The Relationship Between the Disposition and Immunogenicity of Sulfamethoxazole in the Rat

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ABSTRACT

Idiosyncratic toxicity associated with sulfamethoxazole (SMX) is thought to be a consequence of bioactivation to the hydroxylamine metabolite (SMX-NOH) and further oxidation to the ultimate reactive metabolite, nitroso-sulfamethoxazole (SMX-NO). To establish the link between the formation of the ultimate reactive metabolite and SMX hypersensitivity, we have undertaken metabolism and immunogenicity studies in the rat by use of SMX and its metabolites. SMX was excreted in urine as N₂-acetyl SMX and SMX-NOH, with ~10% remaining unchanged as parent amine. After administration of SMX-NOH (54 mg kg⁻¹) and SMX-NO (10 mg kg⁻¹), 38.3% and 46.1% of the doses, respectively, were excreted in urine as SMX and N₂-acetyl SMX, which indicated extensive reduction of these metabolites in vivo. The immunogenic potential of SMX and its metabolites, SMX-NOH and SMX-NO, were assessed in rats by analyzing serum samples for the presence of anti-SMX IgG antibodies during a 4-week dosing period. No antibodies to SMX were detected in either control or SMX-treated rats. In contrast, a high titer of SMX-specific IgG antibody was present in sera from all the rats administered SMX-NO, reaching a maximum 14 to 21 days after the initial dose. Rats administered SMX-NOH only produced a weak IgG response after 3 weeks of dosing. These findings indicate that SMX-NO is highly immunogenic and may be responsible for the hypersensitivity reactions associated with SMX. Both SMX-NOH and SMX-NO undergo extensive reduction in vivo which may afford protection against SMX toxicity.

ABSTRACT

Sulfonamide antimicrobial agents have been associated with ADR since their introduction in the 1930s. A variety of idiosyncratic reactions including fever, rash, hepatitis and blood dyscrasias have been reported in up to 8% of patients (Jick, 1982; Lawson and Paice, 1982). In recent years, cotrimoxazole (SMX in combination with trimethoprim) has been found to be the most effective agent for the treatment of PCP in HIV-infected patients. However, its use in HIV-positive patients has been hampered by a much higher incidence of hypersensitivity reactions, ranging from 30 to 80% in different studies (Carr and Cooper, 1995; Pirmohamed and Park, 1995). The frequency of hypersensitivity is lower when the drug is used for prophylaxis when compared with its use for the treatment of PCP. The clinical spectrum of reactions reported in HIV-positive patients is similar to that seen in HIV-negative patients, although in general, the reactions tend to be more severe. The reasons for the higher frequency of hypersensitivity reactions in HIV-positive patients is unclear, but is likely to be caused by multiple factors including drug dosage, drug-drug interactions, virus-induced changes in drug metabolism and drug detoxication and immune dysregulation (Carr and Cooper, 1995; Pirmohamed and Park, 1995).

The development of strategies to prevent hypersensitivity reactions to SMX requires elucidation of the mechanism of sulfonamide toxicity which is presently poorly understood. It has been postulated that bioactivation of the parent drug to a chemically reactive intermediate is an important step in the pathogenesis of toxicity (Carr et al., 1993). Several in vitro studies have demonstrated metabolism-dependent activation of SMX to cytotoxic and protein-reactive metabolites (Rieder et al., 1988, 1995a; Riley et al., 1991, Carr et al., 1993). In these studies SMX-NOH was identified as the chemically reactive and potentially toxic species, although its further oxidation product, SMX-NO, has more recently been postulated to be the ultimate toxic species (Rieder et al., 1995b; Cribb et al., 1991).

