cell CD19 and T-cell CD2 antigens, respectively (Vartdal *et al.*, 1987). The isolated MNL (Riley *et al.*, 1988) suspended at 1×10^7 cells ml⁻¹ in PBS were mixed with antibody-coated microspheres, corresponding to a microsphere : cell ratio of approximately 1 : 1, for 30 min at room temperature by tilting and rotating. After 30 min the mixture was diluted 1:5 with PBS and immediate magnetic separation carried out by placing the tube on a six-tube magnetic particle concentrator (MPC-6, Dynal (UK) Ltd, Wirral, UK) for 1 min. The microsphere-free fluid phase was aspirated into a universal container. The rosetted cells, i.e. the positively selected cells, were then resuspended in 3 ml PBS and magnetic separation repeated. The rosetted cells were discarded, while the negatively selected cell populations were resuspended in HEPES-buffered medium to obtain approximately 1×10^6 cells ml⁻¹. The cells upon isolation were of > 95% viability. The purity of the negatively selected T- and B-cell suspensions were 95% and 90%, respectively.

Determination of direct and metabolism dependent cytotoxicity

Direct cytotoxicity was determined by incubating MNL (1×10^6 cells ml⁻¹) in HEPES-buffered medium with

varying concentrations of SPS and its principal metabolites, including SPy-NOH, in polystyrene tubes at 37° C for 2 h in a shaking water bath. The drugs were added in 10 µl dimethylsulphoxide (DMSO), which, as 1% (v/v) solution, was non-toxic. The total incubation volume was 1 ml. After 2 h, the cells were sedimented and resuspended in a drug-free medium (HEPES-buffered medium containing 5 mg ml⁻¹ HSA). Incubations were continued for 16 h, following which cell viability was assessed by trypan blue exclusion (0.2% (w/v) trypan blue) as previously reported (Riley *et al.*, 1988).

Metabolism (NADPH)-dependent cytotoxicity was determined in the same manner as above, except that the 2 h incubation included murine or human hepatic microsomes (0.5 mg protein) in the presence or absence of NADPH (1 mM). Some incubations contained ascorbic acid (100 µM), *N*-acetyl cysteine (50 µM) and glutathione (500 µM) which were added to the cells 10 min prior to the addition of the drug and microsomes.

The direct cell-selective toxicity of SPy-NOH was determined in the same manner as that described for direct cytotoxicity above, except that the final incubation volume was 0.5 ml rather than 1 ml.

Determination of direct and metabolism-dependent red cell toxicity (methaemoglobinaemia)

Methaemoglobin formation by SPS and its metabolites was determined using an equilibrium dialysis (Dianorm) apparatus (Tingle *et al.*, 1990), consisting of bicompartamental cells separated from each other by a semi-permeable membrane made of cellulose with a molecular weight cut-off equivalent to 10 kDa (Diachema, Munich, FRG). To investigate the role of metabolism in methaemoglobin formation, varying concentrations (100 µM–2 mM) of the compounds in DMSO (5 µl) and human liver microsomes (liver 1; 2 mg protein) were placed in compartment A. Compartment B contained 500 µl human RBC washed three times with PBS and then resuspended to approximately 50% haematocrit. Metabolism was initiated by the addition of NADPH (2 mM; omitted from controls) to compartment A and conducted for 1 h at 37° C with constant rotation (8 rev min⁻¹). The final volume in each half-cell was 500 µl. The RBC were then expelled from compartment B, and methaemoglobin content measured by the method of Harrison & Jollow (1986). The ability of the compounds to cause methaemoglobin formation directly, i.e. in the absence of a microsomal system, was examined in a similar manner, except that the microsomes and NADPH were omitted from compartment A.

The effect of reduced glutathione, ascorbic acid, *N*-acetylcysteine and the cytochrome P450 inhibitor, ketoconazole (100 µM; Sheets & Mason, 1984), on metabolism-dependent methaemoglobinaemia was investigated as described above, except that these compounds were co-incubated with the test drug and microsomal system in compartment A.

