Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 70 (2005) 275–286
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

# Reactive oxygen species generation and its role in the differential cytotoxicity of the arylhydroxylamine metabolites of sulfamethoxazole and dapsone in normal human epidermal keratinocytes

Piyush M. Vyas [a,b], Sanjoy Roychowdhury [a], Patrick M. Woster [b], Craig K. Svensson [a,b,*]

[a] *Division of Pharmaceutics, College of Pharmacy, The University of Iowa, 115 S. Grand Avenue, S213 PHAR, Iowa City, IA 52242, USA*
[b] *Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI, USA*

Received 19 February 2005; accepted 20 April 2005

**Abstract**

Cutaneous drug reactions (CDR) are responsible for numerous minor to life-threatening complications. Though the exact mechanism for CDR is not completely understood, evidence suggests that bioactivation of drugs to reactive oxygen or nitrogen species is an important factor in the initiation of these reactions. Several CDR-inducing drugs having an arylamine functional group, such as sulfamethoxazole (SMX) and dapsone (DDS), undergo bioactivation to reactive arylhydroxylamine metabolites. These metabolites can generate cellular oxidative stress by forming reactive oxygen species (ROS). Several studies have demonstrated a higher cytotoxicity with DDS hydroxylamine (DDS-NOH) compared to SMX hydroxylamine (SMX-NOH). To investigate the role of differential ROS generation in the higher cytotoxicity of DDS-NOH, hydroxylamine metabolites of SMX and DDS were synthesized and ROS formation by these metabolites determined. DDS-NOH and its analogues/metabolites consistently resulted in higher ROS formation as compared to SMX-NOH. However, comparison of the ROS generation and cytotoxicity of a series of arylhydroxylamine analogues of DDS did not support a simple correlation between ROS generation and cell death. Numerous ROS scavengers were found to reduce metabolite-induced ROS formation, with differences in the potency between the agents. The decrease in DDS-NOH-induced ROS generation in NHEK with ascorbic acid, *N*-acetylcysteine, Trolox, and melatonin was 87, 86, 44, and 16%, respectively. Similarly, the cytotoxicity and adduct formation of DDS-NOH in NHEK was reduced in the presence of ascorbic acid. In summary, these studies show that arylhydroxylamine metabolites of SMX/DDS induce ROS generation in NHEK, though such generation is not directly related to cytotoxicity.


## 1. Introduction

Sulfamethoxazole (SMX) is an effective therapeutic agent in the treatment of *Pneumocystis carinii* pneumonia (PCP), resulting in the recovery of approximately 75% of patients suffering from this ailment [1,2]. Dapsone (DDS) is a useful alternative treatment for PCP, used either alone or in combination with other agents, in patients that cannot tolerate SMX [3–5]. Introduction of the use of these agents for the treatment of PCP in patients infected with the human immunodeficiency virus (HIV) was associated with a high level of adverse reactions, including delayed-type hypersensitivity reactions [6–10]. Numerous studies have suggested that these idiosyncratic reactions result from the bioactivation of these agents to arylhydroxylamine metabolites [11–13].

The in vitro cytotoxicity of arylhydroxylamine metabolites of SMX (SMX-NOH) and DDS (DDS-NOH) towards peripheral blood mononuclear cells (PBMCs) has been proposed as a marker for delayed-type hypersensitivity reactions associated with these drugs [14–16]. Although SMX-NOH and DDS-NOH both cause concentration-dependent increases in cell death, DDS-NOH is significantly more toxic [17]. The in vitro cytotoxicity in human PBMCs exhibits an $LC_{50}$ of 325 μM for DDS-NOH and 1752 μM for SMX-NOH. Studies in normal human epidermal keratinocytes (NHEK) demonstrate an $LC_{50}$ of 293 μM for DDS-NOH and $LC_{50}$ of greater than 1500 μM for SMX-NOH [18]. We have recently observed similar differences in the cytotoxicity of these two arylhydroxylamines in normal human dermal fibroblasts (unpublished observations). Differential toxicity is also observed with methemoglobin formation in vitro, with an $EC_{50}$ of 26.5 μM for DDS-NOH and of 463 μM for SMX-NOH in human red blood cells [19].

* Corresponding author. Tel.: +1 319 335 8823; fax: +1 319 335 9349.
*E-mail address:* craig-svensson@uiowa.edu (C.K. Svensson).

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.04.023

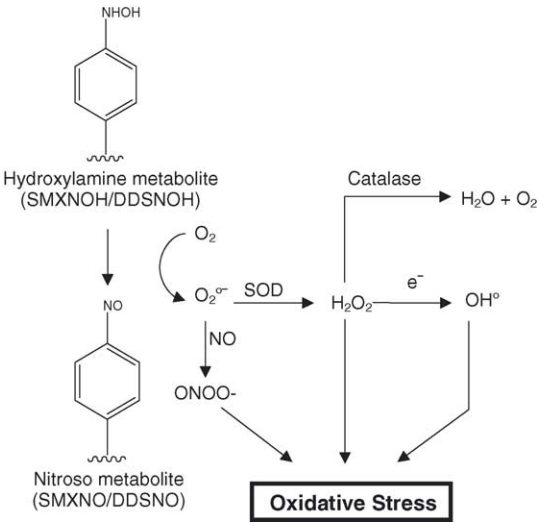



Fig. 1. Scheme for ROS generation and increase in oxidative stress by arylhydroxylamines. DDS-NO, nitroso metabolite of DDS; SMX-NO, nitroso metabolite of SMX; SOD, superoxide dismutase.

Since exposure of cells to SMX-NOH and DDS-NOH have been shown to cause a significant depletion of the major cellular antioxidant (glutathione) [18], ROS accumulation following metabolite exposure may play a causal role in the cytotoxicity observed with these compounds. As other xenobiotics have been shown to be cytotoxic due to their ability to generate reactive oxygen species (ROS) [20–23], we hypothesized that the differential cytotoxicity of DDS-NOH and SMX-NOH may be due to differences in their ability to induce ROS. Arylhydroxylamines such as SMX-NOH and DDS-NOH auto-oxidize to their respective nitroso compounds (Fig. 1). During this auto-oxidation, molecular oxygen reduces to superoxide, which is subsequently reduced to hydrogen peroxide ($H_2O_2$) either spontaneously or catalyzed by superoxide dismutase (SOD). The formed $H_2O_2$ undergoes the Fenton reaction to form highly cytotoxic hydroxide radicals [24,25].

We hypothesized that ROS formation is directly correlated to the presence of hydroxylamine (NHOH) functional groups in the molecule. Thus, compounds having the potential to form two NHOH functional groups may generate a higher level of intracellular ROS. As DDS has two primary amine groups in its molecular structure, while SMX has only one, the former has the *potential* to give rise to a metabolite with two hydroxylamines (Fig. 2). Thus, to probe the proposed hypothesis, in addition to SMX-NOH and DDS-NOH, we synthesized three hydroxylamine metabolites/analogues of DDS: DDS-dihydroxylamine (DDS-diNOH), 4-nitrophenyl-*p*-tolyl sulfone hydroxylamine (NPTS-NOH) and monoacetyl dapsone hydroxylamine (MADDS-NOH) (Fig. 3). The cytotoxicity and ability to form ROS was then determined for each of these compounds.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Chemicals for synthesis, including 4-nitrobenzene sulfonyl chloride, 3-amino-5-methyl-isoxazole, 4-(4-nitrophenyl sulfonyl)-aniline, 4-nitrophenylsulfone, 4-nitrophenyl-*p*-tolylsulfone (NPTS), 4-acetamidophenyl-4′-nitrophenyl sulfone, triethyl phosphite, and platinum oxide, were obtained from Sigma–Aldrich (St. Louis, MO). Rabbit anti-sera were raised against SMX- and DDS-keyhole limpet hemocyanine conjugates and characterized as described previously [18]. Microtiter ELISA plates (96 well) were obtained from Rainin Instruments (Woburn, MA). Goat anti-rabbit antibody conjugated with alkaline phosphatase was purchased from Molecular Probes (Eugene, OR), as was the fluorescent dye Yo-Yo-1. Bradford assay reagent was purchased from PIERCE (Rockford, IL). Trolox was obtained from Oxis International Inc. (Portland, OR). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) or Fisher Scientific (Chicago, IL).

