Metabolic and Chemical Origins of Cross-Reactive Immunological Reactions to Arylamine Benzenesulfonamides: T-Cell Responses to Hydroxylamine and Nitroso Derivatives

J. Luis Castrejon,[†] Sidonie N. Lavergne,[†] Ayman El-Sheikh,[†] John Farrell,[†]
James L. Maggs,[†] Sunil Sabbani,[‡] Paul M. O'Neill,[‡] B. Kevin Park,[†] and Dean J. Naisbitt*[,†]

*MRC Centre for Drug Safety Science, Department of Pharmacology and Therapeutics, School of Biomedical
Sciences, The University of Liverpool, Liverpool L69 3GE, United Kingdom, and Department of Chemistry, The
University of Liverpool, Liverpool L69 7ZD, United Kingdom*

Exposure to sulfamethoxazole (SMX) is associated with T-cell-mediated hypersensitivity reactions in human patients. T-cells can be stimulated by the putative metabolite nitroso SMX, which binds irreversibly to protein. The hydroxylamine and nitroso derivatives of three arylamine benzenesulfonamides, namely, sulfamethozaxole, sulfadiazine, and sulfapyridine, were synthesized, and their T-cell stimulatory capacity in the mouse was explored. Nitroso derivatives were synthesized by a three-step procedure involving the formation of nitro and hydroxylamine sulfonamide intermediates. For immune activation, female Balb-c strain mice were administered nitroso sulfonamides four times weekly for 2 weeks. After 14 days, isolated splenocytes were incubated with the parent compounds, hydroxylamine metabolites, and nitroso derivatives to measure antigen-specific proliferation. To explore the requirement of irreversible protein binding for spleen cell activation, splenocytes were incubated with nitroso derivatives in the presence or absence of glutathione. Splenocytes from nitroso sulfonamide-sensitized mice proliferated and secreted interleukin (IL)-2, IL-4, IL-5, and granulocyte monocyte colony-stimulating factor following stimulation with nitroso derivatives but not the parent compounds. Splenocytes from sensitized mice were also stimulated to proliferate with hydroxylamine and nitroso derivatives of the structurally related sulfonamides. The addition of glutathione inhibited the nitroso-specific T-cell response. Hydroxylamine metabolites were unstable in aqueous solution: Spontaneous transformation yielded appreciable amounts of nitroso and azoxy compounds as well as the parent compounds within 0.1 h. T-cell cross-reactivity with nitroso sulfonamides provides a mechanistic explanation as to why structurally related arylamine benzenesulfonamides are contraindicated in hypersensitive patients.

Introduction

It is estimated that up to 5% of hospital admissions are caused by adverse drug reactions (*1*). Most of these are dose-dependent (type A), on-target toxicities that represent an exaggeration of the known primary and/or secondary pharmacology of the drug. They are normally avoidable through a reduction in dose or improved therapeutic drug monitoring (*2–4*). Off-target toxicity is unpredictable and, although less common, has a tendency to be more severe due to the immune system and its ability to disseminate the initial chemical signal (*5, 6*). A proportion of these off-target toxicities—so-called hypersensitivity reactions—are provoked by drug-specific T-cells (*7–9*). Of the drugs associated with a high incidence of hypersensitivity reactions, sulfamethoxazole (SMX;[1] **1a**) is the model selected by many research groups to explore the phenotype and function of antigen-specific T-cells and mechanisms of antigen presentation. This is because of the drug's well-defined metabolism, the

availability of synthetic stable and reactive metabolites for functional studies, and the accessibility of clinical samples and animal models of immunogenicity.

SMX (**1a**) is an aromatic amine and is metabolized to an *N*-acetylated form, which is rapidly excreted in urine (*10*). Moreover, in humans, approximately 2% of the drug is metabolized by CYP2C8/9 to a hydroxylamine (sulfamethoxazole hydroxylamine, SMX-NHOH; **3a**), an oxidative metabolite that circulates in the blood and is eliminated in urine (*11–14*). Spontaneous oxidation of SMX-NHOH in aqueous solution generates nitroso sulfamethoxazole (SMX-NO; **4a**), an unstable product prone to further oxidation generating nitro SMX (**2a**) (*15*), thiol-mediated reduction (*16–18*), and conjugation with SMX-NHOH (**3a**), forming azo and azoxy dimers (*19*). In spite of these competing reactions, SMX-NO (**4a**) has been shown to bind covalently to serum proteins (*20–22*) at cysteine residues (*20, 23*), to liver tissue (*24*), and to the surface of immune cells (*14, 25, 26*). A complex and partly metastable series of haptenic structures, including cysteine-linked *N*-hydroxysulfenamide (semimercaptal), sulfinamide, *N*-hydroxysulfinamide, and *N*-hydroxysulfonamide adducts, have recently been characterized on model proteins and peptides exposed to SMX-NO (**4a**) (*23*).

SMX-NO (**4a**) is a potent immunogen in experimental animals (*20, 25, 27, 28*). T-cells are stimulated by SMX-NO (**4a**) via a classical hapten mechanism involving protein−complex formation, processing, and the presentation of derived peptides

---

* To whom correspondence should be addressed. Tel: 0044 151 7945346. Fax: 0044 151 7945540. E-mail: dnes@liv.ac.uk.
† Department of Pharmacology and Therapeutics.
‡ Department of Chemistry.
[1] Abbreviations: GM-CSF, granulocyte monocyte colony-stimulating factor; IL, interleukin; SD, sulfadiazine; SD-NHOH, sulfadiazine hydroxylamine; SD-NO, nitroso sulfadiazine; SMX, sulfamethoxazole; SMX-NHOH, sulfamethoxazole hydroxylamine; SMX-NO, nitroso sulfamethoxazole; SP, sulfapyridine; SP-NHOH, sulfapyridine hydroxylamine; SP-NO, nitroso sulfapyridine; THF, tetrahydrofuran.
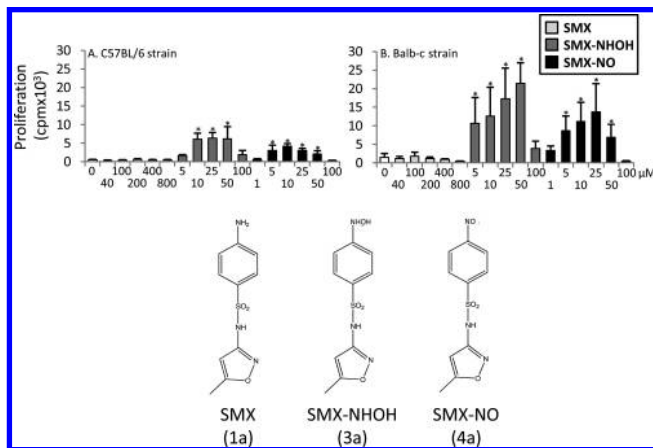
Figure 1. Proliferative response of splenocytes isolated from (A) C57BL/6 and (B) Balb-c strain mice. The mice were immunized with SMX-NO (**4a**) (5 mg/kg), and the cells were cultured in the presence of SMX (**1a**), SMX-NHOH (**3a**), and SMX-NO (**4a**) for 72 h. Proliferation was measured by incorporation of [³H]-thymidine. Results represent the mean ± SD for three animals, with incubations carried out in triplicate. Statistical analysis compares drug-treated splenocytes with cell incubations containing DMSO alone (*$p < 0.05$). Splenocytes from naïve mice were not stimulated with SMX (**1a**), SMX-NHOH (**3a**), or SMX-NO (**4a**).

in the context of MHC molecules (*19*). SMX-NO also stimulates T-cells isolated from blood and blister fluid of hypersensitive patients. In contrast to animal models, incubation of SMX (**1a**) with peripheral blood mononuclear cells and T-cell clones from allergic patients stimulates a proliferative response (*9, 27, 29, 30*).

The fine specificity of the interaction of drugs with MHC and specific T-cell receptors and their ability to stimulate a T-cell response is an important consideration with respect to patient management and administration of structurally related drugs. It is well-established that nonantimicrobial (nonarylamine) sulfonamides, even if they possess a free amino group on the sulfonamide moiety and are associated with hypersensitivity reactions (*31*), are safe to use in SMX (**1a**) hypersensitive patients (*33–35*) in the absence of cross-reactivity (*35*). However, antimicrobial (arylamine) benzenesulfonamides, often related closely to SMX in structure, should be avoided. Indeed, the interaction of T-cells with a panel of antimicrobial sulfonamides containing the same core structure has been explored in detail; T-cells obtained from blood and blister fluid in patients with toxic epidermal necrolysis respond to as many as six antimicrobial sulfonamides and display clear differences in the minimum concentration needed for T-cell stimulation (*9, 32, 34–38*). The lack of a panel of synthetic nitroso sulfonamide derivatives has thus far precluded structure–activity analysis of T-cell responses to protein-reactive drug antigens. Therefore, the aim of this study was to synthesize hydroxylamine and nitroso derivatives of three antimicrobial sulfonamides, namely, SMX (**1a**), sulfapyridine (SP; **1b**), and sulfadiazine (SD; **1c**) (Figure 1; Scheme 1), and to study their T-cell stimulatory capacity in a mouse model.