Metabolism of sulphapyridine by human liver microsomes

Sulphapyridine (100 µM) was incubated with human liver microsomes (0.5 mg protein) and 1 × 10⁶ MNL in

HEPES-buffered medium as described above. Ascorbic acid (2 mM) was added to prevent auto-oxidation of any SPy-NOH formed to nitrososulphapyridine. The reactions were started by the addition of NADPH (omitted in control incubations, *n* = 6) followed by incubation in a shaking water bath for 2 h at 37° C. The reactions were terminated by the addition of 1 ml methanol in each tube. The tubes were left at -20° C overnight and then centrifuged (650 *g* for 10 min) to precipitate all the protein.

Aliquots (50 µl) of the methanol extract were analysed by h.p.l.c. SPy-NOH, SPy and 5-OH SPy were identified by comparison of their retention times with those of co-injected authentic compounds. Chromatography was performed on a Spectra-Physics chromatograph using a method based on that of Fischer & Klotz (1978). Separation was achieved using a µ Bondapak C₁₈ column (30 cm × 0.39 cm i.d., 10 µm, Waters Assoc., Hartford, Cheshire, UK). The mobile phase consisted of acetonitrile : 0.1 M sodium acetate buffer (12 : 88 v/v) flowing at 1.5 ml min⁻¹. Eluate was monitored at 254 nm. Under these conditions, the retention times of SPy-NOH, SPy and 5-hydroxy sulphapyridine were 4.6, 6.5 and 7.1 min, respectively.

Determination of the half-life of sulphapyridine hydroxylamine

The half-life of SPy-NOH was determined by repeated sampling over 2 h of an aqueous 100 µg ml⁻¹ solution (67 mM phosphate buffer; pH 7.4) of the metabolite which was maintained at 37° C. The samples were analysed by h.p.l.c. (using the method described above) immediately on collection. For comparison, the half-life of dapson-hydroxylamine, a structurally related compound, was determined in the same manner with the samples being analysed by the h.p.l.c. method of Uetrecht *et al.* (1988).

Statistical analysis

Statistical analysis was performed by the use of one way analysis of variance (on a MINITAB statistical software package) accepting *P* < 0.05 as significant. Data are presented as mean ± s.e. mean.

Results

Methaemoglobin formation from sulphasalazine and its stable metabolites

Metabolism (NADPH)-dependent methaemoglobin formation (with human liver microsomes) which was investigated in the two-compartment system was observed only with SPy but not with SPS, 5-ASA or 5-OH SPy (Table 1). Incubation of SPy across a concentration range (100 µM–2 mM) with microsomes (2 mg) and NADPH (2 mM) resulted in a concentration-dependent increase in methaemoglobin formation (Figure 1).

Ascorbic acid (100 µM), glutathione (500 µM) and *N*-acetyl cysteine (50 µM) did not reduce metabolism-dependent methaemoglobin formation (11.4 ± 1.3%,

Table 1 Metabolism (NADPH)-dependent methaemoglobin (MetHb) formation from sulphasalazine (2 mM) and its principal metabolites with human liver microsomes (liver 1) after co-incubation with RBC at 37° C for 1 h

Compound	-NADPH % MetHb	+NADPH % MetHb
Sulphasalazine	2.2 ± 0.4	2.0 ± 0.4
Sulphapyridine	2.4 ± 0.7*	8.6 ± 0.7*
5-hydroxy sulphapyridine	3.7 ± 0.1	3.2 ± 0.3
5-aminosalicylic acid	2.4 ± 0.3	1.8 ± 0.1

Results represent the mean ± s.e. mean with each incubation performed in triplicate. The control methaemoglobin values (in the absence of drug) were $3.4 \pm 0.4\%$, $2.7 \pm 0.8\%$, $3.9 \pm 0.3\%$ and $3.4 \pm 0.4\%$ for sulphasalazine, sulphapyridine, 5-hydroxy sulphapyridine and 5-aminosalicylic acid, respectively. Statistical analysis performed by comparing the difference between -NADPH and +NADPH incubations for the same drug: * $P < 0.005$.