The mechanism by which either the hydroxylamine or nitroso metabolites of SMX lead to toxicity is not known, al-

ABBREVIATIONS: ADR, adverse drug reactions; SMX, sulfamethoxazole; SMX-NOH, sulfamethoxazole hydroxylamine; SMX-NO, nitroso sulfamethoxazole; CYP, cytochrome P450; GSH, reduced glutathione; DMSO, dimethyl sulfoxide; IC₅₀, concentration producing 50% inhibition; PBS, phosphate-buffered saline; PCP, Pneumocystis carinii pneumonia; NAT, N-acetyltransferase.
though both direct and immune-mediated forms of toxicity have been implicated (Rieder et al., 1988, 1989, 1995b; Carr et al., 1993; Meekins et al., 1994; Cribb et al., 1996). The most widely accepted view is that generation of the reactive metabolite is followed by covalent binding to target proteins leading to the formation of immunogenic adducts, which ultimately produce the toxicity. However, evidence supporting this view remains inconclusive, and the causal link between the ultimate reactive metabolite and SMX hypersensitivity has yet to be established.

The aim of this study was to investigate the relationship between the metabolism and immunogenicity of SMX in vivo using the rat as a model for man. First, the metabolism of SMX and the fate of its potentially reactive metabolites was examined in vivo with particular emphasis placed on the identification of detoxification products of SMX-NOH and SMX-NO. Second, the immunogenic potential of SMX, SMX-NOH and SMX-NO was assessed in vivo.

Materials and Methods

Chemicals. SMX, urethane, DMSO, ascorbic acid, Tween 20 and o-phenylenediamine hydrochloride were obtained from Sigma Chemicals (Poole, UK). Sodium pentobarbitone was purchased from Rhone Merieux Limited (Essex, UK). SMX-NOH and SMX-NO were prepared by the method of Naisbitt et al. (1996) and were >95% pure as assessed by NMR and elemental analysis. Previous studies have shown that certain nitro compounds are capable of dimerization (Sorriso, 1982). However, this would not appear to be the case for nitroso-sulfamethoxazole based on both the analytical studies (NMR, elemental analysis and mass spectrometry) and the fact the compound synthesized can form stable conjugates with GSH in a 1:1 ratio (Naisbitt et al., 1996). N4-acetyl SMX was prepared by a standard synthesis for acetylated compounds with 2 equivalents of acetic anhydride under reflux. The remaining metabolite standards were gifts from Dr. A.J.A.M. van der Ven (University Hospital St Ramoud, Nijmegen, Netherlands). All HPLC-grade solvents were purchased from Fisher Scientific (Loughborough, UK).

Rabbit anti-SMX IgG antisera (used as positive control) was kindly donated by Dr. A.E. Cribb (Merek Research Laboratories, West Point, PA), and the SMX-HSA adduct was prepared by the method of Cribb et al. (1996). Peroxidase-linked anti-rat and anti-rabbit IgG antibodies were obtained from Sigma Immunochemicals (Poole, UK).

Metabolism of sulfamethoxazole and its metabolites in rats. Male Wistar rats (200–250 g) were anesthetized with urethane (1.4 g ml⁻¹ in isotonic saline, 1.0 ml kg⁻¹ i.p.) and cannulae were inserted into the jugular vein and common bile duct. SMX (50 or 250 mg kg⁻¹), SMX-NOH (54 mg kg⁻¹) and SMX-NO (54 mg kg⁻¹) in DMSO were administered intravenously. Bile was collected for 5 h. In separate experiments, rats were housed in metabolism cages, and SMX (10, 50 or 250 mg kg⁻¹), SMX-NOH (54 mg kg⁻¹) and SMX-NO (10 or 54 mg kg⁻¹) were administered i.p.; the urine was collected for 24 h. Ascorbic acid (25 mg) was added to each urine collecting vessel. Samples of bile and urine were analyzed by electrospray LC-MS as described previously (Gill et al., 1996). Identification of metabolites was performed by co-chromatography with authentic standards. SMX, SMX-NOH, N4-acetyl SMX, SMX N4-glucuronide and the internal standard, sulfadoxine, were assayed by monitoring their ratio (Naisbitt et al., 1996). N4-acetyl SMX was prepared by a standard synthesis for acetylated compounds with 2 equivalents of acetic anhydride under reflux. The remaining metabolite standards were gifts from Dr. A.J.A.M. van der Ven (University Hospital St Ramoud, Nijmegen, Netherlands). All HPLC-grade solvents were purchased from Fisher Scientific (Loughborough, UK).