Experimental Procedures

Caution: *Sulfonamides and their oxidation derivatives are sensitizing compounds. Therefore, they must be handled carefully, and skin contact should be avoided.*

Materials. 4-Nitrobenzylsulfonylchloride, 2-aminopyrimidine, anhydrous pyridine, 2-aminopyridine, 3-amino-5-methylisoxazole, hydrazine, Raney nickel, dichloroethane, sodium hypophosphite, palladium, tetrahydrofuran (THF), methanol, ethanol, chloroform, magnesium sulfate, iron chloride, dichloromethane, ethyl acetate,

dimethyl sulfoxide, Hank's balanced salt solution, RPMI-1640, streptomycin, L-glutamine, HEPES, and penicillin were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). Lymphoprep was obtained from Nycomed (Birmingham, United Kingdom), and [³H]-thymidine was obtained from Moravek (CA). The medium consisted of RPMI, supplemented with 10% fetal bovine serum, HEPES buffer (25 mM), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL).

NMR Spectroscopy and HPLC. The sulfonamide metabolites were characterized using NMR spectroscopy, and the purity was obtained by HPLC. NMR data were obtained with a Bruker, Inc., spectrometer (Coventry, United Kingdom). ¹H NMR spectra were obtained at 400 MHz. All spectra were recorded in 120 μL of DMSO-d_6. Chemical shifts (δ) are expressed in ppm relative to residual peak 2.50 (ppm). For HPLC analysis, samples were eluted at room temperature on a Prodigy 5 μm ODS-2 column (150 mm × 4.6 mm i.d.; Phenomenex, Macclesfield, Cheshire, United Kingdom) using linear gradients of acetonitrile in 0.1% (v/v) formic acid: 20−50% over 20 min for SMX derivatives and 5−50% over 20 min for SD and SP derivatives. The eluent flow rate was 0.9 mL/min.

Synthesis of Nitroso SMX. SMX-NO (**4a**) was synthesized by a three-step procedure described previously (*15*). 4-Nitrobenzyl-sulfonylchloride (10 g) was added slowly to a cooled solution of 3-amino-5-methylisoxazole (20 mL of pyridine, 0 °C). After 24 h, the reaction mixture was filtered, and the resultant brown residue was washed with an excess of distilled water. The crude product was recrystallized from ethyl acetate:toluene (1:3 v/v) to give **2a** as a white powder (nitro SMX; **2a**). To synthesize SMX-NHOH (**3a**), nitro SMX (1 g; **2a**) was suspended in THF (100 mL) and distilled water (10 mL). Sodium hypophosphite (1.12 g; 10 mL of distilled water) and palladium/carbon catalyst (0.1 g) were then added to the stirred solution to reduce the nitro moiety. The reaction was followed by TLC using dichloromethane−ethyl acetate (65: 35, v/v), until all of the starting material had reacted (approximately 20 min). The product was extracted with diethyl ether (200 mL), and the combined organic extracts were dried with magnesium sulfate and filtered, and then, the solvent was evaporated under reduced pressure. The resulting crude yellow oil was recrystallized using trichloromethane to give a white powder [SMX-NHOH (**3a**)]. To synthesize SMX-NO (**4a**), SMX-NHOH (1 g; **3a**) was dissolved in ethanol (70 mL) and added to an iron(III) chloride solution (5.99 g in 50 mL of water) at room temperature. After 5 min, the reaction mixture was filtered. A bright yellow powder (SMX-NO; yield, 30−40%) was collected as a final product. Analytical data have been described previously (*9*).

Synthesis of Nitroso SP. Similar to the synthesis of SMX-NO (**4a**), three-step procedures were used for the synthesis of nitroso sulfapyridine (SP-NO; **4b**) and nitroso sulfadiazine (SD-NO; **4c**). However, palladium/carbon was replaced as the catalyst for nitro reduction because it was associated with excessive reduction and liberation of the parent amine. Several catalysts (palladium, zinc, and Raney nickel) and hydrogen donors (sodium phosphinite, ammonium chloride, and hydrazine) were compared in different solvent systems (THF/water, DMF/ethanol/water, and dichloroethane) to define the most appropriate experimental conditions.

A Raney nickel catalyst and hydrazine in dichloroethane/ethanol were used for the synthesis of SP hydroxylamine (**3b**). Nitro SP (**2b**) [2.5 g, 100 mL of ethanol/dichloroethane (1:1, v/v)] was cooled (0 °C) and mixed with Raney nickel catalyst (≈0.1 g), and hydrazine (0.9 g) was added dropwise. The temperature was maintained between 0 and 10 °C for the duration of the experiment. The reaction was followed by TLC (1:9; methanol/dichloromethane). After 3 h, the solution was filtered through a Celite bed, and the crude solid was washed with acetone to extract the hydroxylamine, and then, the solvent was evaporated. The product was dried under vacuum for 2 h and recrystallized using methanol/dichloromethane (1:9, v/v). The solution was left overnight in an ice bath, and the resultant white precipitate was filtered and dried under vacuum to give **3b** as a final product. SP-NO (**4b**) was synthesized by following the procedure described above for SMX-NO (**4a**), with

the following modifications. First, the reaction was stirred for 30 min. Second, on completion of the reaction, the volume was reduced by half to promote precipitation of the product.

Nitro SP (2b). White solid (7.70 g), mp = 160 °C. ^{1}H NMR (400 MHz, *d*-DMSO): δ 8.37 (dt, *J* = 9.3 and 2.5 Hz, 2H), 8.13 (dt, *J* = 9.3 and 2.5 Hz, 2H), 7.98 (dd, *J* = 5.9 and 1.1 Hz, 1H), 7.85 (ddd, *J* = 8.9, 7.0, and 1.9 Hz, 1H), 7.31 (d, *J* = 8.9 Hz, 1H), 6.9 (ddd, *J* = 7.0, 6.0, and 0.94 Hz), 3.47 (br s, 1H). IR (KBr, cm^{-1}): 2970, 1631, 1350, 1142, 964, 733. HRMS: Found [M + Na]$^+$, 302.0206; $C_{11}H_9N_3O_4SNa$ required, 302.0211.

Sulfapyridine Hydroxylamine (SP-NHOH, 3b). White solid (0.473 g), mp = 160 °C. ^{1}H NMR (400 MHz, *d*-DMSO): δ 8.93 (br s, 1H), 8.63 (br s, 1H), 8.09 (dd, *J* = 5.1 and 1.1 Hz, 1H), 7.66−7.71 (m, 3H), 7.12 (d, *J* = 8.5 Hz, 1H), 6.92 (ddd, *J* = 7.0, 5.3, and 0.7 Hz, 1H), 6.86 (d, *J* = 8.7 Hz, 2H). IR (KBr, cm^{-1}): 2670, 1631, 1389, 1242, 1130, 1080, 879, 775. HRMS: Found [M + Na]$^+$, 288.0418; $C_{11}H_{11}N_3O_3SNa$ required, 288.0419.

SP-NO (4b). Green compound (0.074 g), mp = 150 °C. ^{1}H NMR (400 MHz, *d*-DMSO): δ 8.21 (d, *J* = 8.5 Hz, 2H), 8.09 (d, *J* = 8.5 Hz, 2H), 7.98 (d, *J* = 5.3 Hz, 1H), 7.84 (ddd, *J* = 8.8, 7.2, and 1.6 Hz, 1H), 7.32 (d, *J* = 8.7 Hz, 1H), 6.89 (t, *J* = 6.5 Hz, 1H). IR (KBr, cm^{-1}): 3352, 2974, 1631, 1450, 1385, 1354, 1296, 1146, 999, 883, 775. HRMS: Found [M + H]$^+$, 264.0438; $C_{11}H_{10}N_3O_3S$ required, 264.0443.

Synthesis of Nitroso SDZ. As described above for SP, a Raney nickel catalyst and hydrazine in dichloroethane/ethanol were used for the synthesis of SD hydroxylamine (**3c**). The reaction was followed by TLC (1:9; methanol/dichloromethane), and after 1.5 h, the solution was filtered through a Celite bed, the solvent was evaporated, and the crude solid was washed with acetone to extract the hydroxylamine. The product was then dried under vacuum for 2 h and recrystallized using 2-propanol. The solution was left overnight in an ice bath, and the resultant white precipitate was filtered and dried under vacuum to give **3c** as a final product. SD-NO (**4c**) was synthesized by following the procedure described above for SP-NO (**4b**).

Nitro SD (2c). Yellow solid (8.37 g), mp = 270 °C. ^{1}H NMR (400 MHz, *d*-DMSO): δ 8.53 (d, *J* = 4.9 Hz, 2H), 8.4 (dt, *J* = 9.4 and 2.5 Hz, 2H), 8.22 (dt, *J* = 9.4 and 2.5 Hz, 2H), 7.07 (t, *J* = 4.9 Hz, 1H), 3.42 (br s, 1H). IR (KBr, cm^{-1}): 2970, 1581, 1520, 1442, 1346, 1165, 1088, 957, 798, 737. HRMS: Found [M + H]$^+$, 281.0332; $C_{10}H_9N_4O_4S$ required, 281.0345.