Figure 1 Effect of varying concentrations of sulphapyridine on methaemoglobin formation in the absence (●) and presence (▲) of NADPH (2 mM) and human liver microsomes (liver 1). Each point represents the mean ± s.e. mean of triplicate incubations. The control methaemoglobin value (in the absence of the drug) was $2.7 \pm 0.8\%$. Significant differences between -NADPH and +NADPH incubations: * $P < 0.05$, ** $P < 0.005$. **Figure 2** The ability of varying concentrations of sulphasalazine (+), sulphapyridine (■), 5-hydroxy sulphapyridine (*), 5-aminosalicylic acid (∇) and sulphapyridine hydroxylamine (□) to cause methaemoglobinaemia in the absence of a microsomal system. Each point represents the mean of triplicate determinations (error bars omitted for clarity), except for sulphapyridine hydroxylamine where each point represents the mean ± s.e. mean of two separate experiments (performed in triplicate).

$10.7 \pm 0.9\%$, and $14.5 \pm 3.6\%$, respectively) compared with control, i.e. in the presence of NADPH (2 mM) only, ($10.4 \pm 0.7\%$). In contrast, ketoconazole ($100 \mu\text{M}$) significantly reduced methaemoglobin formation ($3.8 \pm 0.2\%$; $P < 0.001$).

In the absence of a metabolising system, only SPy-NOH caused a concentration-dependent increase in methaemoglobin formation; the other compounds did not show any detectable red cell toxicity (Figure 2).

To investigate the stability of SPy-NOH, the compound was incubated with erythrocytes (in the absence of microsomes) in 10 ml polystyrene tubes (total volume: $500 \mu\text{l}$) for 1 h at 37° C in a shaking water bath. This resulted in significantly higher methaemoglobin levels when directly compared with incubations carried out in the two-compartment system ($P < 0.001$ at all concentrations; Figure 3).

Bioactivation of sulphasalazine and its stable metabolites to cytotoxic products

Incubation of SPy with MNL at a concentration of $100 \mu\text{M}$ in the presence of human liver microsomes and NADPH resulted in a small, but significant degree of bioactivation ($P < 0.005$) to a cytotoxic metabolite, while SPS, 5-OH SPy and 5-ASA did not undergo activation to cytotoxic metabolites (Table 2).

There was a significant ($P < 0.05$) bioactivation of SPy ($100 \mu\text{M}$) by four of the six different liver samples tested (data not shown). Overall, the difference between the mean data for these 6 samples just reached statistical significance (Table 3).

In contrast to metabolism-dependent methaemoglobinaemia, increasing the concentration of SPy ($100 \mu\text{M}$ – 2 mM) did not increase cytotoxicity (data not shown). NADPH-dependent cytotoxicity of SPy was greatest with phenobarbitone-induced mouse microsomes, whereas β -naphthoflavone induced mouse microsomes did not bioactivate SPy to a significant degree (Table 3).

Metabolism-dependent cytotoxicity of SPy with human liver microsomes (liver 1) was significantly reduced by the addition of ascorbic acid, glutathione and *N*-acetyl cysteine (at the same concentrations used for

Figure 3 A comparison of the ability of sulphapyridine hydroxylamine to cause methaemoglobinaemia in the bicompartamental system (▼) and in 10 ml polystyrene tubes (△). Each point represents the mean ± s.e. mean of triplicate (bicompartamental system) or quadruplicate (polystyrene tubes) determinations.

Table 2 Metabolism (NADPH)-dependent cytotoxicity of sulphasalazine (100 μM) and its metabolites after co-incubation of mononuclear leucocytes with human liver microsomes (liver 1) for 16 h at 37° C

Compound	–NADPH % cell death	+NADPH % cell death
Sulphasalazine	6.6 $\pm$ 0.4	6.3 $\pm$ 0.4
Sulphapyridine	5.0 $\pm$ 0.5*	8.3 $\pm$ 0.7*
5-hydroxy sulphapyridine	6.4 $\pm$ 0.6	8.1 $\pm$ 0.5
5-aminosalicylic acid	8.0 $\pm$ 0.5	7.9 $\pm$ 0.3

Results presented as mean $\pm$ s.e. mean.