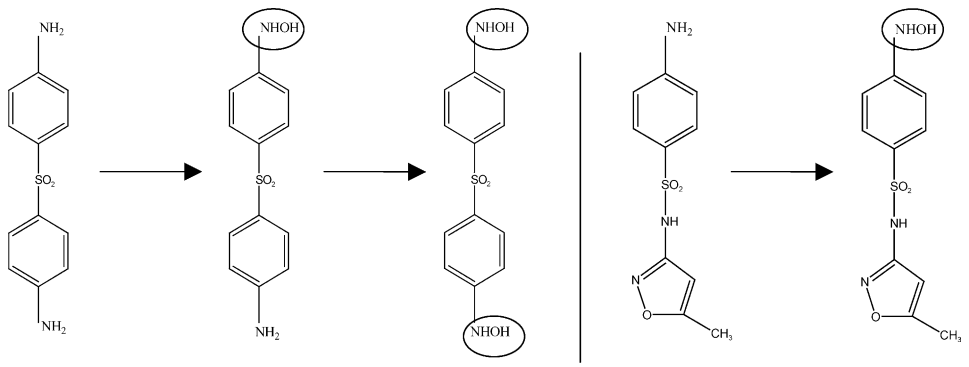
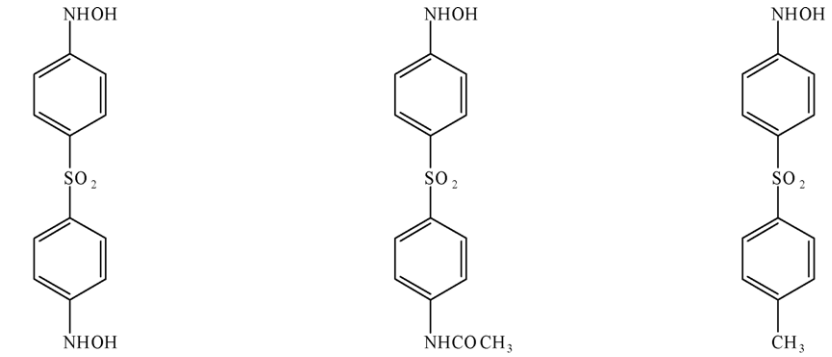



Fig. 2. Identified and potential metabolites of DDS and SMX which may give rise to ROS. To date, DDS-NOH and SMX-NOH have been isolated from humans, while dapsone dihydroxylamine (DDS-diNOH) has not.




**DDS-diNOH** **MADDS-NOH** **NPTS-NOH**

Fig. 3. Structures of dapsone dihydroxylamine (DDS-diNOH), monoacetyldapsone hydroxylamine (MADDS-NOH), and 4-nitrophenyl-*p*-tolyl sulfone hydroxylamine (NPTS-NOH).

### 2.2. Synthesis of arylhydroxylamine metabolites of SMX and DDS

SMX-NOH and DDS-NOH were synthesized as described previously, with modifications to the purification methods [14,17,26]. Briefly, 4-nitrobenzene sulfonyl chloride (4-NSC, 1 mol) was mixed with 3-amino-5-methylisoxazole (3-A-5-MI, 1 mol) to obtain the nitro derivative of SMX (Nitro-SMX) which was recrystallized with ethyl acetate and toluene (1:1). Nitro-SMX (4 mmol) was reduced to SMX-NOH using triethyl phosphite and platinum oxide in a hydrogenator at 40 psi for 4 h. The synthesized SMX-NOH was purified by preparative TLC using hexane:chloroform:methanol (25:69:6). For synthesis of DDS-NOH, 4-(4-nitrophenyl sulfonyl)-aniline (NPSA, 7.12 mmol) was reduced as described above. For purification, DDS-NOH was dissolved in water:ethyl acetate (50:50) and the mixture acidified by hydrochloric acid in a separatory funnel. The acidified water layer containing pure DDS-NOH was separated and then neutralized by addition of sodium carbonate. DDS-NOH was then extracted in ethyl acetate from the water layer and evaporated to dryness to obtain a light yellow solid compound.

For the synthesis of DDS-diNOH, NPTS-NOH and MADDS-NOH, 4-nitrophenylsulfone, 4-nitrophenyl-*p*-tolylsulfone (NPTS) and 4-acetamidophenyl-4′-nitrophenyl sulfone were reduced using triethyl phosphite and platinum oxide as a reducing agent in a hydrogenator. The purity and identity of all metabolites was confirmed by NMR, MS and HPLC techniques.

### 2.3. Cell culture

Adult normal human epithelial keratinocytes (NHEK) (as 1st passage cells), keratinocyte culture media, trypsin, and trypsin neutralizing solution were obtained from CAMBREX (Walkersville, MD). NHEK were grown as we have described previously [18]. Briefly, cells were cultured in 75 $cm^2$ flasks using basal media (KBM-2) supplemented with bovine pituitary extract (7.5 mg/ml), human epidermal growth factors (0.1 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), epinephrine, transferrin, gentamicin (50 μg/ml) and amphotericin (50 ng/ml) at 37 °C in an atmosphere containing 5% $CO_2$. Media was replaced every 2–3 days. When cell cultures reached near confluency (70–90%), cells were disaggregated using 0.025% Trypsin/0.01% EDTA in HEPES followed by neutralization with 2 volumes of Trypsin neutralizing solution. Cell suspension was then centrifuged, followed by washing in basal media and re-suspension in KGM-2 (supplemented growth medium). Cells were then either subjected to sub-culturing or cryo-preservation for later experiments. All experiments were performed using third to fourth passage cells.

### 2.4. Determination of ROS formation

To investigate the differential oxidative stress induced by these arylhydroxylamine metabolites, ROS formation was determined in a cell-free system or in NHEK cells using the fluorescent dye 2′-7′-dichlorodihydrofluorescein diacetate (DCHF-DA) [27–29]. For the cell-free assay, 2.5 μM of metabolite or parent drug were dissolved in dimethyl sulfoxide (DMSO) and incubated either in sodium phosphate buffer (9.5 mM, pH 7.4) or sodium phosphate buffer containing hydrogen peroxide ($H_2O_2$, 100 μM) and peroxidase (0.02 U/ml) at room temperature; followed by addition of 2′,7′-dichlorodihydrofluorescein (DCHF) (5 μM). The DCHF used for the cell-free assay was obtained by alkaline hydrolysis of DCHF-DA [27]. For the complete hydrolysis of DCHF-DA, 5 μM of DCHF-DA was dissolved in DMSO and incubated with 200 μM of sodium hydroxide (NaOH) for 30 min on ice in a dark cabinet. At the completion of the incubation period, 250 μM of sodium dihydrogen phosphate ($NaH_2PO_4$) was added to the reaction mixture. Ten microlitres of this DCHF reaction mixture was added to the final assay

volume of 200 μl in 96-well plates. The total concentration of DMSO was not more than 1% (v/v) in the final assay. ROS formation was measured by the oxidation of DCHF to 2′,7′-dichlorofluorescein (DCF), producing fluorescence which was measured every 5 min for 30 min using a multiwell fluorescence plate reader (Cytofluor®) with an excitation wavelength of 480 nm and emission wavelength of 530 nm. The rate of change in DCF fluorescence was also calculated for these incubations by determining the rate of increase in fluorescence per minute over the 30 min period from a plot of fluorescence versus time.

Metabolite-induced ROS formation in NHEK was determined by adding $10^5$ cells to each well of a 96-well plate and incubating in 200 μl of KBM-2 media at 37 °C, 5% $CO_2$ for 24 h. After 24 h, KBM-2 was replaced by 200 μl PBS (10 mM) (PBS composition—0.8% NaCl, 0.02% KCl, 0.01% $MgCl_2{\cdot}6H_2O$, 0.02% $KH_2PO_4$, 0.114% $Na_2HPO_4$, 0.01% $CaCl_2{\cdot}2H_2O$, pH 7.4). ROS formation was measured in two different conditions. First (designated as preloaded), cells were preloaded with DCHF-DA (20 μM) and after 30 min the plate was washed to discard extracellular DCHF-DA, followed by the addition of 500 μM of metabolite or parent drug dissolved in DMSO. Alternatively, 500 μM of metabolite or parent drug were added to the cells followed by the addition of 20 μM DCHF-DA (designated as postloaded). DCF fluorescence was measured as described above.

### 2.5. Effect of ROS scavengers on metabolite-induced ROS formation

The effect of melatonin, Trolox, ascorbic acid, and *N*-acetylcysteine (NAC) on the ROS formation induced by these metabolites was determined in NHEK. Briefly, $10^5$ cells were plated as mentioned above. After 24 h, melatonin, Trolox, ascorbic acid or NAC (1 mM) were added to cells and the plate was incubated for 1 h at 37 °C prior to addition of metabolites (500 μM) and DCHF-DA (20 μM). DCF fluorescence was determined as previously described. Similarly, the effect of these antioxidants in a cell-free system were evaluated using the cell-free system described previously.

