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EFFECTS OF AMINOSALICYLATES AND IMMUNOSUPPRESSIVE AGENTS ON NITRIC OXIDE-DEPENDENT *N*-NITROSATION REACTIONS

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Abstract—Recent studies have demonstrated that nitric oxide (NO) rapidly and spontaneously decomposes in oxygenated solutions to generate potent *N*-nitrosating agents. These electrophilic substances have been shown to mediate mutagenesis and carcinogenesis via the formation of aliphatic and aromatic nitrosamines. We have also demonstrated that extravasated neutrophils and macrophages produce significant amounts of *N*-nitrosating agents derived exclusively from NO. During the course of these studies, we found that certain antioxidants, including 5-aminosalicylic acid (5-ASA), inhibited the leukocyte-mediated *N*-nitrosation reaction. Because 5-ASA and other anti-inflammatory and immunosuppressive drugs are used to treat inflammatory bowel disease, we wondered if any of these other compounds might also modulate *N*-nitrosation reactions *in vitro*. Therefore, the objectives of this study were to assess the ability of aminosalicylates and certain immunosuppressive agents to inhibit NO-dependent *N*-nitrosation of a model aromatic amine (2,3-diaminonaphthalene) and to determine whether this inhibitory activity correlated with their oxidation potential. We found that the concentrations necessary to inhibit the *N*-nitrosation reaction by 50% (IC_{50}) were 25, 50 and 100 μM for 5-ASA, olsalazine (dimeric 5-ASA) and sulfasalazine, respectively. In contrast, sulfapyridine, 4-ASA, *N*-acetyl-5-ASA, 6-mercaptopurine, azathioprine, and methotrexate were either much less effective or inactive at inhibiting the *N*-nitrosation reaction. Although 5-ASA was able to fully scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl, neither olsalazine nor sulfasalazine was found to be effective at scavenging this weak oxidant. We did find that olsalazine possessed an oxidation potential substantially less than that of sulfasalazine, suggesting that it may, in fact, scavenge more potent oxidizing agents such as the *N*-nitrosating agent. We conclude that 5-ASA and olsalazine inhibit NO-dependent *N*-nitrosation reactions by scavenging or decomposing the nitrosating agent(s). We propose that the secondary nitrogen unique to sulfasalazine interacts with the nitrosating agent to yield a secondary nitrosamine, thereby competing for *N*-nitrosation of our detector.

Key words: nitrosamine; mutagenesis; colitis; inflammation; 5-aminosalicylate; antioxidants

Chronic inflammation of the colon and rectum is known to be associated with an increased incidence in colorectal cancer [1]. Although the mechanisms by which chronic inflammation promotes malignant transformation remain undefined, there is a growing body of experimental data to suggest that certain leukocyte-derived metabolites may be important mediators of mutagenesis and carcinogenesis (reviewed in Ref. 2). Although much of this work has focused on the role of leukocyte-derived reactive oxygen metabolites, there is substantial evidence to suggest that reactive nitrogen intermediates released by the phagocytic leukocytes known to accumulate within the inflamed colonic interstitium may also be important mediators of mutagenesis and carcinogenesis [3–5]. Recent studies from our laboratory have demonstrated that extravasated but not circulating neutrophils (PMNs) and macrophages synthesize potent *N*-nitrosating agents via the L-arginine-dependent formation of NO† [6]. It is known that NO rapidly and spontaneously decomposes in the presence of molecular oxygen to yield potent *N*-nitrosating agents such as NO_2, N_2O_3 and possibly N_2O_4 [7]. These nitrosating agents have been shown to be mutagenic and carcinogenic *in vitro* by virtue of their ability to *N*-nitrosate aliphatic and aromatic amines to yield nitrosamine derivatives. For example, NO-derived *N*-nitrosating agents will react rapidly with secondary aliphatic amines to yield potentially carcinogenic nitrosamines [8], whereas the *N*-nitrosation of primary aromatic amines (e.g. DNA bases) produces a variety of base substitution mutations via the nitrosative deamination of these bases [3, 4]. We have found that certain antioxidants, including 5-ASA, inhibit leukocyte-mediated *N*-nitrosation reactions *in vitro* [6]. Because aminosalicylates, as well as other anti-inflammatory and immunosuppressive drugs are used to treat inflammatory bowel disease (IBD), and contain functional groups that could interact with *N*-nitrosating agents, we wondered whether some of