The results for sulphapyridine represent the results of six separate experiments (performed in quadruplicate). The results for the other compounds represent the results of four separate experiments (performed in quadruplicate). The control cell death values (in the absence of drug) were 7.0 $\pm$ 1.0%, 4.6 $\pm$ 0.3%, 7.0 $\pm$ 1.0% and 8.7 $\pm$ 1.2% for sulphasalazine, sulphapyridine, 5-hydroxy sulphapyridine and 5-amino salicylic acid, respectively. Statistical analysis performed by comparing the difference between between –NADPH and +NADPH incubations for the same drug: * P < 0.005.

Table 3 Variability in the bioactivation of sulphapyridine (100 μM) to a cytotoxic metabolite in the presence of human and mouse liver microsomes

Incubation microsomes	–NADPH %	+NADPH %	P value (ANOVA)
Human microsomes	3.2 $\pm$ 0.8	6.6 $\pm$ 1.3	0.05
Phenobarbitone- induced mouse microsomes	5.0 $\pm$ 0.8	12.1 $\pm$ 0.4	< 0.001
β -naphthoflavone- induced mouse microsomes	3.7 $\pm$ 0.4	4.5 $\pm$ 0.6	NS

For human liver microsomes, the results represent the mean $\pm$ s.e. mean for six human livers (incubations in quadruplicate for each human liver). For mouse liver microsomes, the results represent the mean $\pm$ s.e. mean for quadruplicate incubations with pooled microsomes. The control cell death values (in the absence of drug) were 3.3 $\pm$ 0.6% (human liver microsomes), 5.3 $\pm$ 0.7% (phenobarbitone-induced mouse microsomes) and 2.5 $\pm$ 0.3% (β -naphthoflavone induced mouse microsomes). NS-not significant.

the methaemoglobin assay; Figure 4). The concentrations chosen have been shown to reduce the metabolism-dependent cytotoxicity of dapsone (Coleman *et al.*, 1989) and carbamazepine (unpublished data).

Direct cytotoxicity of sulphasalazine and its stable metabolites

Incubation of SPy-NOH with MNL in the absence of a metabolising system resulted in a concentration-dependent (10 μM –500 μM) increase in cytotoxicity (LC_{50} value of 475 μM), while SPS, SPy, 5-OH SPy and 5-ASA did not significantly increase the cytotoxicity above background values (Figure 5).

Sulphapyridine hydroxylamine also produced a concentration-dependent increase in cytotoxicity of separated T- and B-lymphocyte subpopulations; however, there was no significant difference in sensitivity between the two cell types (Figure 6). In contrast,

Figure 4 Effect of various compounds on metabolism (NADPH)-dependent bioactivation of sulphapyridine (100 μM) to a cytotoxic metabolite in the presence of human liver microsomes (liver 1). Results represent the mean $\pm$ s.e. mean of quadruplicate incubations. The control cell death (in the absence of drug) was 3.2 $\pm$ 0.3%. Asc: ascorbic acid (100 μM); GSH: glutathione (500 μM); NAC: *N*-acetyl cysteine (50 μM); □ –NADPH, ▨ +NADPH. Significant difference from incubations containing NADPH (1 mM) only: * P < 0.05, ** P < 0.005.

Figure 5 Direct cytotoxicity of sulphasalazine (+), sulphapyridine (■), 5-hydroxy sulphapyridine (*), 5-amino-salicylic acid (▽) and sulphapyridine hydroxylamine (□) after incubation with mononuclear leucocytes for 16 h at 37° C. Each point represents the mean of quadruplicate determinations (error bars omitted for clarity), except for sulphapyridine hydroxylamine where each point represents the mean $\pm$ s.e. mean of four separate experiments (performed in quadruplicate).

Figure 6 Cell selective cytotoxicity of varying concentrations of sulphapyridine hydroxylamine after incubation with T-lymphocytes, B-lymphocytes and neutrophils. Each point represents the mean $\pm$ s.e. the mean of quadruplicate incubations.

neutrophils were relatively insensitive to SPy-NOH, with cell death not exceeding background values even at the highest concentration (500 μM) tested ($7.8 \pm 1.5\%$ vs $6.3 \pm 1.8\%$; Figure 6).