### 2.6. Cytotoxicity of arylhydroxylamine metabolites and analogues

To assess the relationship between ROS generation and cytotoxicity of these arylhydroxylamines, the cytotoxicity of analogues towards NHEK was compared at an equimolar concentration (500 μM). In addition, the effect of ascorbic acid as an antioxidant on the cytotoxicity was evaluated. Cytotoxicity was determined using an impermeable DNA binding dye, as we have described previously [18]. Briefly, $10^5$ NHEK cells were plated on 96-well plates and incubated overnight in 200 μl of KGM-2 media at 37 °C, 5% $CO_2$. After 24 h, KGM-2 was replaced by KBM-2 and 1 mM ascorbic acid or vehicle and the plate incubated for 3 h. After this pre-incubation period, 500 μM of metabolite or parent drug was added and cells were incubated for an additional 3 h. After 3 h incubation with metabolites, the plate was washed once with KBM-2 followed by the addition of fresh KBM-2 containing 4 μM of Yo-Yo-1. Fluorescence was measured for 16 h in a temperature controlled fluorescent plate reader as described above. After 16 h, Triton-X (0.1%) was added to all wells and a final reading was taken after a further incubation of 3 h (total incubation time was 19 h). The cytotoxicity was determined using the following formula:

$$\%\,\text{cell death} = \frac{(\text{Yo} - \text{Yo fluorescence})_{16\,\text{h}} - (\text{Yo} - \text{Yo fluorescence})_{0\,\text{h}}}{(\text{Yo} - \text{Yo fluorescence})_{19\,\text{h}} - (\text{Yo} - \text{Yo fluorescence})_{0\,\text{h}}} \times 100$$

### 2.7. Adduct formation by arylhydroxylamine metabolites in NHEK with and without ROS scavenger

Formation of metabolite-protein adducts in NHEK following incubation with arylhydroxylamine metabolite in the presence or absence of 1 mM of ascorbic acid was determined as previously described [18]. Briefly, NHEK cells ($1 \times 10^6$ cells) were cultured for 24 h in 50 ml centrifuge tubes containing 10 ml of KGM-2. Cells were then incubated for 3 h in the presence or absence of 1 mM ascorbic acid prior to the addition of metabolite (50 μM). After 24 h, tubes were centrifuged at 220 × *g* for 5 min to pellet the cells. The supernatant containing the medium was drained off and cell pellets were lysed in 1 ml of deionized water, using repeated cycles of freezing and thawing (three times) and ultrasonication, to ensure complete lysis. The cell suspension was then thoroughly vortexed and centrifuged at 220 × *g* for 5 min and the pellet containing the cell debris discarded. The supernatant containing cellular soluble proteins was collected for protein assay and subsequent ELISA.

ELISA analysis for detection of covalent adduct formation was performed as described previously [18], with minor modifications. Following protein content measurement using the Bradford reagent kit, all samples were diluted to contain 250 μg/ml protein. An aliquot of 100 μl was adsorbed onto 96-well polystyrene microtiter plates for 16 h at 4 °C. Wells were washed three times using Tris–casein buffer (0.5% casein, 0.9% NaCl, 0.01% Thimerosal, 10 mM Tris–HCl, pH 7.6) and then blocked with Tris–casein buffer for 1 h. After an additional wash, wells were incubated for 16 h at 4 °C with 100 μl of anti-SMX or anti-DDS rabbit serum (1:500 diluted with Tris–casein buffer). Wells were subsequently washed four times with Tris–casein buffer and incubated with alkaline phosphatase conjugated goat anti-rabbit antibody (1:1000 diluted in Tris–casein buffer) for 2 h at room temperature. After washing four times with Tris–casein buffer, antibody

binding was detected with colorimetric alkaline phosphatase substrate reagent. After 1 h of incubation at room temperature, optical density was measured at 405 nm using a $V_{max}$ kinetic micro plate reader (Molecular Devices).

### 2.8. Statistical analysis

Data is presented as mean (S.D.). Data were analyzed using the Friedman rank sum test or ANOVA with the Holm–Sidak test for multiple comparisons, as appropriate. A value of $p < 0.05$ was considered significant.

## 3. Results

### 3.1. Synthesis of arylhydroxylamine metabolites of SMX and DDS

The product yield for Nitro-SMX, SMX-NOH and DDS-NOH were 95, 79 and 78%, whereas the determined purities were 99, 89 and 88%, respectively. As the obtained purities for SMX-NOH and DDS-NOH were not optimal for biological use, metabolites were further purified using preparative TLC and acidified extraction methods to obtain a purity of greater than 98% for both metabolites. Synthesized DDS-diNOH, NPTS-NOH and MADDS-NOH were found to have yields of 83, 93 and 85%, with purities of 96, 99 and 97%, respectively.

### 3.2. ROS generation by arylhydroxylamine metabolites and analogues

In a cell-free system, ROS generation was increased by all arylhydroxylamines (Fig. 4). The DCF fluorescence in the presence of SMX-NOH, DDS-NOH, DDS-diNOH, NPTS-NOH or MADDS-NOH was increased by 80, 365, 144, 355 and 395%, respectively, as compared to control. All DDS metabolites/analogues exhibited a higher level of ROS generation than SMX-NOH. DCF fluorescence was not increased in incubations containing the parent compounds. The rate of change in fluorescence for each metabolite (which was found to be linear over the 30-min incubation period) was consistent with the cumulative fluorescence observed at 30 min (Fig. 5).

To more closely mimic the intracellular conditions and to increase the catalytic cycle for the redox reaction of the oxidation of the arylhydroxylamine metabolites, while at the same time avoiding variability that might arise due to permeability differences in the metabolites, metabolites were also incubated in sodium phosphate buffer with hydrogen peroxide and peroxidase. As expected, in the presence of peroxidase/$H_2O_2$, the basal level of ROS generation was considerably higher than that observed in buffer alone. The ROS generation by SMX-NOH, DDS-NOH, DDS-diNOH, NPTS-NOH and MADDS-NOH was increased by 55, 135, 75, 165 and 150%, respectively, as compared to control (Fig. 6). While the absolute DCF fluorescence in the presence of peroxidase/$H_2O_2$ was markedly elevated compared to the results in the absence of peroxidase/$H_2O_2$ (Fig. 4), the rank order of ROS generation between metabolites was the same.

In NHEK, ROS formation in the presence of the various arylhydroxylamines at a concentration of 2.5 μM was not different from NHEK incubated with vehicle alone (data not shown), which is likely due to the high level of antioxidants in these cells (e.g., glutathione and ascorbic acid). Evaluation of a range of concentrations of SMX-NOH and DDS-NOH (5, 50 and 500 μM) showed that at a

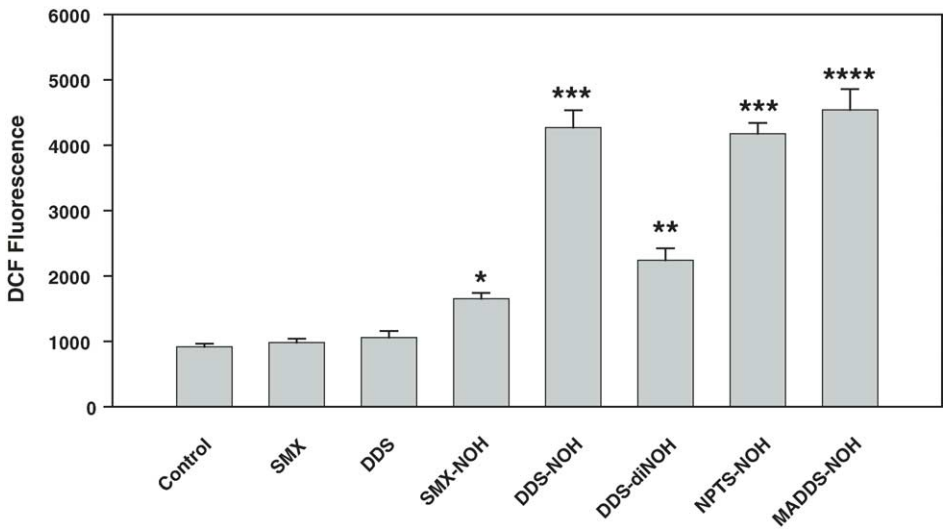



Fig. 4. Fluorescence of 2′,7′-dichlorofluorescein (DCF) in the presence of various compounds in a cell-free system. Parent compounds or metabolites (2.5 μM) were incubated in phosphate buffer to which 2′,7′-dichlorodihydrofluoroscein (5 μM) was added. Fluorescence was measured every 5 min for 30 min. Data shown are the mean (S.D.) fluorescence of five incubations for each metabolite at 30 min which were analyzed statistically using ANOVA with Holm–Sidak test for multiple comparisons. $^{*}p < 0.05$ compared to control, SMX and DDS; $^{**}p < 0.05$ compared to control, SMX, DDS and SMX-NOH; $^{***}p < 0.05$ compared to control, SMX, DDS, SMX-NOH and DDS-diNOH; $^{****}p < 0.05$ compared to control, SMX, DDS, SMX-NOH, DDS-diNOH and NPTS-NOH.