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† Abbreviations: NO, nitric oxide; NO_2, nitrogen dioxide; N_2O_3, dinitrogen trioxide; N_2O_4, dinitrogen tetroxide; 5-ASA, 5-aminosalicylic acid; Olz, olsalazine; SAZ, sulfasalazine; SP, sulfapyridine; 4-ASA, 4-aminosalicylic acid; NASA, *N*-acetyl-5-aminosalicylic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; Sp/NO, spermine/NO adduct; DAN, 2,3-diaminonaphthalene; and IBD, inflammatory bowel disease.

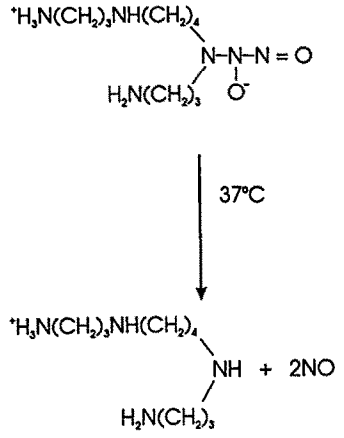

Fig. 1. Structure and decomposition of the nitric oxide-releasing spermine/NO adduct. Spermine/NO decomposes at a known and constant rate at 37° and pH 7.4 to yield NO and spermine.

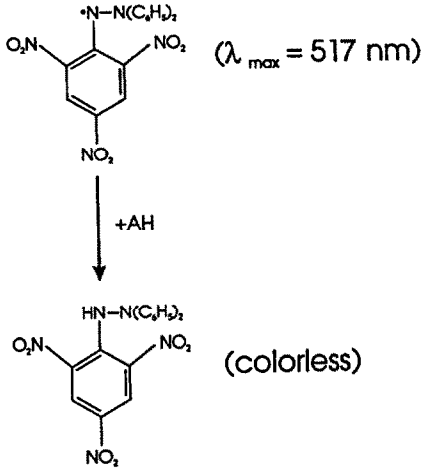

Fig. 2. Structure and spectophotometric properties of the stable free radical 1,1-diphenyl-2-picrylhydrazyl. AH represents an electron-donating compound, such as an antioxidant.

these other compounds might also modulate potentially mutagenic reactions *in vitro*. Therefore, the objectives of this study were to assess the ability of aminosalicylates and certain immunosuppressive agents used in the treatment of IBD to inhibit NO-dependent *N*-nitrosation reactions, using a chemically defined NO generator, and to determine whether this inhibitory activity correlated with their oxidation potentials.

MATERIALS AND METHODS

Materials. 2,3-Diaminonaphthalene (DAN) was purchased from the Sigma Chemical Co. (St. Louis, MO), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) and *N*-1-naphthylethylenediamine dihydrochloride (NEDD) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). 2,3-Naphthotriazole was synthesized using the method of Wheeler *et al.* [9]. 5-ASA, olsalazine (Olz; dimeric 5-ASA) and *N*-acetyl-5-ASA (NASA) were provided by Dr. Thomas Berglind of Kabi-Pharmacia (Uppsala, Sweden) and the NO-releasing spermine/NO adduct (Sp/NO) was obtained from Dr. Larry Keefer (NCI, Frederick, MD). All other drugs and reagents were purchased from the Sigma Chemical Co.