Results

Biliary excretion of sulfamethoxazole and its metabolites. Biliary excretion after i.v. administration of either SMX, SMX-NOH or SMX-NO in rats was low with less than 5% of each of the compounds being excreted in 5 h (table 1).

![Fig. 1](https://jpet.aspetjournals.org)
No glutathione (GSH) conjugates or further metabolites of GSH conjugates, which we have synthesized previously (Naisbitt et al., 1996), were detected in the bile after administration of any of the compounds.

**Urinary metabolites of sulfamethoxazole.** There was extensive urinary excretion of SMX and its metabolites with ~10% of the dose being eliminated unchanged at all dose levels (table 2). After i.p. administration of SMX (10, 50 and 250 mg kg⁻¹), 61.3 ± 7.2%, 64.7 ± 7.1% and 40.7 ± 5.9% of the dose, respectively, was excreted by this route. Even though acetylation was the predominant route of metabolism at all dose levels, recovery of the N₄-acetyl SMX at the higher dose (250 mg kg⁻¹) was significantly lower than both the 10 mg kg⁻¹ (P < .05) and 50 mg kg⁻¹ doses (P < .01). Acetyl-sulfonamides are well known for their insolubility in water (Dorfman and Smith, 1970) and it is possible that, at these high concentrations, precipitation of the metabolite may have occurred within the kidney. The proportion of SMX excreted as the hydroxylamine was equivalent to that seen in man (Gill et al., 1996). SMX-N₁-glucuronide represented less than 0.5% of the dose in all groups of animals, and no 5-hydroxylated metabolites were detected in the urine.

**Urinary metabolites of sulfamethoxazole hydroxylamine.** In rats administered SMX-NOH (54 mg kg⁻¹), 64.3 ± 11.9% of the dose was recovered in urine. SMX-NOH underwent extensive reduction with only 25.9 ± 8.1% of the dose being excreted unchanged. Reduction to the parent amine and further metabolism to N₄-acetyl SMX accounted for 15.4 ± 3.3% and 22.9 ± 3.2% of the dose, respectively.

**Urinary metabolites of nitroso-sulfamethoxazole.** SMX-NO underwent extensive reduction after i.p. administration, with 59.6 ± 16.2% (10 mg kg⁻¹) and 39.9 ± 3.8% (54 mg kg⁻¹) being excreted as products of reduction. At the 10 mg kg⁻¹ dose, 18.4 ± 4.6%, 27.6 ± 6.7% and 13.6 ± 5.5% were excreted as the parent amine, N₄-acetyl SMX and the hydroxylamine respectively. This compared with 4.9 ± 0.6%, 24.8 ± 6.8% and 10.1 ± 4.5%, respectively, at the higher dose of 54 mg kg⁻¹. A lower recovery was evident at the higher dose of SMX-NO (P < .05), which may be attributed to saturation of the reductive mechanisms involved in its detoxification.

**Detection of antisulfamethoxazole antibodies.** All rats dosed with SMX-NOH displayed high titers of anti-SMX IgG antibodies (fig. 2). These titers remained elevated throughout the dosing schedule and were still present in the serum 1 week after dosing had terminated. Maximum titers were detected 14 to 21 days after the initial dose. Although rats given SMX-NOH failed to produce a response initially, during the last week of the dosing schedule, two of the rats displayed weak IgG responses (fig. 3). These were shown to be drug-specific by hapten inhibition with SMX. No anti-SMX antibodies were detected in any of the rats administered SMX alone or the vehicle control (DMSO).

The specificity of the antibodies was determined by using hapten inhibition (fig. 4). The antibodies produced after dosing with SMX-NOH were specific for the SMX structure. Sulfamerazine, sulfanilamide and sulfaguanidine did not inhibit the rat IgG antibody, whereas sulfisoxazole only inhibited at high concentrations (IC₅₀ >1000 µg/ml). In contrast, SMX, SMX-NOH and SMX-NO strongly inhibited IgG binding with IC₅₀ values of 8.4 µg/ml, 1.1 µg/ml and 0.5 µg/ml, respectively.