Metabolism of sulphapyridine by human liver microsomes to sulphapyridine hydroxylamine

Chromatographic analysis of the human liver microsomal incubations containing SPy (100 μM) and NADPH (1 mM) in the presence of ascorbic acid (2 mM) revealed that $6.8 \pm 0.3\%$ ($n = 6$ microsomal incubations) of SPy was converted to a metabolite, the retention time (4.6 min) of which corresponded to synthetic SPy-NOH. In additional experiments, the half-lives of SPy-NOH and dapsone hydroxylamine were found to be 8.1 min and 37.0 min, respectively.

Discussion

The adverse effects associated with chronic SPS therapy can be divided into two types: dose-dependent and dose-independent (Riley & Turnberg, 1990). Apart from nausea and vomiting which have been associated with high circulating SPy levels (Das *et al.*, 1973), none of the other adverse effects (dose-dependent and -independent) has been consistently correlated with circulating levels of SPS or any of its metabolites.

Sulphasalazine consists of two moieties: SPy and 5-ASA. Based on clinical impressions, it is thought that the sulphonamide moiety is responsible for the side effects associated with SPS (Pullar, 1989; Riley & Turnberg, 1990). Recently, this view has been supported by a clinical study which showed that patients who had previously experienced severe side-effects or allergic reactions with SPS were able to tolerate 5-ASA (Mulder *et al.*, 1988).

In the present study, in order to investigate which component of SPS is responsible for the reported toxicity, we have used human cells as targets to assess the toxicity of sulphasalazine and its metabolites in the presence of human liver microsomes. Methaemoglobin formation and cytotoxicity have been used as the toxic end-points to provide indirect markers for the *in situ* formation of reactive metabolites. The results indicate that only SPy was bioactivated by NADPH-dependent human liver microsomes to a metabolite which caused both methaemoglobinaemia and cytotoxicity (Tables 1 and 2). Chromatographic analysis of the microsomal incubation supernatant showed that SPy was converted to a metabolite, the retention time of which corresponded to synthetic SPy-NOH. Moreover, the cellular toxicity of this metabolite was confirmed by using synthetic SPy-NOH which caused a concentration dependent increase in methaemoglobin formation (Figure 2) and cytotoxicity (Figure 5). This metabolite, to the best of our knowledge, has not previously been identified in either *in vivo* or *in vitro* studies involving SPS. It is highly likely that this metabolite (or its further oxidation product, the nitroso compound) is responsible for many of the adverse effects associated with SPS. This is in agreement with the proposed mechanisms of toxicity of other sulphona-

mides (Reider *et al.*, 1989; Shear & Spielberg, 1985), dapsone (Coleman *et al.*, 1989; Uetrecht *et al.*, 1988) and procainamide (Uetrecht, 1985); the hydroxylamines of the latter two compounds have, as with SPy-NOH in the present study, been shown to cause cytotoxicity (Coleman *et al.*, 1989; Rubin *et al.*, 1987) and methaemoglobin formation (Roberts *et al.*, 1989; Tingle *et al.*, 1990).