Methods. Various concentrations of each drug were incubated for 60 min at 37° in 0.5-mL reaction volumes containing PBS (pH 7.4), 200 μM DAN and 40 μM Sp/NO. Spermine/NO will spontaneously decompose at pH 7.4 to produce 2 mol of NO and 1 mol of spermine with a half-life of 39 min at 37° (Fig. 1; [10]). Following the incubation period, 2.5 mL of 10 mM NaOH was added to each tube to stop the reaction [6]. The NO-dependent *N*-nitrosation of DAN to yield its highly fluorescent *N*-nitrosated derivative, 2,3-naphthotriazole, was quantified by measuring the fluorescence of each sample using an excitation wavelength of 375 nm and an emission wavelength of 450 nm [6]. The concentration of triazole was determined using standards of the pure 2,3-naphthotriazole. All fluorescence measurements were corrected for the background fluorescence contributed by the various anti-inflammatory and immunosuppressive drugs. The antioxidant activity (i.e. electron donating potential) of the various drugs was quantified by measuring the decrease in absorbance at 517 nm of the stable free radical DPPH as described by Smith *et al.* [11]. This purple-colored, stable free radical possesses a wavelength maximum at 517 nm and will react with avid electron donating substrates (antioxidants) to yield the colorless product 1,1-diphenyl-2-picrylhydrazine (Fig. 2; [11]). Small aliquots (20–100 μL) of each drug were prepared in 95% ethanol and were incubated with 100 μM DPPH prepared in 95% ethanol for 30 min at 37°. The absorbance at 517 nm was recorded before and after the addition of each drug.

Cyclic voltammetry measurements were made using an EG & G Princeton Applied Research Potentiostat/Galvanostat model 273 with model 270 electrochemical analysis system software. Measurements were made at 25° using a glassy carbon working electrode, a platinum mesh counter electrode and a saturated calomel (SCE) reference electrode at a sweep rate of 0.05 V/sec from a potential of 0.0 to 1.1 V. The appropriate compound was dissolved in 100 mM phosphate buffer (pH 7.4) to achieve a final concentration of 10 mM. The azo dye coupling reaction was performed in 100 mM phosphate buffer (pH 7.4) containing a 10 mM concentration of the appropriate compound, 3 mM NEDD, and an aliquot (20 μM) of NO as prepared by Wink *et al.* [12].

Statistics. All data were analyzed using standard statistical analyses, i.e. analysis of variance with the Least Significant Difference (posthoc) test for comparisons of more than two groups. Statistical significance was set at $P < 0.05$.

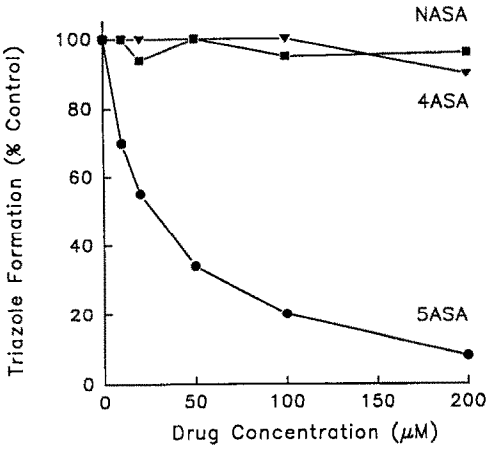

Fig. 3. Effects of aminosalicylates on the nitric oxide-dependent *N*-nitrosation of 2,3-diaminonaphthalene to yield 2,3-naphthotriazole. Assay conditions are described in Materials and Methods. 5-ASA, 4-ASA and NASA represent 5-aminosalicylate, 4-aminosalicylate and *N*-acetyl-5-ASA, respectively. Each data point is the mean from at least five determinations and varied by less than ±5%. A mean value of 9 μM 2,3-naphthotriazole (Triazole) was obtained in the absence of drug and was designated as 100%.