**Discussion**

Bioactivation of SMX initially to the hydroxylamine (Rieder et al., 1988, 1995a; Riley et al., 1991, Carr et al., 1993) and subsequently to the nitroso metabolite (Rieder et al., 1995b) has been postulated to be the mechanism by which SMX causes hypersensitivity reactions. To test this hypothesis, we have investigated the relationship between the metabolism of SMX to its chemically reactive metabolites, SMX-

**TABLE 1**

<table>
<thead>
<tr>
<th>Administered Dose Present As</th>
<th>SMX</th>
<th>N₄-Acetyl SMX</th>
<th>SMX-NOH</th>
<th>SMX N₁-Glucuronide</th>
<th>Glutathione Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>SMX (50 mg kg⁻¹)</td>
<td>1.2 ± 0.3</td>
<td>0.5 ± 0.2</td>
<td>n.d.²</td>
<td>1.4 ± 0.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>SMX (250 mg kg⁻¹)</td>
<td>1.3 ± 0.4</td>
<td>0.4 ± 0.1</td>
<td>n.d.²</td>
<td>1.3 ± 0.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>SMX-NOH (54 mg kg⁻¹)</td>
<td>0.8 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>0.7 ± 0.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>SMX-NO (54 mg kg⁻¹)</td>
<td>0.8 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>n.d.²</td>
<td>0.8 ± 0.3</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* Anesthetized and cannulated rats were dosed intravenously. Bile was collected for 5 h and analyzed by LC-MS without prior treatment. Values are mean ± SD from four animals. 

**: Not detected.

**TABLE 2**

<table>
<thead>
<tr>
<th>Administered Dose Present As</th>
<th>SMX</th>
<th>N₄-Acetyl SMX</th>
<th>SMX-NOH</th>
<th>SMX N₁-Glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>SMX (10 mg kg⁻¹)</td>
<td>11.5 ± 0.2</td>
<td>48.1 ± 7.0</td>
<td>1.8 ± 0.7</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>SMX (50 mg kg⁻¹)</td>
<td>12.6 ± 2.2</td>
<td>49.5 ± 6.0</td>
<td>2.4 ± 0.9</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>SMX (250 mg kg⁻¹)</td>
<td>13.5 ± 1.5</td>
<td>26.0 ± 5.8*</td>
<td>1.0 ± 0.6</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

* Animals were administered drug intraperitoneally. Urine was collected for 24 h and analyzed by LC-MS without prior treatment. Values are mean ± SD from four animals. Statistical analysis was performed by comparing the urinary excretion of the different metabolites at the different doses by the Mann-Whitney test; * P < .05 (comparison of 250 mg kg⁻¹ against the 10 mg kg⁻¹ and 50 mg kg⁻¹ doses).
NOH and SMX-NO, and their immunogenicity in a rat model.

The metabolic profile of SMX in rats was similar to what has been reported previously in man (Vree et al., 1995; Gill et al., 1996), with more than 60% of the administered dose being excreted in the urine over 24 h. Only minor differences were noted between man and rats; these were the lack of 5-hydroxylation and decreased glucuronidation in the rat. The degree of bioactivation, as assessed by the urinary excretion of SMX-NOH (1–2%), was similar in the two species. The rat thus appears to be a good model for the pattern of metabolism seen in man in vivo, especially with respect to detoxification and oxidative bioactivation.

N4-Acetyl SMX was the major urinary metabolite in both rats and man (Vree et al., 1995; Gill et al., 1996) accounting for up to 50% of the dose, which indicates that acetylation plays a key role in the detoxication and elimination of SMX. In a clinical context, it is interesting to note that the slow acetylator phenotype and genotype has been considered to be a risk factor for sulfonamide hypersensitivity in both HIV-negative and HIV-positive patients (Shear et al., 1986; Rieder et al., 1991; Lee et al., 1993; Wolkenstein et al., 1995). This is rather surprising given that NAT-1, and not the polymorphically expressed NAT-2, is the predominant enzyme involved in N4-acetyl SMX formation (Cribb et al., 1993). A more recent study, however, has suggested that NAT-2 may protect against SMX hypersensitivity by preventing the conversion of the proximate toxin, SMX-NOH, to the ultimate toxin, SMX-NO (Cribb et al., 1996).