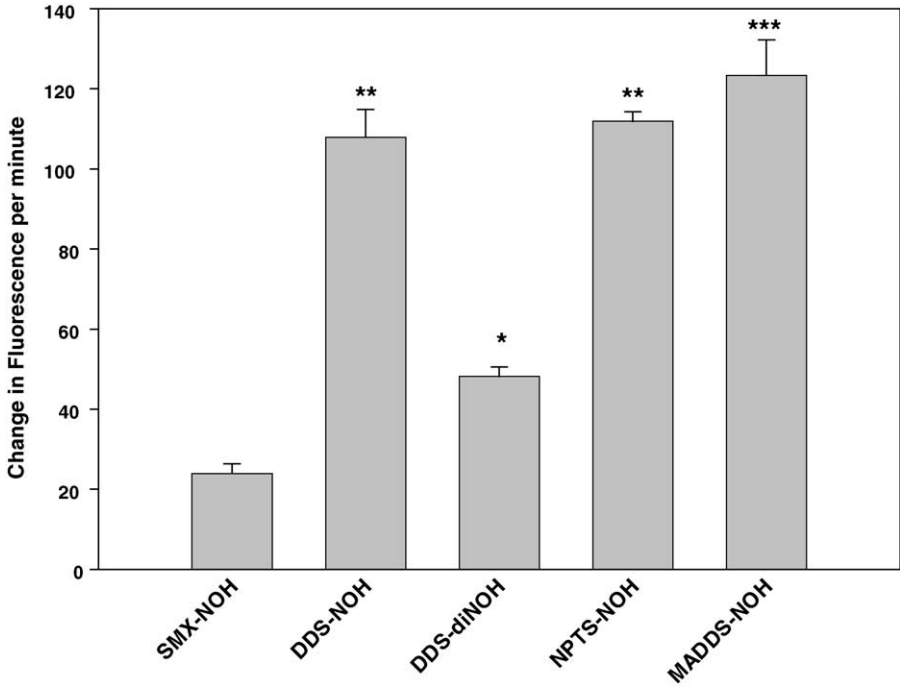



Fig. 5. Increase of DCF fluorescence per minute in presence of various compounds in a cell-free system. See Fig. 4 for experimental details. Data shown are the mean (S.D.) of the rate of change in the fluorescence measured over 30 min. Data were analyzed statistically using ANOVA with the Holm–Sidak test for multiple comparisons. $^{*}p < 0.05$ compared to SMX-NOH; $^{**}p < 0.05$ compared to SMX-NOH and DDS-diNOH; $^{***}p < 0.05$ compared to SMX-NOH, DDS-NOH, DDS-diNOH and NPTS-NOH.

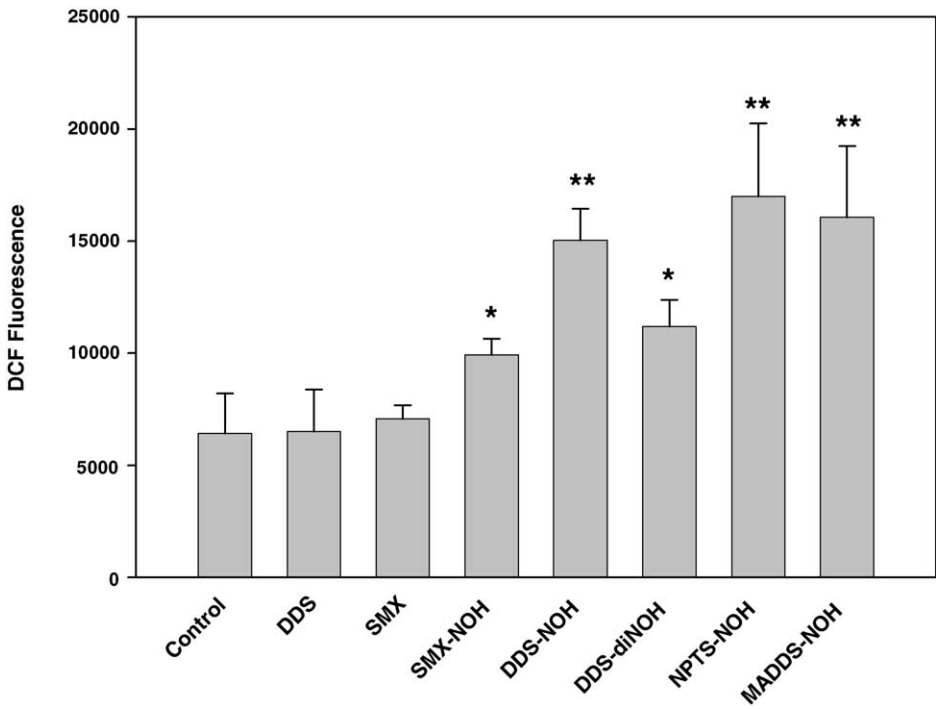



Fig. 6. Fluorescence of 2′,7′-dichlorofluorescein (DCF) in the presence of various compounds and horseradish peroxidase in a cell-free system. Parent compounds or metabolites (2.5 μM) were incubated in phosphate buffer containing $H_2O_2$ (100 μM) and horseradish peroxidase (0.02 U/mL) to which 2′,7′-dichloro-dihydrofluoroscein (5 μM) was added. Fluorescence was measured every 5 min for 30 min. Data shown are the mean (S.D.) fluorescence of five incubations at 30 min. (Note difference in scale from Fig. 4). Data were analyzed statistically using ANOVA with the Holm–Sidak test for multiple comparisons. $^{*}p < 0.05$ compared to control, SMX and DDS; $^{**}p < 0.05$ compared to control, SMX, DDS, SMX-NOH and DDS-diNOH.

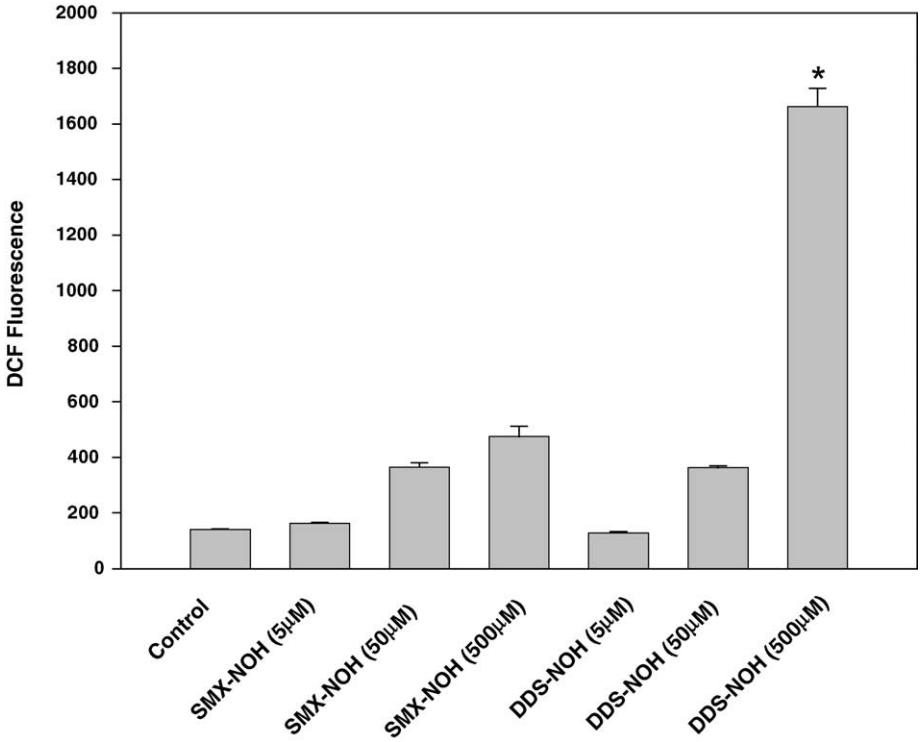



Fig. 7. Fluorescence of 2′,7′-dichlorofluorescein (DCF) in NHEK incubated with SMX-NOH and DDS-NOH. Metabolites were added to suspensions of NHEK in phosphate buffer to which 2′,7′-dichlorodihydro-fluoroscein diacetate (20 μM) was added. Fluorescence was measured every 5 min for 30 min. Data shown are the mean (S.D.) fluorescence of three incubations at 30 min and were analyzed by Friedman rank sum test. $^{*}p < 0.05$ compared to same concentration of SMX-NOH.