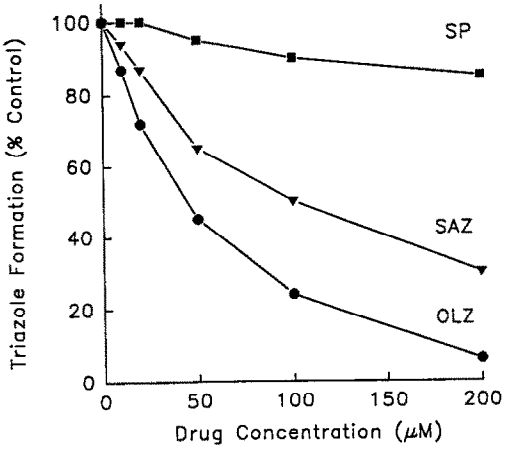

Fig. 4. Effects of olsalazine (Olz), sulfasalazine (SAZ) and sulfapyridine (SP) on the nitric oxide-dependent *N*-nitrosation of 2,3-diaminonaphthalene to yield 2,3-naphthotriazole. Assay conditions are described in Materials and Methods. Each data point is the mean from at least four determinations and varied by less than ±5%. A mean value of 9 μM 2,3-naphthotriazole (Triazole) was obtained in the absence of drug and was designated as 100%.

RESULTS

Figure 3 illustrates the effects of 5-ASA, 4-ASA and NASA on the NO-dependent *N*-nitrosation of DAN. We found that 5-ASA was very effective at inhibiting the nitrosation reaction [i.e. the concentration necessary to inhibit the reaction by 50% (IC_{50}) was approximately 25 μM]. Neither 4-ASA nor NASA was an effective inhibitor of this reaction. Both of the azo precursors to 5-ASA (SAZ and Olz) were effective at inhibiting the *N*-nitrosation reaction, possessing IC_{50} values of 100 and 50 μM, respectively (Fig. 4). Sulfapyridine inhibited triazole formation by less than 10%. The immunosuppressive agents were much less effective than the aminosalicylates in their ability to inhibit the NO-dependent *N*-nitrosation of DAN. For example, 200 μM azathioprine and methotrexate inhibited the nitrosation reaction only 40%, whereas addition of 6-mercaptopurine inhibited NO-dependent triazole formation by only 25% (data not shown). Previous reports from our laboratory, as well as others, have demonstrated that certain antioxidants (i.e. electron donors) are potent inhibitors of NO-dependent *N*-nitrosation reactions, suggesting their effectiveness as possible inhibitors of carcinogen formation *in vivo* [6, 13]. Thus, we wished to ascertain whether the ability of certain aminosalicylates and/or immunosuppressive drugs to inhibit the nitrosation reaction correlated with their antioxidant properties. We found that 5-ASA, but neither SAZ nor Olz (data not shown), was able to scavenge to the stable free radical DPPH (Fig. 5). 6-Mercaptopurine was able to reduce the steady-state concentrations of the stable free radical by only 15%, indicating that it had only modest antioxidant activity (Fig. 5). None of the other immunosuppressive drugs were effective antioxidants in this system. Because DPPH is a relatively weak oxidant and Wink *et al.* [12] have determined recently that the NO-derived nitrosating agent is a potent oxidizing agent that appears to be a more potent oxidant than DPPH, we could not rule out the possibility that SAZ and/or Olz inhibit

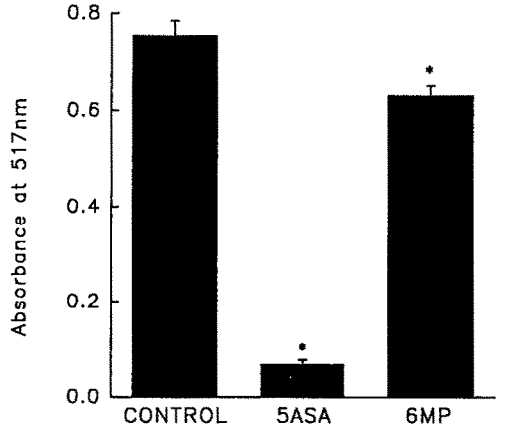

Fig. 5. Antioxidant activity of 5-aminosalicylate (5-ASA) and 6-mercaptopurine (6-MP). Assay conditions are described in Materials and Methods. Data are the means ± SD from three determinations. A mean absorbance value of 0.754 represents a concentration of 1,1-diphenyl-2-picrylhydrazyl of 100 μM. Key: (*) $P < 0.05$ compared with control.