In previous studies of drugs which undergo bioactivation in vivo such as clozapine (Maggs et al., 1995), amodiaquine (Jewell et al., 1995) and carbamazepine (Madden et al., 1996), bile has been identified as the major route of excretion for thioether conjugates formed from the respective chemically reactive metabolites of the drugs. In the present study, however, the bile was only a minor route of excretion for the metabolites of SMX, SMX-NOH and SMX-NO. No GSH conjugates or further rearrangement products of such conjuga-

Fig. 2. Anti-SMX IgG antibody titers in rats (n = 4) given SMX-NO (10 mg·kg⁻¹) as per the protocol illustrated in figure 1.

Fig. 3. Anti-SMX IgG antibody titers in rats (n = 4) given SMX-NOH (10 mg·kg⁻¹) as per the protocol illustrated in figure 1.
gates, which we previously synthesized (Naisbitt et al., 1996), were present. Thus, it was not possible to quantify the extent of bioactivation in vivo by measurement of products of bioinactivation.

A notable finding in our study was that direct administration of the oxidative metabolites (SMX-NOH and SMX-NO) resulted in extensive reduction to the parent compound and the inactive acetate. This was seen with both metabolites, although with SMX-NO, 10% of the dose was also reduced to the hydroxylamine. Reduction of SMX-NOH to the parent drug has been reported previously with human liver microsomes (Cribb et al., 1995). Thus, with in vivo administration as in this study, the liver may be the site of primary reduction. However, preliminary studies from our laboratory suggest red blood cells may also play a critical role in reducing the hydroxylamine back to the parent drug (Gill, H.J., unpublished data). Furthermore, rats dosed with SMX display a much higher degree of acetylation than the rats dosed with SMX-NOH or SMX-NO. Reduction of the hydroxylamine or nitroso to SMX must occur before acetylation, and therefore, SMX is more likely to escape acetylation by the liver if reduction takes place extrahepatically.

Two mechanisms have been implicated for the reduction (fig. 5). First, in the presence of GSH, SMX-NO can be reduced to the parent amine and hydroxylamine via the formation of an unstable semimercaptal conjugate (Cribb et al., 1991; Ellis et al., 1992; Naisbitt et al., 1996). This may be of significance in vivo because it may also provide a mechanism for the detoxification of SMX-NO. Further evidence for this is provided by in vitro cytotoxicity assays which show that both N-acetylcysteine and GSH markedly attenuate the cytotoxic effects of SMX-NOH (Rieder et al., 1988, 1995a; Carr et al., 1993). A deficiency of GSH may thus explain the higher incidence of hypersensitivity reactions in patients with AIDS (van der Ven et al., 1991). However, whether or not there is a deficiency of GSH in HIV-positive patients is controversial (Aukrust et al., 1995; Pirmohamed et al., 1996). Second, microsomal studies have suggested that reduction of the hydroxylamine can be catalyzed by two separate enzyme systems (Cribb et al., 1995), one involving cytochrome P450 (NADPH-dependent) and the other involving an NADH-dependent reductase system. Alterations in the activity of drug-metabolizing enzymes has been observed in patients with AIDS (Lee et al., 1993), although whether this is responsible for the increased susceptibility of patients with HIV requires further study. Irrespective of the principal reaction that is responsible for the reduction in vivo, it is evident that there are defense mechanisms against the potential toxicity of SMX-NOH and SMX-NO. The balance between bioactivation and reduction may thus be an important determinant of individual susceptibility to SMX hypersensitivity. After SMX administration, about 1 to 2% of the dose is excreted as the hydroxylamine (Gill et al., 1996). Given the extensive reduction observed in this study, it is likely that bodily tissues are

Fig. 4. Hapten inhibition of serum anti-SMX IgG antibody from rats administered SMX-NO (10 mg·kg⁻¹) by structurally related compounds illustrated at the bottom of the figure.