Sulfadiazine Hydroxylamine (SD-NHOH, 3c). Orange solid (0.701), mp = 220 °C. ^{1}H NMR (400 MHz, *d*-DMSO): δ 8.97 (br s, 1H), 8.65 (br s, 1H), 8.48 (d, *J* = 4.7 Hz, 2H), 7.76 (dt, *J* = 9.5 and 2.0 Hz, 2H), 7.01 (t, *J* = 4.9 Hz, 1H), 6.85 (dt, *J* = 9.5 and 2.3 Hz, 2H). IR (KBr, cm^{-1}): 3321, 2974, 1577, 1493, 1442, 1315, 1146, 1088, 945, 795. HRMS: Found [M + Na]$^+$, 289.0359; $C_{10}H_{10}N_4O_3SNa$ required, 289.0371.

SD-NO (4c). Brown solid (0.05 g), mp = 230 °C. ^{1}H NMR (400 MHz, *d*-DMSO): δ 8.44−8.60 (m, 2H), 8.25−8.36 (m, 2H), 8.06−8.17 (m, 2H), 6.99−7.12 (m, 1H). IR (KBr, cm^{-1}): 2970, 1581, 1500, 1442, 1412, 1346, 1169, 1088, 953, 795. HRMS: Found [M + Na]$^+$, 287.0203; $C_{10}H_8N_4O_3SNa$ required, 287.0215.

Immunizing Protocol. Female Balb-c (16−20 g) and C57/Bl6 (20−30 g) mice were purchased from Charles River U.K. Ltd. (Kent, United Kingdom). Mice were administered SMX-NO (**4a**) (5 mg/kg; *n* = 4), SP-NO (**4b**) (5 mg/kg; *n* = 3), or SD-NO (**4c**) (5 mg/kg; *n* = 3) in DMSO (100 μL) via i.p. injection four times weekly for 2 weeks according to a protocol described previously (*25*). On completion of this dosing regimen, animals were killed, and the spleen was removed for analysis of splenocyte proliferation.

Assessment of the ex Vivo Proliferative Response of Splenocytes to Sulfonamides and Sulfonamide Derivatives. Spleens were homogenized in Hank's balanced salt solution (10 mL) and filtered to liberate a single cell suspension. Red cells were removed by centrifugation with Lymphoprep. The splenocytes were washed, and viability was assessed using Trypan Blue. Cells were suspended in culture medium at a concentration of 1.5×10^6 cells/mL and placed in a round-bottom 96-well plate (1.5×10^5/well) with SMX-NO (**4a**), SP-NO (**4b**), SD-NO (**4c**) (all 1−25 μM), SMX-NHOH

(**3a**), SP-NHOH (**3b**), SD-NHOH (**3c**) (all 5−50 μM), SMX (**1a**), SP (**1b**), and SD (**1c**) (all 40−800 μM) for 72 h (37 °C, 5% CO$_2$). To measure proliferation, [^{3}H]-thymidine (0.5 μCi) was added to each well for the final 16 h. Cells were harvested, and [^{3}H]-thymidine incorporation was measured as counts per min on a β-counter (PerkinElmer Life Sciences, Cambridge, United Kingdom).

Assessment of the ex Vivo Proliferative Response of Splenocytes to Sulfonamide Derivatives in the Presence of Glutathione. Splenocytes from sensitized mice were incubated with SMX-NO (**4a**), SP-NO (**4b**), SD-NO (**4c**) [25 μM; except for SD-NO (**4c**), 10 μM], SMX-NHOH (**3a**), SD-NHOH (**3c**), or SP-NHOH (**3b**) [25 μM; except for SMX-NHOH (**3a**), 50 μM] for analysis of proliferation using the conditions described above. Glutathione (1 mM) was added to certain incubations. After 72 h (37 °C, 5% CO$_2$), [^{3}H]-thymidine (0.5 μCi) was added for the last 16 h of the experiment to measure antigen-specific spleen cell proliferation.

Assessment of the Stability of Sulfonamide Hydroxylamines by Liquid Chromatography−Mass Spectrometry. Aliquots (75 μL) of solutions derived from cell incubations treated with SMX-NHOH (**3a**), SP-NHOH (**3b**), or SD-NHOH (**3c**) (0.1, 4, 24, and 72 h) were chromatographed at room temperature on a Prodigy 5 μm ODS-2 column (150 mm × 4.6 mm i.d.; Phenomenex) using linear gradients of acetonitrile in 0.1% (v/v) formic acid: 20−50% over 20 min for SMX and 5−50% over 20 min for SD and SP. The eluent flow rate was 0.9 mL/min. The LC system consisted of two Jasco PU980 pumps (Jasco UK, Great Dunmow, Essex, United Kingdom) and a Jasco HG-980-30 mixing module. Eluted compounds were monitored at 254 nm with a Jasco UV-975 spectrophotometer. Eluate split-flow to the LC-MS interface was ca. 40 μL/min. A Quattro II mass spectrometer (Waters Corp., Manchester, United Kingdom) fitted with the standard coaxial electrospray source was operated using nitrogen as the nebulizing and drying gas. The interface temperature was 80 °C, the electrospray capillary voltage was 3.8 kV, and the cone voltage was 40 V. The instrument was set up for selected ion monitoring in the positive-ion mode as follows: channel dwell time, 200 ms; cycle time, 230 ms; and span, 0.5 amu.

Assessment of Cytokine Secretion. Supernatant from incubations of immunized splenocytes stimulated with SMX-NO (**4a**), SP-NO (**4b**), or SD-NO (**4c**) (all 25 μM) was collected after 72 h and stored at −70 °C for analysis of cytokine secretion. Concentrations of interleukin (IL)-2, IL-4, IL-5, TNF-α, granulocyte monocyte colony-stimulating factor (GM-CSF), and IFN-γ were measured in the supernatant using a mouse cytokine/chemokine LINCOplex multiplex assay kit (LINCO Research Inc., Hampshire, United Kingdom). Cytokine secretion (pg/mL) was measured using a Bio-Plex Suspension Array System (model Luminex100) operating with BioRad Bio-Plex Manager 3.0 Software (BioRad, Hertfordshire, United Kingdom).

Statistical Analysis. Values to be compared were initially analyzed for non-normality by the Shapiro-Wilks test. Because the data were found to be non-normally distributed, we used the Mann−Whitney test for comparison of the two groups. A *p* value <0.05 was considered statistically significant.
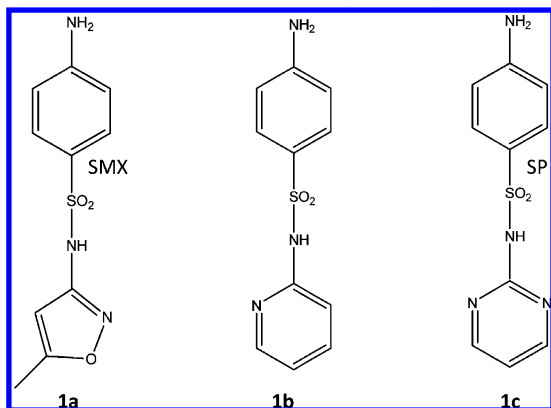
Results

Synthesis of Sulfonamide Hydroxylamine and Nitroso Derivatives. Nitroso sulfonamides can be synthesized via a three-step procedure involving: (1) nucleophilic coupling of 4-nitrobenzenesulfonyl chloride to the relevant side chain, (2) catalytic hydrogenation of nitro intermediates, and (3) oxidation of the hydroxylamine intermediates with iron(III) chloride (Schemes 1 and 2).
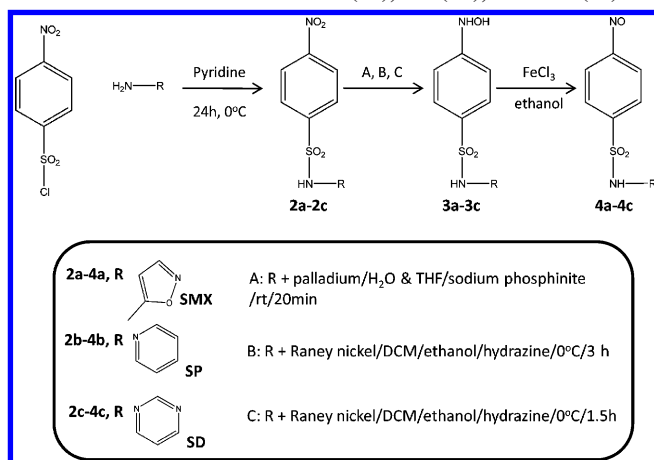
Nitro-SMX (**2a**), SP (**2b**), and SD (**2c**) were synthesized in high yields and purity using an established literature method (*15*). Nitro-SMX (**2a**) reduction and the liberation of SMX-NHOH (**3a**) were performed using an established procedure with palladium−carbon as the catalyst and sodium phosphinite as

T-Cell Responses to N-Oxygenated Sulfonamides

Chem. Res. Toxicol., Vol. 23, No. 1, 2010 **187**

Scheme 1. Structures of SMX (1a), SP (1b), and SD (1c)

Scheme 2. Three-Step Synthesis of the Hydroxylamine and Nitroso Derivatives of SMX (1a), SP (1b), and SD (1c)

SP-NO (**4b**) and SD-NO (**4c**). For SP-NHOH (**3b**), the reaction mixture was filtered after 30 min, yielding a bright green product that was found to be SP-NO (**4b**) on analysis. For SD-NHOH (**3c**), a pale yellow product, SD-NO (**4c**) was detected after 15−20 min.