the nitrosation reaction by scavenging this oxidant. Therefore, we quantified the oxidation potentials of 5-ASA, Olz, 4-ASA and SAZ, and found them to be +0.319, 0.556, 0.779 and >0.93, vs SCE respectively. These data suggest that Olz but not SAZ could be interacting with and scavenging the *N*-nitrosating agent.

DISCUSSION

We have demonstrated recently that extravasated PMNs and macrophages synthesize and release large quantities of NO via the five-electron oxidation of L-arginine [6]. In addition, we found that extravasated phagocytes possess potent NO-dependent *N*-nitrosating activity [6]. Although NO *per se* is not a nitrosating agent, it will interact rapidly and spontaneously with molecular oxygen (O_2) to yield potent nitrosating agents such as NO_2 and N_2O_3:

$$2NO + O_2 \leftrightarrow 2NO_2$$

$$NO + NO_2 \rightleftharpoons N_2O_3$$

NO-dependent *N*-nitrosation reactions have been suggested to promote mutagenesis and carcinogenesis by at least two possible mechanisms. One mechanism involves the *N*-nitrosation of secondary aliphatic amines (e.g. dimethylamine) to yield nitrosamines. It is well known that certain nitrosamines are activated into mutagenic and carcinogenic species via the action of the cytochrome P450 system [8]. Cytochrome P450-mediated metabolism of nitrosamines produces electrophilic agents that will rapidly alkylate a variety of different nucleophilic sites in cellular components, including protein, DNA and RNA. Experimental data suggest that O^6-alkylation of guanine bases in DNA represents one of the most important reactions because there is a better correlation of the extent of O^6-alkylation with carcinogenicity and mutagenicity than with any other type of alkylation reaction [14, 15].

A second mechanism by which NO-derived *N*-nitrosating agents may mutagenize cellular DNA is via the nitrosative deamination of primary aromatic amines such as DNA bases [3–5]. It has been demonstrated that *N*-nitrosation of cytosine, methylcytosine, guanine and adenine results in the formation of unstable nitrosamine intermediates that spontaneously deaminate to yield their hydroxy derivatives of uracil, thymine, xanthine and hypoxanthine, respectively [3, 4]. These types of reactions have been shown to produce base substitution mutations *in vitro* [3, 4].

It is well known that certain electron-donating compounds (antioxidants), such as ascorbate and α-tocopherol, are potent inhibitors of *N*-nitrosation reactions [13]. Furthermore, we have found that 5-ASA, the pharmacologically active metabolite of SAZ used to treat patients with active colitis, is both a potent antioxidant [16] and an inhibitor of PMN-mediated *N*-nitrosation [6]. These data suggested that other anti-inflammatory aminosalicylates and possibly other immunosuppressive agents used to treat IBD may be effective not only in controlling the inflammatory response but also in inhibiting potentially mutagenic and carcinogenic reactions *in vivo*.