concentration of 500 μM, DDS-NOH formed a substantially higher amount (4.5-fold) of ROS (1079% increase as compared to control) compared to 500 μM of SMX-NOH (237% increase as compared to control). At lower concentrations, 5 μM and 50 μM, there was no significant difference in ROS formation between DDS-NOH and SMX-NOH (Fig. 7). When each of the arylhydroxylamines was added to NHEK at a concentration of 500 μM, all metabolites/analogues of DDS induced a similar level of ROS, which was at least two-fold higher than that induced by SMX-NOH (Fig. 8). Importantly, at the concentrations examined, none of the metabolites induced significant cell death within the 30 min time frame of ROS generation assessment (data not shown). Though DDS-diNOH consistently resulted in a lower level of ROS generation than other metabolites/analogues of DDS in a cell-free system, it induced a similar level of ROS in NHEK. While the ROS measured was slightly higher using postloaded DCHF than preloaded DCHF, the pattern between metabolites was similar in both paradigms.

### 3.3. Effect of various ROS scavengers on ROS formation by SMX-NOH and DDS-NOH in NHEK

To further probe the role of ROS generation in the differential cytotoxicity of SMX-NOH and DDS-NOH, we assessed the ability of various antioxidants to suppress the generation of ROS induced by arylhydroxylamine metabolites (Fig. 9). The reduction in DDS-NOH and SMX-NOH induced ROS generation in NHEK with trolox was found to be 44 and 43%, respectively. In contrast, melatonin did not reduce ROS generation compared to incubation of either metabolite alone. Ascorbic acid reduced DDS-NOH- and SMX-NOH-induced ROS generation by 87 and 69%, respectively. NAC was also found to markedly attenuate arylhydroxylamine-induced ROS generation. The effect of these antioxidants on ROS generation in a cell-free system paralleled that observed in NHEK (data not shown).

### 3.4. Cytotoxicity of arylhydroxylamine metabolites and analogues

Cytotoxicity of the compounds toward NHEK was evaluated in presence and absence of ascorbic acid (Fig. 10). As previously observed, DDS-NOH was found to cause a higher level of cell death compared to SMX-NOH at an equimolar concentration (500 μM). Among the other arylhydroxylamine analogues of DDS, NPTS-NOH was found to be highly toxic compared to all other arylhydroxylamine metabolites evaluated. Ascorbic acid attenuated, but did not completely block, the cytotoxicity of DDS-NOH and NPTS-NOH towards NHEK. Interestingly, neither MADDS-NOH or DDS-diNOH was cytotoxic at this concentration.

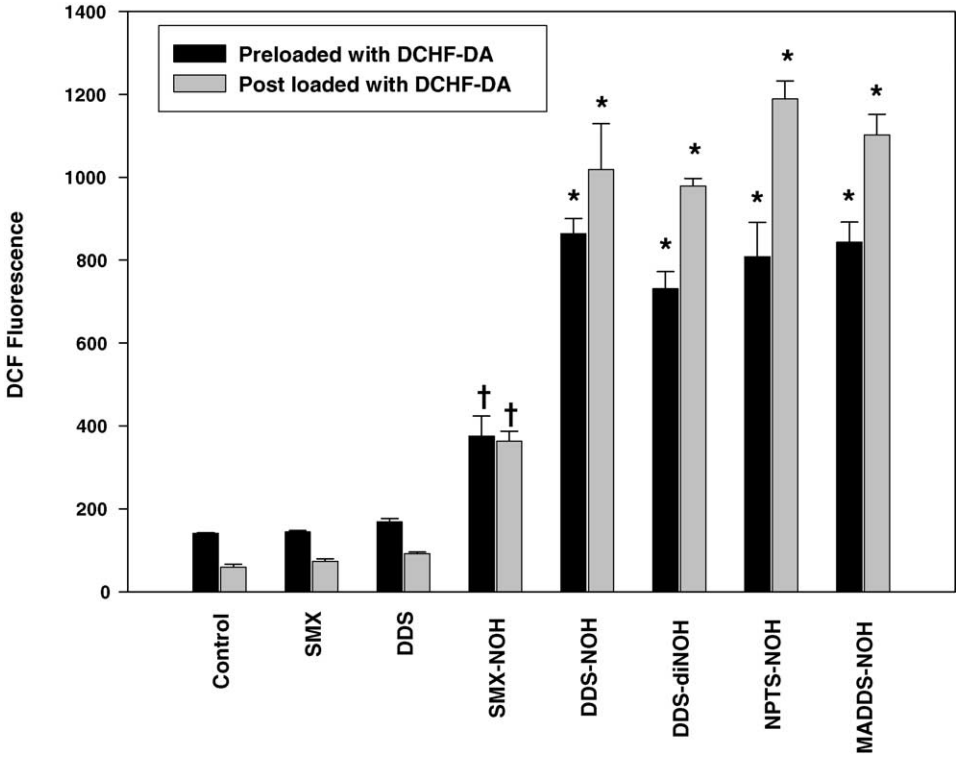



Fig. 8. Fluorescence of 2′,7′-dichlorofluorescein (DCF) in NHEK preloaded/postloaded with DCHF-DA exposed to various metabolites and analogues. NHEK cells were preloaded or postloaded with 2′,7′-dichlorodihydro-fluoroscein diacetate (20 μM) and exposed to metabolites (500 μM) in phosphate buffer. Fluorescence was measured every 5 min for 30 min. Data shown are the mean (S.D.) fluorescence of three incubations at 30 min and were analyzed statistically using ANOVA with the Holm–Sidak test for multiple comparisons. $^{*}p < 0.05$ compared to control, SMX, DDS and SMX-NOH under same condition; $^{\dagger}p < 0.05$ compared to control, SMX and DDS under same condition.

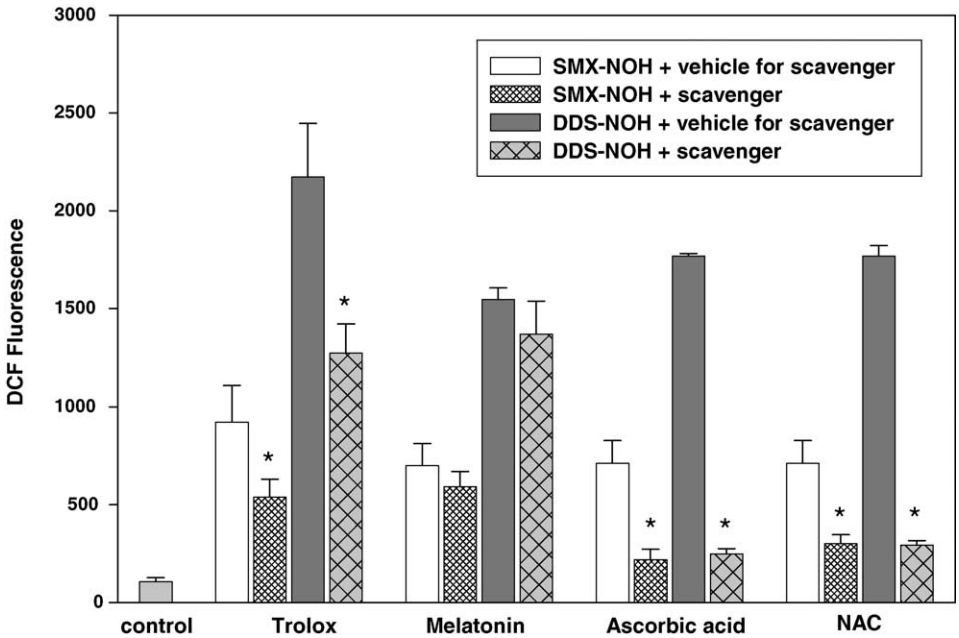



Fig. 9. ROS generation in NHEK incubated with SMX-NOH or DDS-NOH in presence and absence of ROS scavengers. ROS scavengers, Trolox, melatonin, ascorbic acid and NAC (1 mM), were added to $10^5$ NHEK prior to addition of metabolites (500 μM) and DCHF-DA (20 μM). Data presented represent the mean (S.D.) fluorescence at 30 min of three experiments with four replicates of each condition in each experiment. Data were analyzed statistically using ANOVA with the Holm–Sidak test for multiple comparisons. $^{*}p < 0.05$ compared to metabolite without scavenger.