***N*-Oxygenated Derivatives of SMX, SD, and SP Stimulate a T-Cell Response in Naïve Mice.** Preliminary experiments were designed to explore the immunogenicity of SMX-NO (**4a**) (5 mg/kg; 4 × weekly for 2 weeks) in BALB/c and C57BL/6 strain mice. Higher SMX-NO (**4a**) concentrations were not used due to overt toxicity following repeated dosing (data not shown). Splenocytes isolated from BALB/c and C57BL/6 strain mice administered SMX-NO (**4a**) were stimulated to proliferate with SMX-NO (**4a**) (Figure 1). The response to SMX-NO (**4a**) was significantly stronger with splenocytes from Balb/c strain mice; thus, this strain of mouse was selected for all subsequent investigations.

As described previously (*27*), splenocytes from mice exposed to SMX-NO (**4a**) (5 mg/kg; 4 × weekly for 2 weeks) proliferated following stimulation with SMX-NO (**4a**) and SMX-NHOH (**3a**) but not SMX (**1a**) (Figure 2A). Similar results were obtained with SP-NO (**4b**)- and SD-NO (**4c**)-immunized mice. Splenocytes were stimulated to proliferate with nitroso and hydroxylamine derivatives but not the parent compounds (Figure 2B,C). Hydroxylamine- and nitroso-specific proliferation was concentration-dependent up to 25−50 μM. Concentrations above 50 μM inhibited splenocyte proliferation (data not shown).

The strength of the maximum proliferative response of splenocytes from SD-NO- (**4c**) immunized mice to SD-NO (**4c**) was significantly lower than that observed with SMX-NO (**4a**)-stimulated and SP-NO (**4b**)-stimulated splenocytes from SMX-NO (**4a**)- and SP-NO (**4b**)-immunized mice, respectively [SMX-NO (**4a**)-immunized: 0 μM, 2967 ± 1073.14 cpm; SMX-NO (**4a**): 25 μM, 26120.41 ± 8891.24 cpm; $p < 0.05$. SP-NO (**4b**)-immunized: 0 μM, 3225.11 ± 2386.57 cpm; SP-NO (**4b**): 25 μM, 35592.11 ± 11310.10 cpm; $p < 0.05$. SD-NO (**4c**)-immunized: 0 μM, 193.55 ± 127.77 cpm; SD-NO (**4c**): 5 μM, 4120.55 ± 3529.75 cpm; $p < 0.05$]. Splenocytes from naïve mice, that is, mice not exposed to any of the sulfonamides or their derivatives, were not stimulated specifically with SMX (**1a**), SP (**1b**), SD (**1c**), or their respective hydroxylamine or nitroso derivatives.

***N*-Oxygenated Derivatives of SMX, SD, and SP Stimulate a T-Cell Response in Mice Immunized with Other Nitroso Derivatives.** Splenocytes from mice immunized with SMX-NO (**4a**) were additionally stimulated to proliferate in the presence of SD-NHOH (**3c**) and SD-NO (**4c**). Hydroxylamine and nitroso derivatives of SP (**3b** or **4b**) stimulated a weak response and only at the highest concentration tested (Figure 3A). A proliferative response of splenocytes from SD-NO (**4c**)- and SP-NO (**4b**)-immunized mice was detected with hydroxylamine and nitroso derivatives of all three sulfonamides (Figure 3B,C). The proliferative response was stronger when splenocytes from SMX-NO (**4a**)-, SP-NO (**4b**)-, and SD-NO (**4c**)-treated mice were stimulated with the compound against which the animals had been immunized.

the hydrogen donor (*15*). The syntheses of SP-NHOH (**3b**) and SD-NHOH (**3c**) were more challenging as the initial experiments using palladium−carbon and sodium phosphinite in THF−water were unsuccessful (data not shown). Nitro-SP (**2b**) and nitro-SD (**2c**) were, indeed, largely insoluble in THF. Furthermore, as hydrogen transfer occurred almost immediately, it was not possible to prevent excessive reduction. To resolve these issues, several experimental approaches used previously in the reduction of nitrobenzene derivatives were tested (*39–43*). Choices of solvent, catalyst, hydrogen donor, and temperature were all found to influence the rate of hydrogen transfer (Table 1). The selective reduction of nitro-SP (**2b**) and nitro-SD (**2c**) was eventually performed using Raney nickel catalysis with hydrazine as the hydrogen donor in a dichloroethane/ethanol solvent system maintained at 0 °C. The reactions were stopped as soon as the starting material was consumed. Following recrystallization using methanol/chloroform or 2-propanol, both SP-NHOH (**3b**) and SD-NHOH (**3c**) were obtained in high purity.

As reported previously, SMX-NHOH (**3a**) oxidizes rapidly in the presence of iron(III) chloride (*44, 45*) SMX-NO (**4a**), a bright yellow insoluble product, was detected in high yields and purity after 5 min. A similar procedure was used to synthesize

Table 1. Reagents and Conditions Used To Optimize the Synthesis of SP and SD Hydroxylamines (3b and 3c)

|	catalyst	hydrogen donor	solvent, conditions	duration	result summary
A	palladium	sodium phosphinite	THF, water, room temperature	7 min	hydroxylamine, amine and nitro
B	zinc	ammonium chloride	DMF, water, room temperature	30 min	hydroxylamine, amine and nitro
C	palladium	hydrazine	DMF, water, ethanol, THF, room temperature	24 h	no reaction
D	zinc	ammonium chloride	DMF, water, ethanol, room temperature	20 min	hydroxylamine and nitro
E	Raney nickel	hydrazine	dichloroethane, ethanol, 0 °C	3 h	SP hydroxylamine (95% pure)
F	Raney nickel	hydrazine	dichloroethane, ethanol, 0 °C	1.5 h	SD hydroxylamine (95% pure)
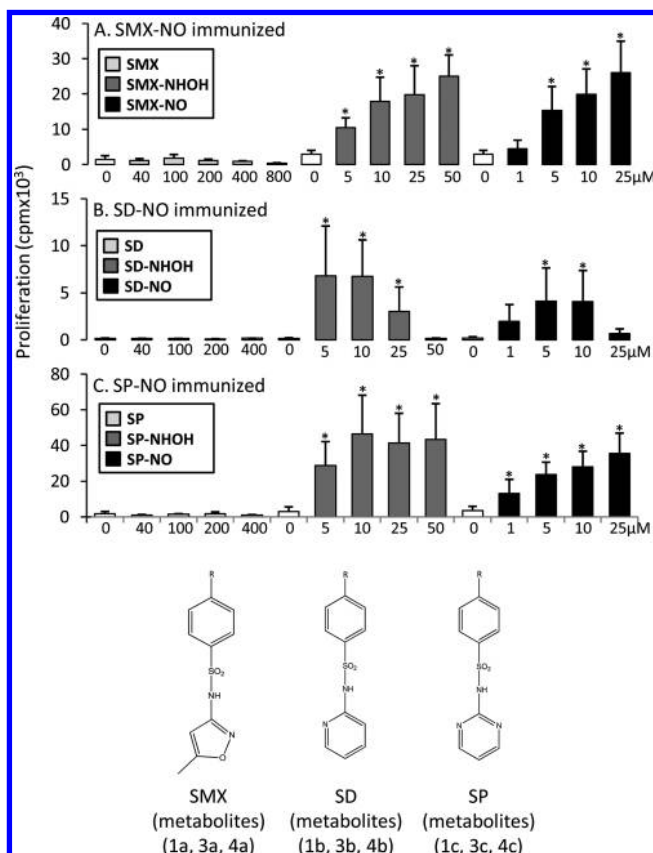
Figure 2. Proliferative response of splenocytes from Balb-c strain mice immunized with (A) SMX-NO (**4a**), (B) SD-NO (**4c**), or (C) SP-NO (**4b**) (all 5 mg/kg) and cultured with their respective hydroxylamine and nitroso derivatives for 72 h. Proliferation was measured by incorporation of [³H]-thymidine. Results represent the mean ± SD for three animals, with incubations carried out in triplicate. Statistical analysis compares drug-treated splenocytes with cell incubations containing DMSO alone (*p < 0.05). Splenocytes from naïve mice were not stimulated specifically with SMX (**1a**), SP (**1b**), SD (**1c**), or their respective hydroxylamine or nitroso derivatives.
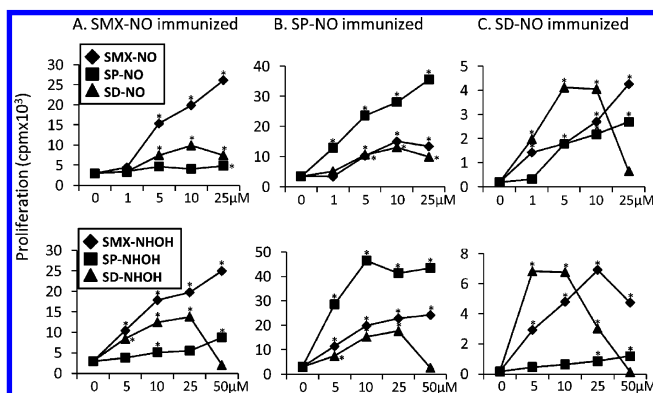
Figure 3. Proliferative response of splenocytes from Balb-c strain mice immunized with (A) SMX-NO (**4a**), (B) SP-NO (**4b**), or (C) SD-NO (**4c**) (all 5 mg/kg) and cultured with different hydroxylamine or nitroso derivatives. Proliferation was measured by incorporation of [³H]-thymidine. Results represent the mean ± SD for three animals, with incubations carried out in triplicate. Statistical analysis compares drug-treated splenocytes with cell incubations containing DMSO alone (*p < 0.05).