Fig. 5. Postulated mechanisms for the detoxification of SMX-NOH and SMX-NO.
exposed to much higher levels of the hydroxylamine than would be anticipated from the urinary metabolite profile.

Drug bioactivation to chemically reactive intermediates is widely postulated as being a prerequisite for many forms of idiosyncratic toxicity. In general, the reactive intermediate may cause toxicity either directly by interfering with essential cellular processes, or by acting as a hapten to initiate an immune-mediated reaction. With SMX, although bioactivation to the hydroxylamine and nitroso metabolites occurs, the mechanism by which toxicity ensues is unclear. Both direct and immune-mediated forms of toxicity have been postulated (Rieder et al., 1988, 1989, 1995b; Carr et al., 1993; Meekins et al., 1994; Cribb et al., 1996). We therefore investigated the immunogenic potential of SMX and its metabolites, SMX-NOH and SMX-NO, by chronic administration of pharmacologically relevant doses without administration of an immunological stimulant such as Freund’s adjuvant.

Administration of SMX itself to the rat did not result in the production of anti-SMX IgG antibodies, even when given at doses in excess of the maximum therapeutic dose. This finding is consistent with the fact that hypersensitivity reactions associated with sulfamethoxazole are idiosyncratic, and that most patients administered the drug display a lack of general immunogenicity even when given large doses. This study demonstrates that the rat is able to efficiently detoxify sulfamethoxazole and so prevent sufficient levels of nitroso being formed to generate an immune response. In contrast, however, high titers of anti-SMX IgG antibodies were present in the serum of the rats administered SMX-NO. This provides direct evidence that SMX-NO is immunogenic in the rat in vivo. The antibody titers in the animals reached a maximum on the third week of dosing, and were still high after dosing was terminated. Administration of SMX-NOH to rats did not result in an antibody response until the last week of dosing when two of the two rats were found to have weak IgG responses. It is possible that in these two rats, chronic dosing may have led to the conversion of SMX-NOH to SMX-NO such that the threshold required to initiate an immune response may have been achieved. Hapten inhibition experiments with a range of structurally similar sulfonylaminines showed that the anti-SMX IgG antibody was highly specific for the sulfamethoxazole structure. The highest degree of inhibition of binding was observed when the antibody was preincubated with SMX-NO, which provides further evidence for the role of this metabolite in the immune response.

Evidence for the immunological nature of SMX has been largely based on the clinical symptoms of hypersensitivity. However, more recently, anti-SMX antibodies have been detected in HIV-positive patients with a history of SMX hypersensitivity (Daftarian et al., 1995). In addition, a specific T-cell response to SMX has been detected in patients who experience hypersensitivity reactions to this drug, with both CD4+ and CD8+ cells being activated (Mauri-Hellweg et al., 1995). However, antidrug antibodies have also been detected in a large proportion of patients receiving SMX without a history of hypersensitivity, although the antibody titers were lower when compared with those of hypersensitive patients. From the results of the present study, there can be little doubt about the immunogenicity of SMX-NO; however, the mechanism by which such an immunogenic response results in pathogenicity requires further study. The role of cellular immunity, in particular, needs further investigation and studies are underway to determine an appropriate test species for this purpose.

In summary, this study provides evidence that SMX-NO is the ultimate immunogen responsible for the immune response associated with SMX administration. The mechanism by which such an immunogenic response results in pathogenicity and thus tissue injury requires further investigation. An important finding of the study is that there are defense mechanisms by which the toxic metabolites can be reduced back to the parent compound or inactive metabolites, and this suggests that immunogenicity and subsequent hypersensitivity will only ensue when these detoxification processes are overwhelmed. Further studies are ongoing in our laboratory to identify individual susceptibility factors relevant to man, and in particular, to determine why patients who are HIV-positive have an increased predisposition to developing drug hypersensitivity reactions.

Acknowledgments

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