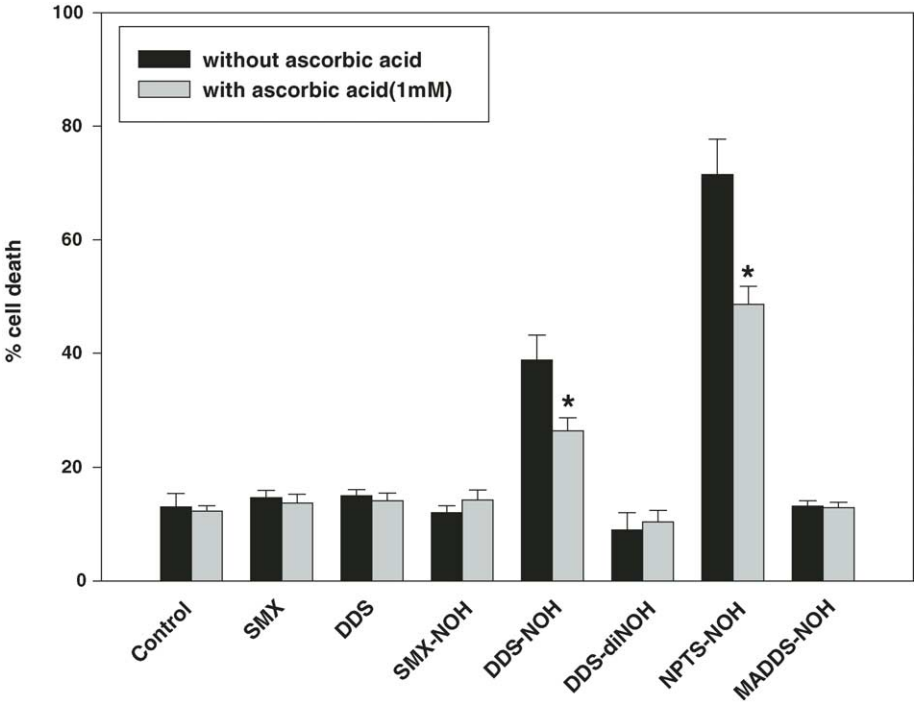



Fig. 10. Cytotoxicity of various compounds in presence and absence of ascorbic acid in NHEK. $10^5$ NHEK cells were plated on 96-well plates and 1 mM ascorbic acid or vehicle was added to cells 3 h prior to addition of 500 μM of metabolite or parent drug. After 3 h incubation with metabolites, the plate was washed with KBM-2 followed by the addition of fresh KBM-2 containing 4 μM of Yo-Yo-1. Fluorescence was measured for 16 h in a temperature controlled fluorescent plate reader followed by the addition of Triton-X (0.1%) for 3 h. The %cytotoxicity was determined as described in Section 2.6. Data presented represent the mean (S.D.) fluorescence of five replicates of each condition. Data were analyzed statistically using Friedman rank sum test. $^*p < 0.05$ compared to metabolite without ascorbic acid.

### *3.5. Adduct formation by arylhydroxylamine metabolites in NHEK with and without ROS scavenger*

Compared with SMX-NOH, DDS-NOH formed a higher amount of metabolite-protein adduct when incubated with NHEK (Fig. 11). Ascorbic acid significantly reduced, but did not prevent, the adduct formation for both of these hydroxylamine metabolites in NHEK cells.

## 4. Discussion

As several studies have suggested that the in vitro cytotoxicity of the arylhydroxylamine metabolites of sulfonamides may serve as an indicator of predisposition to hypersensitivity reactions to these compounds [15,30,31], it is of interest to note that DDS-NOH has consistently been found to be more cytotoxic in vitro than SMX-NOH in cells from normal subjects and those with a history of hypersensitivity to sulfonamides [17,32]. Importantly, the limited number of direct comparisons have found that SMX is associated with a higher frequency of hypersensitivity reactions than DDS [5,33,34]. The basis for the differential cytotoxicity between metabolites is unclear. Studies comparing the methemoglobin forming ability of these two arylhydroxylamines in purified hemoglobin suggest that DDS-NOH more readily oxidizes to reactive species [19]. Hence, we postulated that differences in ROS generation between the metabolites may explain the observed differences in cytotoxicity.

As shown in Fig. 2, both SMX and DDS possess arylamine functional groups that are capable of undergoing catalytic oxidation to the corresponding arylhydroxylamine; both of which have been confirmed as metabolites in man after administration of the respective parent compound [35–40]. In contrast to SMX, DDS possesses a second arylamine group that may undergo oxidation to yield a di-arylhydroxylamine. As some cells (e.g., NHEK) in which DDS-NOH has been shown to exhibit a higher level of cytotoxicity have been demonstrated to be capable of bioactivating SMX and DDS to their respective arylhydroxylamine metabolites, the potential exists for further oxidation of DDS-NOH to its di-arylhydroxylamine. As such a metabolite would possess two functional groups capable of giving rise to ROS (Fig. 1), this is a potential mechanism for the enhanced toxicity of DDS-NOH in this cell type. Hence, in addition to SMX-NOH and DDS-NOH, we synthesized this potential metabolite of DDS. To assess the impact of functional groups that alter the reactivity of the arylhydroxylamine, we also examined two additional metabolites/analogues of DDS-NOH. MADDS-NOH is a metabolite of DDS that has been isolated in man [35], as well as being formed in vitro in liver microsomes [41]. NPTS-NOH is an analogue of DDS-NOH in which the opposing amine has been replaced by a methyl group (Fig. 3).

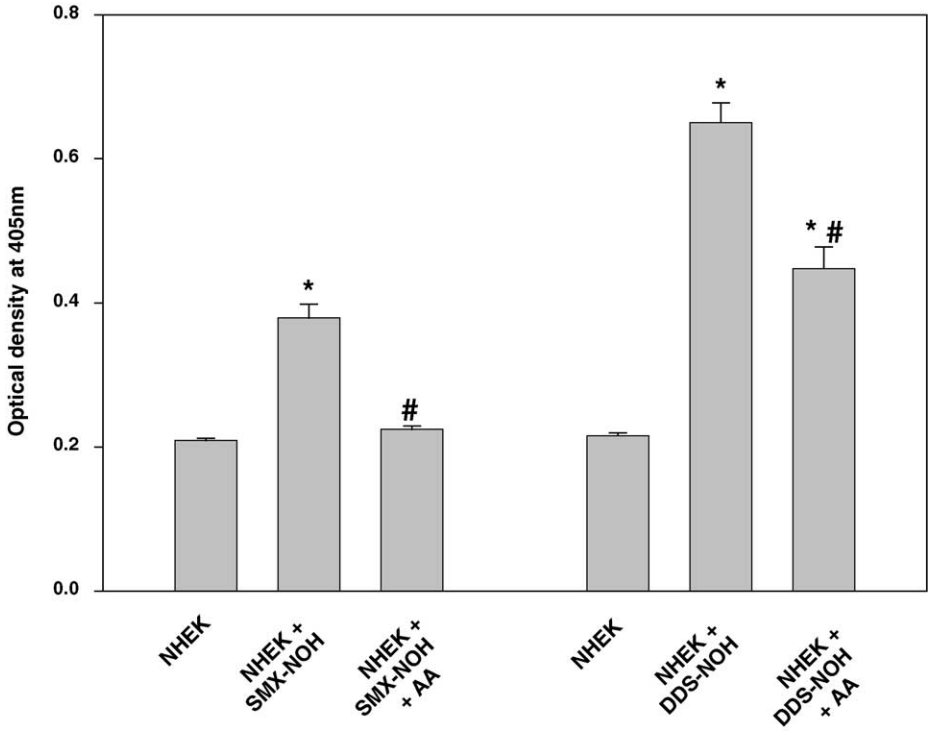



Fig. 11. Metabolite-protein adducts formation by SMX-NOH and DDS-NOH in presence and absence of ascorbic acid (AA) in NHEK. $10^6$ NHEK cells were grown for 24 h in 50 ml centrifuge tubes containing 10 ml of complete growth medium (KGM-2). Cells were then incubated for 3 h in presence or absence of 1 mM ascorbic acid (AA) prior to the addition of metabolite (50 μM). After 24 h, tubes were centrifuged and the cell pellets were lysed in 1 ml of deionized water. Adducts for SMX-NOH and DDS-NOH were determined by the ELISA technique as described in Section 2.7. Data presented represent the mean (S.D.) optical density of five replicates of each condition. Data were analyzed statistically using ANOVA with the Holm–Sidak test multiple pairwise comparisons. $^{*}p < 0.05$ compared to NHEK without metabolite; $^{\#}p < 0.05$ compared to NHEK + metabolite without ascorbic acid.