Glutathione Prevents the Selective Stimulation of Splenocytes by Hydroxylamine and Nitroso Derivatives of SMX, SD, and SP. Glutathione prevents the conversion of hydroxylamine metabolites to nitroso compounds and rapidly reduces synthetic nitroso compounds (*15, 16, 18, 25*). Furthermore, glutathione inhibits SMX-NO (**4a**)-specific stimulation
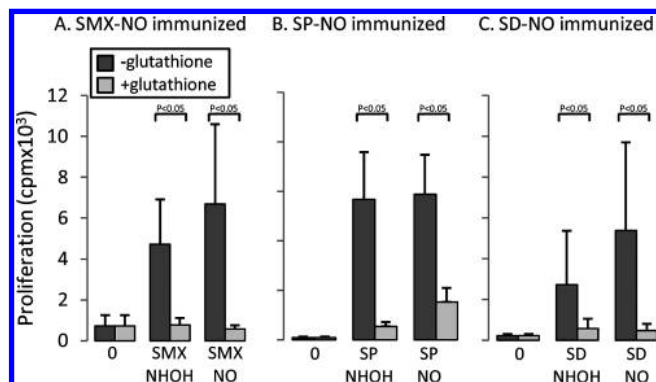
Figure 4. Proliferative response of splenocytes from Balb-c strain mice immunized with (A) SMX-NO (**4a**), (B) SP-NO (**4b**), or (C) SD-NO (**4c**) (all 5 mg/kg) and cultured with hydroxylamine or nitroso derivatives in the presence or absence of glutathione. Proliferation was measured by incorporation of [³H]-thymidine. Results represent the mean ± SD for three animals, with incubations carried out in triplicate. Statistical analysis compares drug-treated splenocytes with cell incubations containing DMSO alone (*p < 0.05*).

of lymphocytes from allergic patients and animal models of SMX (**1a**) immunogenicity (*25, 29*). In this study, glutathione pretreatment significantly reduced the proliferation of splenocytes from SMX-NO (**4a**)-, SP-NO (**4b**)-, and SD-NO (**4c**)-immunized mice stimulated with the corresponding hydroxylamine and nitroso derivatives. [³H]-Thymidine incorporation was decreased almost to control levels (Figure 4).

Rapid Degradation of SMX, SD, and SP Hydroxylamines in Solution. To investigate the fate of SMX-NHOH (**3a**), SP-NHOH (**3b**), and SD-NHOH (**3c**) in solution, splenocytes were incubated in complete medium for 0.1−72 h (the duration of the proliferation assay), and products of oxidation, reduction, and self-conjugation were analyzed by LC-MS.

SMX-NHOH (**3a**) (R_t, 8.2 min; m/z [270]⁺) was detectable at 0.1 h. Weak signals for SMX (**1a**) (R_t, 9.5 min; m/z 254 [M + 1]⁺) and azoxy SMX (R_t, 24.0 min; m/z 519 [M + 1]⁺) were also detected. After 1 h, the signal for SMX-NHOH (**3a**) had all but disappeared. Degradation of SMX-NHOH (**3a**) coincided with the appearance of SMX (**1a**) and azoxy SMX in a time-dependent fashion. Low levels of SMX-NO (**4a**) were detected between 1 and 4 h (R_t, 18.3 min; m/z 268 [M + 1]⁺).

SP-NHOH (**3b**) degraded rapidly in culture. A weak signal (R_t, 9.6 min; m/z 266 [M + 1]⁺) was detectable at the earliest time point analyzed (0.1 h). Interestingly, although low levels of SP (**1b**) (R_t, 9.5 min; m/z 250 [M + 1]⁺) and azoxy SP (R_t, 17.1 min; m/z 511 [M + 1]⁺) were detectable at each time point tested, the intensity of the respective signals did not increase with time.

Incubation of SD-NHOH (**3c**) with splenocytes resulted in a similar degradation profile. SD-NHOH (**3c**) (R_t, 7.9 min; m/z 267 [M + 1]⁺) was detected between 0 and 1 h. A weak signal for azoxy SD (R_t, 17.5 min; m/z 513 [M + 1]⁺) was detected at 0.1 h, and the intensity of the signal increased with time. SD (**1c**) and SD-NO (**4c**) were not detected throughout the incubation period.

Cytokine Secretion from Splenocytes Stimulated by SMX-NO, SD-NO, and SP-NO. Cytokines secreted by antigen-stimulated splenocytes from mice immunized with nitroso sulfonamides were analyzed using a mouse cytokine multiplex assay kit. SMX-NO (**4a**) stimulated splenocytes from SMX-NO (**4a**)-immunized mice and secreted the Th2 cytokines IL-4 and IL-5 and IL-2 and GM-CSF (Table 2). Similar results were obtained following antigen stimulation of splenocytes from SP-NO (**4b**)-immunized mice (Table 2). In contrast, splenocytes

Table 2. Cytokine Secretion by Antigen-Stimulated Splenocytes from Nitroso Sulfonamide-Immunized Mice

immunogen	antigen	cytokine (pg/m)					
		IL-2	IL-4	IL-5	GM-CSF	IFN-γ	TNF-α
SMX-NO (**4a**)	0	<10	ND[b]	ND	ND	25.4 ± 4.8	ND
	SMX-NO	75.6 ± 1.6[a,c]	18.5 ± 0.9[c]	39.1 ± 3.5[c]	82.6 ± 6.5[c]	39.4 ± 8.5	ND
	SP-NO	44.3 ± 1.2[c]	<10	<10	32.2 ± 8.7[c]	16.9 ± 4.7	ND
	SD-NO	67.3 ± 1.3[c]	10.2 ± 0.9[c]	<10	31.2 ± 18.6[c]	16.6 ± 4.7	ND
SP-NO (**4b**)	0	27.9 ± 3.9	10.0 ± 1.2	ND	ND	<10	ND
	SMX-NO	60.4 ± 9.3[c]	23.7 ± 1.8[c]	18.1 ± 2.7[c]	105.5 ± 32.1[c]	<10	ND
	SP-NO	122.4 ± 15.4[c]	94.2 ± 7.9[c]	48.2 ± 5.7[c]	202.9 ± 28.7[c]	25.8 ± 8.9	ND
	SD-NO	104.7 ± 12.7[c]	21.0 ± 2.9[c]	11.7 ± 2.1[c]	45.0 ± 7.9[c]	13.0 ± 1.4	ND
SD-NO (**4c**)	0	10.0 ± 0.8	<10	ND	ND	12.3 ± 0.2	ND
	SMX-NO	68.8 ± 1.3[c]	16.7 ± 0.2[c]	<10	ND	<10	ND
	SP-NO	<10	<10	ND	ND	16.0 ± 2.0	ND
	SD-NO	54.3 ± 3.4[c]	12.3 ± 0.8[c]	<10	ND	21.0 ± 9.5	ND

[a] Mean ± SEM. [b] ND, not detected. [c] $P < 0.05$.

from SD-NO (**4c**)-immunized mice secreted IL-2 and IL-4 alone and only when stimulated with SD-NO (**4c**) or SMX-NO (**4a**) (Table 2). Splenocytes from naïve mice did not secrete detectable cytokine when stimulated with SMX (**1a**), SP (**1b**), SD (**1c**), or their respective hydroxylamine or nitroso derivatives.

Discussion

Drug-specific T-cells have been isolated from the blood and skin of allergic patients but not control subjects exposed to the same drug without any adverse event (*9, 46–48*). The mechanism by which drugs interact with immunological receptors and stimulate T-cells remains an intriguing area of research since the majority of drugs associated with a high incidence of hypersensitivity are low molecular weight chemicals and therefore are not classical antigens, that is, proteins requiring processing prior to the presentation of derived, MHC-restricted peptides to specific T-cell receptors.