We found that 5-ASA was a very effective inhibitor of NO-dependent *N*-nitrosation of DAN, possessing an IC_{50} of approximately 25 μM, a concentration well within those that have been determined to be achieved in the colonic interstitium ([17]; Fig. 3). *N*-Acetylation of the amino group on 5-ASA to yield the pharmacologically inactive NASA completely inactivated 5-ASA as an inhibitor of the *N*-nitrosation reaction (Figs. 3 and 6) and eliminated the antioxidant activity of 5-ASA (data not shown). Interestingly, moving the amino group to the 4 position to yield 4-ASA, a compound with demonstrable anti-inflammatory activity comparable to that of 5-ASA [18], also inactivated the ability of this aminosalicylate to inhibit the *N*-nitrosation reaction and eliminated its antioxidant activity by increasing its oxidation potential (Figs. 3 and 6). These data suggest that the mechanism by which 5-ASA inhibits *N*-nitrosation of DAN is due to its ability to scavenge or decompose the nitrosating agent. Consistent with this conclusion is the observation of Wink *et al.* [12] who determined that the *N*-nitrosating agent produced by the interaction between NO and O_2 (e.g. N_2O_3) is a potent oxidant. Another mechanism by which a compound may block *N*-nitrosation reactions requires that the compound has a free amino group that competes with the detector substrate (DAN) for *N*-nitrosation [19]. This mechanism does not appear to account for the inhibitory activity of 5-ASA, since we found that 5-ASA could not be *N*-nitrosated and coupled to NEDD to yield a chromophoric azo dye as is classically performed in the Griess reaction (data not shown). These observations raise the interesting pharmacological dilemma that although clinical studies suggest that 4-ASA may be as effective as 5-ASA in attenuating colonic inflammation in patients with active IBD, it is not as good as 5-ASA at inhibiting NO-dependent mutagenic and possibly carcinogenic reactions.

In addition to 5-ASA, we found that the azo precursor compounds (SAZ, Olz) inhibited the *N*-nitrosation of DAN with apparent IC_{50} values of 100 and 50 μM, respectively (Fig. 4). The mechanisms by which these drugs inhibit the *N*-nitrosation reaction are less apparent. Unlike 5-ASA, neither azo compound was able to reduce the stable free radical DPPH, suggesting that neither azo compound is a particularly avid electron-donating compound. Although DPPH has been used to assess the antioxidant properties of various compounds [11], it should be remembered that this stable free radical is a relatively weak oxidant that will interact with and be scavenged by only the most avid electron-donating substrates (e.g. ascorbate and 5-ASA) [11, 16]. In view of the recent observations made by Wink *et al.* [12] in which they demonstrated that the NO-derived *N*-nitrosating agent is a potent oxidizing agent (and probably more potent than DPPH), we could not rule out the possibility that SAZ and/or Olz may inhibit the nitrosation reaction by acting as scavengers for the nitrosating agent. Thus, we determined the oxidation potentials of 5-ASA, Olz, 4-ASA and SAZ and found them to be +0.319,