The metabolism-dependent cytotoxicity observed with SPy in the presence of human liver microsomes is low (Table 2); this is partly due to the low *in vitro* conversion (6.8%) of SPy to the hydroxylamine, and thus, the use of activating microsomes with an increased cytochrome P450 content, i.e. phenobarbitone-induced mouse microsomes, results in increased cytotoxicity (Table 3). However, this cannot be the full explanation as studies with the structurally related compound dapsone (Coleman *et al.*, 1989; Tingle *et al.*, 1990), have shown that it causes a greater degree of metabolism-dependent cytotoxicity (and methaemoglobinaemia) than SPy, despite an *in vitro* metabolic conversion to the reactive metabolite which is equivalent to that of SPy. This apparent discrepancy can be accounted for by the much shorter half-life of SPy-NOH (8 min) compared with that of DDS-NOH (37 min). The functional significance of the instability of SPy-NOH is further illustrated in Figure 3, which shows that the ability of SPy-NOH to cause methaemoglobinaemia is significantly greater when the metabolite and red blood cell are in direct contact (20% methaemoglobinaemia at 22 μM) than when they are initially separated by a semi-permeable membrane in the bicompartamental system (20% methaemoglobinaemia at 384 μM). Thus, with MNL, it is possible that the majority of the hydroxylamine of SPy formed by microsomes oxidises to a stable non-toxic product before it reaches the cell. In addition, co-incubation of MNL with reducing agents such as ascorbic acid, glutathione and *N*-acetyl cysteine reduces cytotoxicity (Figure 4) by preventing the auto-oxidation of the hydroxylamine to the ultimately toxic nitroso species. This effect of the reducing agents is not seen with erythrocytes, however, because the hydroxylamine, as with other toxic arylamines (Eyer, 1983, 1988; Kiese, 1966), is readily taken up by the red blood cell to undergo rapid redox cycling to the nitroso species and produce methaemoglobin.

How does the *in vitro* toxicity demonstrated in this study relate to the side effects seen with SPS in patients? While the conversion of SPy to the hydroxylamine *in vitro* is low, the amount of SPy-NOH formed *in vivo* is unknown. It is unlikely that the hydroxylamine would be detectable in the circulation because of its instability and rapid uptake by the red blood cells, but its formation could be estimated by the measurement of stable conjugates in the urine. The ability of SPS to cause adverse effects may in part be a reflection of the high doses (up to 3 g day⁻¹) of the drug which have to be given in order to achieve therapeutic concentrations of SPy (Pullar *et al.*, 1985); this may result in high local concentrations of the hydroxylamine. Thus, red cell toxicity (Pounder *et al.*, 1975) and possibly leucopenia (Farr *et al.*, 1989) have been shown to be dependent on the dose of SPS. In addition, individual susceptibility to the adverse effects of SPS may be due to inadequate detoxification

of its reactive metabolite. Indeed, it has been suggested that a heritable deficiency in cellular detoxification may be responsible for predisposing individuals to sulphonamide hypersensitivity and that such patients could be identified by *in vitro* challenge of their lymphocytes to the reactive metabolite (Reider *et al.*, 1989).

Sulphasalazine causes neutropenia in approximately 1% of patients (Pullar, 1989). Our data suggest that this is unlikely to be due to direct toxicity of the SPy-NOH as the peripheral blood neutrophil was relatively insensitive to this compound *in vitro*. The reason for the insensitivity of the neutrophils is unclear, but could be related to better self-defence mechanisms when compared with non-phagocytic white blood cells. Immune-mediated destruction of neutrophils might be the possible mechanism; in fact, formation of leukoagglutinins has been demonstrated in patients treated with SPS (Evans & Ford, 1958). It is known that activated neutrophils, because of their content of myeloperoxidase and ability to generate oxygen radicals, can activate drugs such as dapsone (Uetrecht *et al.*, 1988) and procainamide (Uetrecht, 1989) to highly reactive metabolites, which by adsorption on to the cell surface could lead to neo-antigen formation and thus, an immune response.

The other component of SPS, 5-ASA is present in low

concentrations in the blood (Riley & Turnberg, 1990). Clinically, it has not been considered to be the toxic moiety of SPS. In our *in vitro* study, 5-ASA did not show either direct or metabolism-dependent cellular toxicity in keeping with the clinical impression. However, more recently, with the increasing use of 5-ASA derivatives in inflammatory bowel disease (Riley & Turnberg, 1990), a small number of patients have been reported to have suffered adverse effects. These include peripheral neuropathy (Woodward, 1989) and pancreatitis (Deprez *et al.*, 1989). The mechanism of these side effects is unknown.

In summary, the results of this *in vitro* study suggest that *N*-oxidation products of sulphapyridine may play a role in the haematological toxicity which has been reported with sulphasalazine. It is therefore important to determine the role of such metabolites in the *in vivo* toxicity of sulphasalazine, and define factors which may influence *N*-hydroxylation, and the further metabolism of sulphapyridine hydroxylamine.

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