Selective covalent modification of nucleophilic residues on protein is thought to be a prerequisite for the activation of an immune response directed against a drug or chemical allergen (*49, 50*). Using SMX (**1a**) as a model compound, several research groups have shown that the protein-reactive derivative SMX-NO (**4a**) (*25, 26, 51*) is at least 3 orders of magnitude more immunogenic than the parent compound (*20, 25, 27, 28*), stimulates blood- and skin-derived T-cells from allergic patients (*9, 29, 30*), and activates naïve T-cells from healthy volunteers (*52*). Furthermore, we have established a causal relationship between oxidative metabolism of SMX (**1a**) and costimulatory signaling in dendritic cells by showing that 1-aminobenzotriazole, a nonspecific P450 and myeloperoxidase inhibitor, attenuates SMX (**1a**)-mediated upregulation of CD40 (*14*). The presentation of a distinct antigenic determinant to T-cells from allergic patients is similarly dependent on metabolism of SMX (**1a**) (unpublished observation). The aim of the current investigation was to evaluate whether nitroso sulfonamide-specific T-cells can be stimulated with structurally related nitroso sulfonamides through T-cell receptor cross-reactivity.

To accomplish this objective, hydroxylamine and nitroso derivatives of SMX (**1a**), SP (**1b**), and SD (**1c**) were synthesized. SP (**1b**) and SD (**1c**) are also antimicrobial, that is, arylamine, benzenesulfonamides that have been associated with hypersensitivity reactions (*53*) and are metabolized to a hydroxylamine by human hepatic microsomes (*54, 55*). However, SP (**1b**) and SD (**1c**) were selected particularly because they are for the most part ineffective at stimulating SMX (**1a**)-specific T-cell clones from allergic patients (*32, 36–38*). The pyrimidine derivative SD (**1c**) stimulated a weak response in 10−15% of SMX (**1a**)-specific T-cell clones, while only one out of 21 clones were

activated with the pyridine derivative SP (**1b**). SMX (**1a**) derivatives were synthesized in high yields and purity using a previously described three-step procedure (*15*) (Scheme 2). first, coupling of 4-nitrobenzenesulfonyl chloride to 3-amino-5-methylisoxazole, liberating nitro-SMX (**2a**); second, reduction of nitro-SMX (**2a**) to SMX-NHOH (**3a**); and finally, oxidation of SMX-NHOH (**3a**) to SMX-NO (**4a**). Essentially, the same approach was used to synthesize SP (**1b**) and SD (**1c**) derivatives with the exception that alternative conditions had to be sought for the nitro reduction. Use of a palladium−carbon catalyst with sodium phosphinate as the hydrogen donor in THF/water to reduce nitro SP (**2b**) and SD (**2c**) gave a mixture of products in low yield. By optimizing the reaction conditions (catalyst, hydrogen donor, and solvent; Table 1), it was eventually possible to synthesize both SP-NHOH (**3b**) and SD-NHOH (**3c**) in acceptable yields and high purity (Table 2). The use of Raney nickel as the catalyst and hydrazine as the hydrogen donor prevented excessive reduction, and the reaction was easily monitored over 1.5−3 h.

SMX-NO (**4a**), SP-NO (**4b**), and SD-NO (**4c**) (5 mg/kg, i.p.) induced an immune response in mice at the same dose. Spleen cells isolated from sensitized mice proliferated following in vitro stimulation with the nitroso compound against which the animals were immunized. The proliferative response of splenocytes was concentration-dependent, a significant response being detected at inducer concentrations between 5 and 100 μM (Figure 2). Inhibition of splenocyte proliferation, at concentrations above 100 μM (results not shown), conformed with the previously described in vitro cytotoxicity of sulfonamide hydroxylamines and nitroso derivatives (*56–58*).

Nitroso sulfonamides are known to degrade readily in solution and are not detectable after 5 min in cell cultures (*19*). Their disappearance from aqueous solutions has been associated with the formation of an azoxy dimer (*19*), adducts of cysteine and cysteinyl sulfoxy acids residues on protein that presumably represent important antigenic determinants for T-cells (*17, 23, 26, 51*), and nitro and hydroxylamine intermediates, products of autoxidation and reduction by glutathione and antioxidants, respectively (*15, 16, 19, 59*). Thus, the exposure of splenocytes to nitroso derivatives in the proliferation assay is probably low for most of the incubation period and likely several orders of magnitude lower than the starting concentration.

The hydroxylamine derivative of SMX (**3a**) is a well-characterized human metabolite; approximately 2% of an oral dose of SMX (**1a**) is excreted as the hydroxylamine in urine (*60, 61*). However, hydroxylamines are susceptible to oxidation, reduction, and self-conjugation reactions (*15, 16, 27, 57*). Thus, when exploring the T-cell stimulatory capacity of hydroxylamine

metabolites, it is important to relate proliferation of splenocyte cultures to analysis of compound stability. Each hydroxylamine [SMX-NHOH (**3a**), SP-NHOH (**3b**), and SD-NHOH (**3c**)] was found to degrade rapidly in the culture medium, and no more than trace amounts were detected after 1 h. Despite this, exposing spleen cells isolated from immunized mice to hydroxylamine stimulated a proliferative response (Figure 2). With SMX-NHOH (**3a**), low levels of SMX-NO (**4a**) were detected even after 4 h. For SP-NHOH (**3b**) and SD-NHOH (**3c**), nitroso formation was confirmed indirectly through the characterization of azoxy dimers, compounds originating from the conjugation of hydroxylamine and nitroso species (*19*). Interestingly, SP-NHOH (**3b**) appeared to be particularly unstable in culture medium. Only a weak signal corresponding to SP-NHOH (**3b**) was detected by mass spectrometry at the earliest time point tested (0.1 h), which is in agreement with the previously described short half-life of SP-NHOH (**3b**) (*58*). Furthermore, unlike SMX-NHOH (**3a**) and SD-NHOH (**3c**), products of reduction and dimerization were only detected at low levels throughout the incubation period. These results indicate that the formation of SP-NO (**4b**) protein adducts may be favored over other reactions. To further investigate this possibility, specific antidrug antibodies are being generated in ongoing experiments.

In agreement with previous studies focusing on the immunogenicity of SMX (**1a**) metabolites (*25, 28*), neither SMX (**1a**), SP (**1b**), nor SD (**1c**) stimulated splenocytes from nitrososulfonamide-immunized mice. Splenocytes from SMX-NO (**4a**)-, SP-NO (**4b**)-, and SD-NO (**4c**)-immunized mice proliferated in the presence of all three nitroso sulfonamides (Figure 3A−C). The concentrations of nitroso metabolite associated with splenocyte proliferation were lower than those needed to deplete intracellular glutathione and/or decrease cell viability (*17, 19*). Glutathione, which is one of a panel of low molecular weight thiols that prevent the spontaneous oxidation of sulfonamide hydroxylamines (*16*), the reaction of nitroso sulfonamides with protein (*16, 17, 59*), and the direct toxicity of sulfonamide metabolites (*58*), effectively inhibited the splenocyte proliferation, indicating that protein adduct formation is needed to stimulate specific T-cells. Collectively, these results imply that although the drug structure per se contributes toward the selectivity of the antigen-T-cell receptor binding interaction, the protein (or the derived peptide) covalently modified at specific cysteine residues provides the principal signal that determines whether or not a T-cell responds.

T-cells' cytokine production promotes the activation and differentiation of immune cells into different effector populations. T-cells can be divided into classes based on their cytokine secretion profile. IFN-γ-secreting T-cells (Th1) are dominant in many forms of allergic contact dermatitis, whereas respiratory allergy is associated with the preferential activation of Th2 cells (secreting IL-4, IL-5, and IL-13) (*62, 63*). More recently, new T-cell populations have been classified in terms of the cytokines that they secrete (IL-10 and IL-17). However, their role in the development (or prevention) of allergic drug reactions has not been defined. In SMX-allergic patients and in Balb/c strain mice immunized with SMX-NO, which are predisposed toward the development of Th2 responses (*64*), antigen-stimulated T-cells display some Th2-like features (high IL-5 production) as well as variable levels of IFN-γ secretion. TNF-α is only occasionally secreted following antigen stimulation (*46, 65, 66*). Data presented herein using the same mouse strain demonstrate that SMX-NO (**4a**)-, SP-NO (**4b**)-, and SD-NO (**4c**)-specific T-cells from immunized mice secreted a mixed Th1/Th2 cytokine profile. Furthermore, splenocytes from SMX-NO (**4a**)- and SP-

NO (**4b**)-immunized mice, but not those from SD-NO (**4c**)-immunized mice, secreted GM-CSF, a cytokine involved in phagocyte production and the differentiation of Langerhans cells into mature dendritic cells.