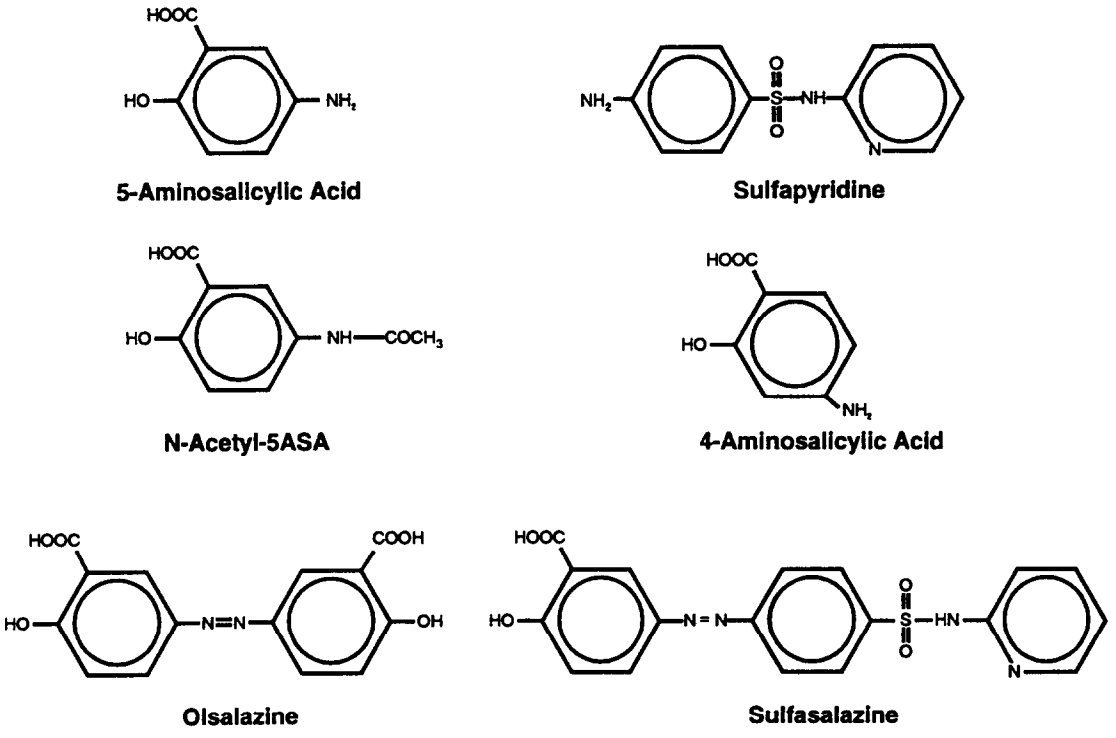

Fig. 6. Structures of aminosalicylates and azo precursors.

0.556, 0.779 and >0.93 vs SCE, suggesting that the inhibitory activity of Olz, but not SAZ, is most probably due to its ability to scavenge the nitrosating agent. In fact, these data suggest that the oxidation potential of the NO-derived nitrosating agent lies somewhere between 0.556 and 0.779 SCE. It is also possible that Olz and SAZ inhibit *N*-nitrosation via their ability to retard the release of NO from Sp/NO. We found, using a NO-sensitive electrode, that neither drug affected NO release of concentrations up to 200 μM (data not shown). The mechanism by which SAZ inhibits *N*-nitrosation is more speculative. We hypothesize that the secondary nitrogen uniquely situated in the SAZ molecule interacts with the nitrosating agent to yield a stable nitrosamine derivative. This type of reaction would compete with the *N*-nitrosation of our detector molecule (DAN), resulting in an apparent inhibition.

The use of immunosuppressive agents, such as azathioprine and its metabolite 6-mercaptopurine as well as methotrexate, has only recently begun to be appreciated as viable modes of therapy for the treatment of IBD. Because these drugs contain functional groups known to react with nitrosating agents (e.g. primary and secondary amines, sulfhydryls; Fig. 7), we wondered whether they could also be effective inhibitors of the potentially mutagenic nitrosation reactions. We found that all three immunosuppressive agents inhibited the NO-dependent *N*-nitrosation reaction; however, this inhibition was substantially less than with 5-ASA, Olz and SAZ. Thus, the efficacy of these drugs as inhibitors of mutagenic nitrosation reactions would be limited. Only 6-mercaptopurine, by virtue of its free sulfhydryl group, demonstrated any significant antioxidant activity, and this was only modest at best (Fig. 5). Taken together, our data suggest that certain aminosalicylates may be useful not only in attenuating intestinal inflammation but also in inhibiting the NO-dependent formation of mutagens.

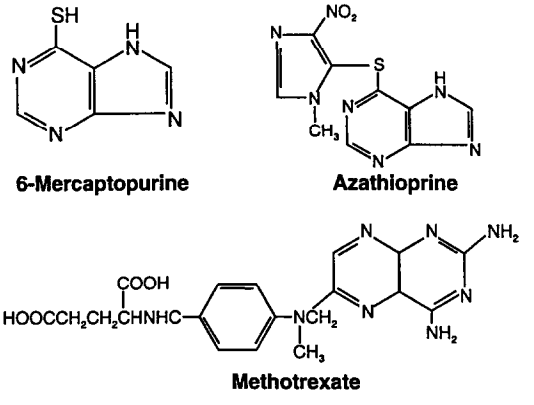

Fig. 7. Structures of three immunosuppressive agents.

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