As demonstrated in Figs. 4–6, there are clear differences in the ability of these metabolites/analogues to induce ROS generation in a cell-free system. Consistent with our hypothesis, ROS generation in the presence of DDS-NOH was substantially greater than that seen with SMX-NOH. MADDS-NOH and NPTS-NOH exhibited a similar level of ROS generation as that seen with DDS-NOH, while the di-arylhydroxylamine analogue induced a level of ROS more similar to that seen with SMX-NOH. This suggests that a second hydroxylamine functional group attenuates rather than enhances the formation of ROS.

The concentration of metabolite needed to induce a measurable increase in ROS in NHEK was considerably higher than that needed in a cell-free system (Fig. 7). This observation is expected, since cellular antioxidants serve as an important defence mechanism and rapidly detoxify cellular generated ROS. At a concentration of 500 μM, DDS-NOH produced substantially more ROS than that seen with SMX-NOH. Comparison of all examined metabolites at this concentration in NHEK demonstrated that each of the arylhydroxylamine metabolites/analogues of DDS formed a significantly higher amount of ROS as compared to SMX-NOH (Fig. 8). Though the ROS generation induced by DDS-diNOH was consistently lower than that seen with other metabolites/analogues of DDS in each of the cell-free systems, it resulted in a similar level of ROS generation in NHEK. This observation may be explained by differences in cellular distribution between the metabolites or the presence of an as yet unidentified enzymatic oxidation of the arylhydroxylamines.

As a component of assessing the role of ROS generation on the differential cytotoxicity of the metabolites examined, we evaluated the ability of several ROS scavengers to attenuate arylhydroxylamine-induced ROS generation in NHEK exposed to either SMX-NOH or DDS-NOH. In particular, trolox, melatonin, ascorbic acid and NAC, which have demonstrated antioxidant properties and scavenge various free radicals [42–47], were assessed for their ability to attenuate metabolite-induced ROS generation. The lack of effect of melatonin was somewhat surprising (Fig. 9), but may be due to its ability to induce superoxide dismutase [48–50]. The induction of superoxide dismutase leads to increased formation of $H_2O_2$ and subsequently to elevated levels of hydroxyl radicals in the cells. This pro-oxidant activity might counteract the generalized antioxidant activity of melatonin and thus reduce its intracellular ROS scavenging activity.

If the ROS generation is responsible for the differential cytotoxicity in NHEK cells for DDS-NOH and SMX-NOH, then the reduction in free radical formation should lead to reduced cytotoxicity. As ascorbic acid was found to be the most potent antioxidant reducing ROS generation,

we determined the cytotoxicity of the arylhydroxylamines toward NHEK cells in the presence and absence of ascorbic acid (Fig. 10). The percent cell death induced by these metabolites was not found to correlate with ROS generation. In particular, though DDS-diNOH and MADDS-NOH induced a similar level of ROS in NHEK as that seen with DDS-NOH and NPTS-NOH, neither of these former metabolites induced significant cell death at a concentration of 500 μM. At this same concentration, DDS-NOH and NPTS-NOH were clearly cytotoxic. Moreover, though DDS-NOH and NPTS-NOH induced a similar level of ROS in NHEK (Fig. 8), the later was substantially more cytotoxic than DDS-NOH (71% versus 39% mean cell death). The difference in relative potencies for ROS generation and cell death between these metabolites suggests that differences ROS generation does not explain the relative cytotoxicity of arylhydroxylamine metabolites. However, the fact that cell death was decreased under conditions of reduced ROS generation for DDS-NOH and NPTS-NOH (i.e., in the presence of ascorbic acid), suggests that ROS may play some role in the cell death observed with these metabolites.

Since previous studies have demonstrated that SMX-NOH and DDS-NOH result in the formation of metabolite-protein adducts in NHEK, we determined whether or not ascorbic acid would reduce the covalent adduct formation. As illustrated in Fig. 11, ascorbic acid was able to reduce the adduction that occurred with direct addition of metabolite. As previous studies have demonstrated that ascorbic acid blocks the auto-oxidation of these arylhydroxylamines, this data supports the suggestion that formation of the nitroso enhances the covalent binding of these metabolites. However, the ability to detect adducts despite concentrations of ascorbic acid shown previously to prevent auto-oxidation, suggests alternative pathways for covalent adduction to cellular proteins.

In summary, our studies do not support the hypothesis that the formation of ROS serves as a primary means for induction of cell death by arylhydroxylamine metabolites of sulfonamides in NHEK. While there are clear differences in the ROS generation observed with SMX-NOH and DDS-NOH, studies with structural analogues of DDS-NOH demonstrate that ROS formation and cytotoxicity are not directly correlated for these compounds. Elucidation of the mechanism of cell death induced by these agents will be necessary to identify the basis for the differential potency observed. While our studies do not support a simple connection between cell death and ROS generation induced by these metabolites, they should not be construed to suggest that the increase in ROS generation in such cells is unimportant. Indeed, induction of oxidative stress may result in the release or surface expression of important danger signals that are critical in the provocation of an immune response—an essential step in the immune-mediated idiosyncratic toxicity observed with these drugs. Studies probing this potential are ongoing.

## Acknowledgements

This work was supported in part by NIH Grant AI 41395 and GM063821 to Dr. Svensson. The authors wish to acknowledge the helpful advice of Dr. Aloke Dutta (Wayne State University) in our synthetic work for the hydroxylamine metabolites of SMX and DDS.

## References

[1] Hughes WT. Treatment and prophylaxis for Pneumocystis carinii pneumonia. Parasitol Today 1987;3:332–5.
[2] Goldie SJ, Kaplan JE, Losina E, Weinstein MC, Paltiel AD, Seage GR, et al. Prophylaxis for human immunodeficiency virus-related Pneumocystis carinii pneumonia: using simulation modeling to inform clinical guidelines. Arch Intern Med 2002;162:921–8.
[3] Hughes W. Use of dapsone in the prevention and treatment of Pneumocystis carinii pneumonia: a review. Clin Infect Dis 1998;27:191–204.
[4] Bozette SA, Finkelstein DM, Spector SA, Frame P, Powderly WG, He W, et al. A randomized trial of three antipneumocystis agents in patients with advanced human immunodeficiency virus infection. NEJM 1995;332:693–9.
[5] Blum RN, Miller LA, Gaggini LC, Cohn DL. Comparative trial of dapsone versus trimethoprim/sulfamethoxazole for primary prophylaxis of Pneumocystis carinii pneumonia. J Acquir Immune Defic Syndr 1992;5:341–7.
[6] Carr A, Cooper DA, Penny R. Allergic manifestations of human immunodeficiency virus (HIV) infection. J Clin Immunol 1991;11:55–64.
[7] Kovacs J, Hiemenz J, Macher A, Stover D, Murray H, Shelhamer J, et al. Pneumocystis carinii pneumonia: a comparison between patients with the acquired immunodeficiency syndrome and patients with other immunodeficiencies. Ann Intern Med 1984;100:663–71.
[8] Roudier C, Caumes E, Rogeaux O, Bricaire F, Gentilini M. Adverse cutaneous reactions to trimethoprim-sulfamethoxazole in patients with the acquired immunodeciency syndrome and Pneumocystis carinii pneumonia. Arch Dermatol 1994;130:1383–6.
[9] Gordin FM, Simon GL, Wofsy CB, Mills J. Adverse reactions to trimethoprim-sulfamethoxazole in patients with the acquired immunodeficiency syndrome. Ann Intern Med 1984;100:495–9.
[10] Jaffe HS, Abrams DL, Ammann AJ, Lewis BJ, Golden JA. Complications of co-trimoxazole in treatment of AIDS-associated Pneumocystis carinii pneumonia in homosexual men. Lancet 1984;2:1009–11.
[11] Cribb AE, Lee BL, Trepanier LA, Spielberg SP. Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: clinical syndromes and pathogenesis. Adv Drug React Toxicol Rev 1996;15:9–50.
[12] Reilly TP, Ju C. Mechanistic perspectives on sulfonamide-induced cutaneous drug reactions. Curr Opin Allergy Clin Immunol 2002;2:307–15.
[13] Svensson CK. Do arylhydroxylamine metabolites mediate the idiosyncratic reactions associated with sulfonamides and sulfones? Chem Res Toxicol 2003;16:1034–43.
[14] Rieder MJ, Uetrecht J, Shear NH, Spielberg SP. Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides. J Pharmacol Exp Ther 1988;244:724–8.
[15] Rieder MJ, Uetrecht J, Shear NH, Cannon M, Miller M, Spielberg SP. Diagnosis of sulfonamide hypersensitivity reactions by in-vitro "rechallenge" with hydroxylamine metabolites. Ann Intern Med 1989;110:286–9.
[16] Rieder MJ. In vivo and in vitro testing for adverse drug reactions. Pediatr Clin North Am 1997;44:93–111.