In conclusion, our data show that splenocytes from mice immunized against the synthetic nitroso derivative of a given antimicrobial (arylamine) benzenesulfonamide can recognize in vitro the nitroso derivative of another antimicrobial sulfonamide. In addition, the inhibitory effect of glutathione on this in vitro proliferation supports the potential role of drug−protein adduct formation in sulfonamide-specific T cell proliferation. These data support the hapten hypothesis of immune recognition of drugs by immune cells and suggest that the response of T-cells from allergic human donors against noncovalently associated parent drugs may be a consequence of T-cell receptor cross-reactivity. They provide additional justification for the contraindication of structurally related antimicrobial sulfonamides in sulfonamide allergic patients.

References

(1) Pirmohamed, M., James, S., Meakin, S., Green, C., Scott, A. K., Walley, T. J., Farrar, K., Park, B. K., and Breckenridge, A. M. (2004) Adverse drug reactions as cause of admission to hospital: Prospective analysis of 18 820 patients. *BMJ 329*, 15–19.

(2) Pirmohamed, M., and Park, B. K. (2001) Genetic susceptibility to adverse drug reactions. *Trends Pharmacol. Sci. 22*, 298–305.

(3) Park, K., Williams, D. P., Naisbitt, D. J., Kitteringham, N. R., and Pirmohamed, M. (2005) Investigation of toxic metabolites during drug development. *Toxicol. Appl. Pharmacol. 207*, 425–434.

(4) Smith, D. A., Obach, R. S., Williams, D. P., and Park, B. K. (2009) Clearing the MIST (metabolites in safety testing) of time: The impact of duration of administration on drug metabolite toxicity. *Chem.-Biol. Interact. 179*, 60–67.

(5) Pichler, W. J. (2003) Delayed drug hypersensitivity reactions. *Ann. Intern. Med. 139*, 683–693.

(6) Lavergne, S. N., Park, B. K., and Naisbitt, D. J. (2008) The roles of drug metabolism in the pathogenesis of T-cell-mediated drug hypersensitivity. *Curr. Opin. Allergy Clin. Immunol. 8*, 299–307.

(7) Wu, Y., Farrell, J., Pirmohamed, M., Park, B. K., and Naisbitt, D. J. (2007) Generation and characterization of antigen-specific CD4+, CD8+, and CD4+CD8+ T-cell clones from patients with carbamazepine hypersensitivity. *J. Allergy Clin. Immunol. 119*, 973–981.

(8) Beeler, A., Engler, O., Gerber, B. O., and Pichler, W. J. (2006) Long-lasting reactivity and high frequency of drug-specific T cells after severe systemic drug hypersensitivity reactions. *J. Allergy Clin. Immunol. 117*, 455–462.

(9) Nassif, A., Bensussan, A., Boumsell, L., Deniaud, A., Moslehi, H., Wolkenstein, P., Bagot, M., and Roujeau, J. C. (2004) Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells. *J. Allergy Clin. Immunol. 114*, 1209–1215.

(10) Masters, P. A., O'Bryan, T. A., Zurlo, J., Miller, D. Q., and Joshi, N. (2003) Trimethoprim-sulfamethoxazole revisited. *Arch. Intern. Med. 163*, 402–410.

(11) Cribb, A. E., and Spielberg, S. P. (1990) An in vitro investigation of predisposition to sulphonamide idiosyncratic toxicity in dogs. *Vet. Res. Commun. 14*, 241–252.

(12) Gill, H. J., Hough, S. J., Naisbitt, D. J., Maggs, J. L., Kitteringham, N. R., Pirmohamed, M., and Park, B. K. (1997) The relationship between the disposition and immunogenicity of sulfamethoxazole in the rat. *J. Pharmacol. Exp. Ther. 282*, 795–801.

(13) Roychowdhury, S., Vyas, P. M., Reilly, T. P., Gaspari, A. A., and Svensson, C. K. (2005) Characterization of the formation and localization of sulfamethoxazole and dapsone-associated drug-protein adducts in human epidermal keratinocytes. *J. Pharmacol. Exp. Ther. 314*, 43–52.

(14) Sanderson, J. P., Naisbitt, D. J., Farrell, J., Ashby, C. A., Tucker, M. J., Rieder, M. J., Pirmohamed, M., Clarke, S. E., and Park, B. K.

(2007) Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. *J. Immunol. 178*, 5533–5542.

(15) Naisbitt, D. J., O'Neill, P. M., Pirmohamed, M., and Park, B. K. (1996) Synthesis and reactions of nitroso sulfamethoxazole with biological nucleophiles—Implications for immune-mediated toxicity. *Bioorg. Med. Chem. Lett. 6*, 1511–1516.

(16) Cribb, A. E., Miller, M., Leeder, J. S., Hill, J., and Spielberg, S. P. (1991) Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione. Implications for idiosyncratic toxicity. *Drug Metab. Dispos. 19*, 900–906.

(17) Naisbitt, D. J., Hough, S. J., Gill, H. J., Pirmohamed, M., Kitteringham, N. R., and Park, B. K. (1999) Cellular disposition of sulphamethoxazole and its metabolites: implications for hypersensitivity. *Br. J. Pharmacol. 126*, 1393–1407.

(18) Trepanier, L. A., Yoder, A. R., Bajad, S., Beckwith, M. D., Bellehumeur, J. L., and Graziano, F. M. (2004) Plasma ascorbate deficiency is associated with impaired reduction of sulfamethoxazole-nitroso in HIV infection. *JAIDS, J. Acquired Immune Defic. Syndr. 36*, 1041–1050.

(19) Naisbitt, D. J., Farrell, J., Gordon, S. F., Maggs, J. L., Burkhart, C., Pichler, W. J., Pirmohamed, M., and Park, B. K. (2002) Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Mol. Pharmacol. 62*, 628–637.

(20) Cheng, L., Stewart, B. J., You, Q., Petersen, D. R., Ware, J. A., Piccotti, J. R., Kawabata, T. T., and Ju, C. (2008) Covalent binding of the nitroso metabolite of sulfamethoxazole is important in induction of drug-specific T-cell responses in vivo. *Mol. Pharmacol. 73*, 1769–1775.

(21) Meekins, C. V., Sullivan, T. J., and Gruchalla, R. S. (1994) Immunochemical analysis of sulfonamide drug allergy: Identification of sulfamethoxazole-substituted human serum proteins. *J. Allergy Clin. Immunol. 94*, 1017–1024.

(22) Gruchalla, R. S., Pesenko, R. D., Do, T. T., and Skiest, D. J. (1998) Sulfonamide-induced reactions in desensitized patients with AIDS—The role of covalent protein haptenation by sulfamethoxazole. *J. Allergy Clin. Immunol. 101*, 371–378.

(23) Callan, H. E., Jenkins, R. E., Maggs, J. L., Lavergne, S. N., Clarke, S. E., Naisbitt, D. J., and Park, B. K. (2009) Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and proteins: Implications for hapten formation. *Chem. Res. Toxicol. 22*, 937–948.

(24) Cribb, A. E., Nuss, C. E., Alberts, D. W., Lamphere, D. B., Grant, D. M., Grossman, S. J., and Spielberg, S. P. (1996) Covalent binding of sulfamethoxazole reactive metabolites to human and rat liver subcellular fractions assessed by immunochemical detection. *Chem. Res. Toxicol. 9*, 500–507.

(25) Naisbitt, D. J., Gordon, S. F., Pirmohamed, M., Burkhart, C., Cribb, A. E., Pichler, W. J., and Park, B. K. (2001) Antigenicity and immunogenicity of sulphamethoxazole: Demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. *Br. J. Pharmacol. 133*, 295–305.

(26) Manchanda, T., Hess, D., Dale, L., Ferguson, S. G., and Rieder, M. J. (2002) Haptenation of sulfonamide reactive metabolites to cellular proteins. *Mol. Pharmacol. 62*, 1011–1026.

(27) Farrell, J., Naisbitt, D. J., Drummond, N. S., Depta, J. P., Vilar, F. J., Pirmohamed, M., and Park, B. K. (2003) Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans. *J. Pharmacol. Exp. Ther. 306*, 229–237.

(28) Choquet-Kastylevsky, G., Santolaria, N., Tedone, R., Aujoulat, M., and Descotes, J. (2001) Induction of delayed-type hypersensitivity to sulfamethoxazole in mice: Role of metabolites. *Toxicol. Lett. 119*, 183–192.

(29) Burkhart, C., von Greyerz, S., Depta, J. P., Naisbitt, D. J., Britschgi, M., Park, K. B., and Pichler, W. J. (2001) Influence of reduced glutathione on the proliferative response of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human CD4+ T-cells. *Br. J. Pharmacol. 132*, 623–630.

(30) Schnyder, B., Burkhart, C., Schnyder-Frutig, K., von Greyerz, S., Naisbitt, D. J., Pirmohamed, M., Park, B. K., and Pichler, W. J. (2000) Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. *J. Immunol. 164*, 6647–6654.

(31) Chamberlin, K. W., and Silverman, A. R. (2009) Celecoxib-associated anaphylaxis. *Ann. Pharmacother. 43*, 777–781.

(32) Depta, J. P., Altznauer, F., Gamerdinger, K., Burkhart, C., Weltzien, H. U., and Pichler, W. J. (2004) Drug interaction with T-cell receptors: T-cell receptor density determines degree of cross-reactivity. *J. Allergy Clin. Immunol. 113*, 519–527.

(33) Dibbern, D. A., Jr., and Montanaro, A. (2008) Allergies to sulfonamide antibiotics and sulfur-containing drugs. *Ann. Allergy Asthma Immunol. 100*, 91–100; quiz 100−103, 111.

(34) Smith, D. A., and Jones, R. M. (2008) The sulfonamide group as a structural alert: A distorted story? *Curr. Opin. Drug Discovery Dev. 11*, 72–79.

(35) Smith, W. B., and Mullins, R. J. (2007) No proof of cross-reactivity between celecoxib and antibiotic sulfonamides. *Intern. Med. J. 37*, 347–348.

(36) von Greyerz, S., Bultemann, G., Schnyder, K., Burkhart, C., Lotti, B., Hari, Y., and Pichler, W. J. (2001) Degeneracy and additional alloreactivity of drug-specific human alpha beta(+) T cell clones. *Int. Immunol. 13*, 877–885.

(37) von Greyerz, S., Zanni, M. P., Frutig, K., Schnyder, B., Burkhart, C., and Pichler, W. J. (1999) Interaction of sulfonamide derivatives with the TCR of sulfamethoxazole-specific human alpha beta+ T cell clones. *J. Immunol. 162*, 595–602.

(38) Schmid, D. A., Depta, J. P., Luthi, M., and Pichler, W. J. (2006) Transfection of drug-specific T-cell receptors into hybridoma cells: Tools to monitor drug interaction with T-cell receptors and evaluate cross-reactivity to related compounds. *Mol. Pharmacol. 70*, 356–365.

(39) Ayyangar, N. R., Lugade, A. G., Nikrad, P. V., and Sharma, V. K. (1981) Catalytic reduction of nitroarenes with hydrazine hydrate in suitable solvents. *Synthesis* 640.

(40) Johnstone, R. A. W., Wilby, A. H., and Entwistle, I. D. (1985) Heterogeneous catalytic transfer hydrogenation and its relation to other methods for reduction of organic compounds. *Chem. Rev. 85*, 129–170.

(41) Ayyangar, N. R., Brahme, K. C., Kalkote, U. R., and Srinivasan, K. V. (1984) Facile transfer-reduction of nitroarenes to N-arylhydroxylamines with hydrazine in the presence of Raney-nickel. *Synthesis* 938–941.

(42) Entwistle, I. D., Gilkerson, T., Johnstone, R. A. W., and Telford, R. P. (1978) Rapid catalytic transfer reduction of aromatic nitro compounds to hydroxylamines. *Tetrahedron 34*, 213–215.

(43) Entwistle, I. D., Jackson, A. E., Johnstone, R. A. W., and Telford, R. P. (1977) Reduction of nitro-compounds. *J. Chem. Soc., Perkin Trans. 1*, 443–444.

(44) Naisbitt, D. J., O'Neill, P. M., Pirmohamed, M., and Park, K. B. (1996) Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: Implications for immune mediated toxicity. *Bioorg. Med. Chem. Lett. 6*, 1511–1516.

(45) Gowenlock, B. G., and Richter-Addo, G. B. (2004) Preparations of C-nitroso compounds. *Chem. Rev. 104*, 3315–3340.

(46) Mauri-Hellweg, D., Bettens, F., Mauri, D., Brander, C., Hunziker, T., and Pichler, W. J. (1995) Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J. Immunol. 155*, 462–472.

(47) Schaerli, P., Britschgi, M., Keller, M., Steiner, U. C., Steinmann, L. S., Moser, B., and Pichler, W. J. (2004) Characterization of human T cells that regulate neutrophilic skin inflammation. *J. Immunol. 173*, 2151–2158.

(48) Wu, Y., Sanderson, J. P., Farrell, J., Drummond, N. S., Hanson, A., Bowkett, E., Berry, N., Stachulski, A. V., Clarke, S. E., Pichler, W. J., Pirmohamed, M., Park, B. K., and Naisbitt, D. J. (2006) Activation of T cells by carbamazepine and carbamazepine metabolites. *J. Allergy Clin. Immunol. 118*, 233–241.

(49) Landsteiner, K., and Jacobs, J. (1935) Studies on the sensitization of animals with simple chemical compounds. *J. Exp. Med. 61*, 643–656.

(50) Park, B. K., Pirmohamed, M., and Kitteringham, N. R. (1998) Role of drug disposition in drug hypersensitivity: A chemical, molecular and clinical perspective. *Chem. Res. Toxicol. 9*, 969–988.

(51) Summan, M., and Cribb, A. E. (2002) Novel non-labile covalent binding of sulfamethoxazole reactive metabolites to cultured human lymphoid cells. *Chem.-Biol. Interact. 142*, 155–173.

(52) Engler, O. B., Strasser, I., Naisbitt, D. J., Cerny, A., and Pichler, W. J. (2004) A chemically inert drug can stimulate T cells in vitro by their T cell receptor in non-sensitised individuals. *Toxicology 197*, 47–56.

(53) McLeod, R., Khan, A. R., Noble, G. A., Latkany, P., Jalbrzikowski, J., and Boyer, K. (2006) Severe sulfadiazine hypersensitivity in a child with reactivated congenital toxoplasmic chorioretinitis. *Pediatr. Infect. Dis. J. 25*, 270–272.

(54) Winter, H. R., and Unadkat, J. D. (2005) Identification of cytochrome P450 and arylamine N-acetyltransferase isoforms involved in sulfadiazine metabolism. *Drug Metab. Dispos. 33*, 969–976.

(55) Pirmohamed, M., Coleman, M. D., Hussain, F., Breckenridge, A. M., and Park, B. K. (1991) Direct and metabolism-dependent toxicity of sulphasalazine and its principal metabolites towards human erythrocytes and leucocytes. *Br. J. Clin. Pharmacol. 32*, 303–310.

(56) Reilly, T. P., Bellevue, F. H., 3rd, Woster, P. M., and Svensson, C. K. (1998) Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. *Biochem. Pharmacol. 55*, 803–810.

(57) Pirmohamed, M., Coleman, M. D., Hussain, F., Breckenridge, A. M., and Park, B. K. (1991) Direct and metabolism-dependent toxicity of sulphasalazine and its principal metabolites towards human erythrocytes and leucocytes. *Br. J. Clin. Pharmacol. 32*, 303–310.

(58) Rieder, M. J., Uetrecht, J., Shear, N. H., and Spielberg, S. P. (1988) Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides. *J. Pharmacol. Exp. Ther. 244*, 724–728.

(59) Lavergne, S. N., Kurian, J. R., Bajad, S. U., Maki, J. E., Yoder, A. R., Guzinski, M. V., Graziano, F. M., and Trepanier, L. A. (2006) Roles of endogenous ascorbate and glutathione in the cellular reduction and cytotoxicity of sulfamethoxazole-nitroso. *Toxicology 222*, 25–36.

(60) Mitra, A. K., Thummel, K. E., Kalhorn, T. F., Kharasch, E. D., Unadkat, J. D., and Slattery, J. T. (1996) Inhibition of sulfamethoxazole hydroxylamine formation by fluconazole in human liver microsomes and healthy volunteers. *Clin. Pharmacol. Ther. 59*, 332–340.

(61) Gill, H. J., Maggs, J. L., Madden, S., Pirmohamed, M., and Park, B. K. (1996) The effect of fluconazole and ketoconazole on the metabolism of sulphamethoxazole. *Br. J. Clin. Pharmacol. 42*, 347–353.

(62) Dearman, R. J., Warbrick, E. V., Skinner, R., and Kimber, I. (2002) Cytokine fingerprinting of chemical allergens: Species comparisons and statistical analyses. *Food Chem. Toxicol. 40*, 1881–1892.

(63) Dearman, R. J., Moussavi, A., Kemeny, D. M., and Kimber, I. (1996) Contribution of CD4+ and CD8+ T lymphocyte subsets to the cytokine secretion patterns induced in mice during sensitization to contact and respiratory chemical allergens. *Immunology 89*, 502–510.

(64) von Stebut, E., and Udey, M. C. (2004) Requirements for Th1-dependent immunity against infection with Leishmania major. *Microbes Infect. 6*, 1102–1109.

(65) Hopkins, J. E., Naisbitt, D. J., Humphreys, N., Dearman, R. J., Kimber, I., and Park, B. K. (2005) Exposure of mice to the nitroso metabolite of sulfamethoxazole stimulates interleukin 5 production by CD4+ T-cells. *Toxicology 206*, 221–231.

(66) Lochmatter, P., Beeler, A., Kawabata, T. T., Gerber, B. O., and Pichler, W. J. (2009) Drug-specific in vitro release of IL-2, IL-5, IL-13 and IFN-gamma in patients with delayed-type drug hypersensitivity. *Allergy 64*, 1269–1278.