[17] Reilly TP, Bellevue FH, Woster PM, Svensson CK. Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. Biochem Pharmacol 1998;55:803–10.
[18] Reilly TP, Lash LH, Doll MA, Hein DW, Woster PM, Svensson CK. A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. J Invest Dermatol 2000;114:1164–73.
[19] Reilly TP, Woster PM, Svensson CK. Methemoglobin formation by hydroxylamine metabolites of sulfamethoxazole and dapsone: implications for differences in adverse drug reactions. J Pharmacol Exp Ther 1999;288:951–9.
[20] Shangari N, O'Brien PJ. The cytotoxic mechanism of glyoxal involves oxidative stress. Biochem Pharmacol 2004;68:1433–42.
[21] Tafazoli S, O'Brien PJ. Prooxidant activity and cytotoxic effects of indole-3-acetic acid derivatives radicals. Chem Res Toxicol 2004;17:1350–5.
[22] Atsumi T, Ishihara M, Kadoma Y, Tonosaki K, Fujisawa S. Comparative radical production and cytotoxicity induced by camphorquinone and 9-fluorenone against human pulp fibroblasts. J Oral Rehabil 2004;31:1155–64.
[23] Yang J, Li H, Chen YY, Wang XJ, Shi GY, Hu QS, et al. Anthraquinones sensitize tumor cells to arsenic cytotoxicity in vitro and in vivo via reactive oxygen species-mediated dual regulation of apoptosis. Free Radic Biol Med 2004;37:2027–41.
[24] Cribb AE, Miller M, Leeder JS, Hill J, Spielberg SP. Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione. Implications for idiosyncratic toxicity. Drug Metab Dispos 1991;19:900–6.
[25] Bradshaw TP, McMillan DC, Crouch RK, Jollow DJ. Formation of free radicals and protein mixed disulfides in rat cells exposed to dapsone hydroxylamine. Free Radic Biol Med 1997;22:1183–93.
[26] Vage C, Saab N, Woster PM, Svensson CK. Dapsone-induced hematologic toxicity: comparison of the methemoglobin-forming ability of hydroxylamine metabolites of dapsone in rat and human blood. Toxicol Appl Pharmacol 1994;129:309–16.
[27] Myhre O, Andersen JM, Aarnes H, Fonnum F. Evaluation of probes 2′,7′-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. Biochem Pharmacol 2003;65:1575–82.
[28] Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM. Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparision with 2′,7′-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. Free Radic Biol Med 1999;27:146–59.
[29] Chignell CF, Sik RH. A photochemical study of cells loaded with 2′,7′-dichlorofluoroscein: implications for detection of reactive oxygen species generated during UVA irradiation. Free Radic Biol Med 2003;34:1029–34.
[30] Carr A, Tindall B, Penny R, Cooper DA. In vitro cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. Clin Exp Immunol 1993;94:21–5.
[31] Wolkenstein P, Charue D, Laurent P, Revuz J, Roujeau JC, Bagot M. Metabolic predisposition to cutaneous adverse drug reactions. Role in toxic epidermal necrolysis caused by sulfonamides and anticonvulsants. Arch Dermatol 1995;131:544–51.
[32] Reilly TP, MacArthur RD, Farrough MJ, Crane LR, Woster PM, Svensson CK. Is hydroxylamine-induced cytotoxicity a valid marker for hypersensitivity reactions to sulfamethoxazole in HIV-infected individuals? J Pharmacol Exp Ther 1999;291:1356–64.
[33] Pertel P, Hirschtick R. Adverse reactions to dapsone in persons infected with human immunodeficiency virus. Clin Infect Dis 1994;18:630–2.
[34] Medina L, Mills J, Leoung G, Hopewell P, Lee B, Modin G, et al. Oral therapy for Pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. NEJM 1990;323:776–82.
[35] Israili ZH, Cucinell SA, Vaught J, Davis E, Lesser JM, Dayton PG. Studies of the metabolism of dapsone in man and experimental animals: formation of *N*-hydroxy metabolites. J Pharmacol Exp Ther 1973;187:138–51.
[36] Gordon GR, Murray JF, Peters JH, Gelber RH, Jacobson RR. Studies on the urinary metabolites of dapsone in man. Inter J Lepr 1979;47:681–2.
[37] Coleman MD, Scott AK, Breckenridge AM, Park BK. The use of cimetidine as a selective inhibitor of dapsone *N*-hydroxylation in man. Br J Clin Pharmacol 1990;30:761–7.
[38] Cribb AE, Spielberg SP. Sulfamethoxazole is metabolized to the hydroxylamine in humans. Clin Pharmacol Ther 1992;51:522–6.
[39] van der Ven AJ, Mantel MA, Vree TB, Koopmans PP, van der Meer JW. Formation and elimination of sulphamethoxazole hydroxylamine after oral administration of sulphamethoxazole. Br J Clin Pharmacol 1994;38:147–50.
[40] van der Ven AJ, Vree TB, van Ewijk-Beneken Kolmer EW, Koopmans PP, van der Meer JW. Urinary recovery and kinetics of sulphamethoxazole and its metabolites in HIV-seropositive patients and healthy volunteers after a single oral dose of sulphamethoxazole. Br J Clin Pharmacol 1995;39:621–5.
[41] Vage C, Svensson CK. Evidence that the biotransformation of dapsone and monoacetyldapsone to their respective hydroxylamine metabolites in rat liver microsomes is mediated by cytochrome P450 2C6/2C11 and 3A1. Drug Metab Dispos 1994;22:572–7.
[42] Carr A, Frei B. Does vitamin C act as a pro-oxidant under physiological conditions? FASEB J 1999;13:1007–24.
[43] Chaudiere J, Ferrari-Iliou R. Intracellular antioxidants: from chemical to biochemical mechanisms. Food Chem Toxicol 1999;37:949–62.
[44] Bolkenius FN, Grisar JM, DeJong W. A water-soluble quaternary ammonium analog of alpha-tocopherol that scavenges lipoperoxyl, superoxyl and hydroxyl radicals. Free Radic Res Commun 1991;14:363–72.
[45] Dinis TC, Maderia VM, Almeida LM, Bolkenius FN, Grisar JM, DeJong W. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys 1994;315:161–9.
[46] Kang JO, Slivka A, Slater G, Cohen G. In vivo formation of hydroxyl radicals following intragastric administration of ferrous salt in rats. J Inorg Biochem 1989;35:55–69.
[47] Pedraza-Chaverri J, Barrera D, Maldonado PD, Chirino YI, Macias-Ruvalcaba NA, Medina-Campos ON, et al. *S*-Allylmercaptocysteine scavenges hydroxyl radical and singlet oxygen in vitro and attenuates gentamicin-induced oxidative and nitrosative stress and renal damage in vivo. BMC Clin Pharmacol 2004;4:5.
[48] Juknat AA, Kotler ML, Quaglino A, Carrillo NM, Hevor T. Necrotic cell death induced by delta-aminolevulinic acid in mouse astrocytes. Protective role of melatonin and other antioxidants. J Pineal Res 2003;35:1–11.
[49] Reiter RJ, Tan DX, Mayo JC, Sainz RM, Leon J, Czarnocki Z. Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. Acta Biochim Pol 2003;50:1129–46.
[50] Rodriguez C, Mayo JC, Sainz RM, Antolin I, Herrara F, Martin V, et al. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res 2004;36:1–9.