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THE SCAVENGING OF OXIDANTS BY SULPHASALAZINE AND ITS METABOLITES

A POSSIBLE CONTRIBUTION TO THEIR ANTI-INFLAMMATORY EFFECTS?

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Abstract—Sulphasalazine (Salazopyrin®) and its metabolites sulphapyridine and 5-aminosalicylate are powerful scavengers of the hydroxyl radical, determined by pulse radiolysis and confirmed by assays based on deoxyribose degradation by hydroxyl radicals. 5-Aminosalicylate can also protect α_1-antiprotease against attack by the myeloperoxidase-derived oxidant hypochlorous acid. The ability to scavenge oxidants produced at sites of inflammation may contribute to the anti-inflammatory action of sulphasalazine and its metabolites.

Phagocytic cells stimulated by opsonized bacteria or by immune complexes produce the oxygen-derived species superoxide (O_2^-) and H_2O_2. In the presence of suitable metal ions, which often exist *in vivo*, O_2^- and H_2O_2 interact to form the highly-reactive and tissue-damaging hydroxyl radical, $^{\cdot}OH$ [1–3], and/or species of comparable reactivity. Stimulated neutrophils also release the enzyme myeloperoxidase, which uses H_2O_2 to oxidize Cl^- ions into a powerful oxidant that has been identified as hypochlorous acid, HOCl [4–6]. It has been proposed that phagocyte-generated O_2^-, H_2O_2 and $^{\cdot}OH$ [2, 3, 7, 8], as well as HOCl [5], contribute to tissue damage at sites of inflammation, such as the inflamed joints of patients with rheumatoid arthritis [3, 7, 9] and the inflamed bowel in ulcerative colitis and Crohn's disease [2].

Inflammatory diseases are treated with a wide range of drugs, including steroids, non-steroidal anti-inflammatory drugs, so-called "disease-modifying" drugs such as gold salts and penicillamine, immunosuppressive compounds and antimalarials. Each of these drugs probably has multiple mechanisms of action. For example, non-steroidal anti-inflammatory drugs not only inhibit cyclooxygenase, but might also scavenge $^{\cdot}OH$ radicals [8, 10, 11]. Many of the above drugs have been reported to influence the production of O_2^- and H_2O_2 by activated phagocytes [12–17] and to act as scavengers of HOCl [18–20]. For example, Pekoe *et al.* [18] found that almost every anti-inflammatory drug tested could react with HOCl. However, HOCl is a powerful oxidant that can attack many different molecules [4, 6, 21, 22]. Scavenging of HOCl by a given molecule will only be biologically significant if, at the concentration of scavenger that can be achieved *in vivo*, its reaction with HOCl is fast enough to protect important biological targets from attack by HOCl. The most important target of HOCl attack *in vivo* is probably the α_1-antiprotease protein, which protects tissues against proteolytic damage [5]. Thus, although dimethylsulphoxide reacts with HOCl [6, 21], this reaction is probably too slow to protect α_1-antiprotease against inactivation by HOCl, which suggests that scavenging of HOCl is unlikely to contribute to the anti-inflammatory effects of dimethylsulphoxide [23]. In the same way, scavenging of $^{\cdot}OH$ radicals by a drug is only likely to be significant if the drug is present at the site of inflammation at concentrations approaching the millimolar range and/or if its rate constant for reaction with $^{\cdot}OH$ is high, certainly $>10^9\ M^{-1}\ sec^{-1}$ [11].

Sulphasalazine (Salicylazosulphapyridine, Salazopyrin®) is effective in the treatment and prophylaxis of ulcerative colitis [24, 25]. It is rapidly cleaved by gut flora into sulphapyridine and 5-aminosalicylate, and it is thought that the latter compound is the true anti-inflammatory agent [24, 26–28]. There is also a resurgence of interest in the use of sulphasalazine as a second-line agent in the treatment of rheumatoid arthritis [29], although in this case the anti-inflammatory effect appears most likely to be due to absorbed sulphapyridine or to the sulphasalazine molecule itself [29, 30]. The mechanism of action of sulphasalazine in arthritis is not understood. In the present paper, we have investigated the possibility that sulphasalazine and its metabolites sulphapyridine and 5-aminosalicylate might scavenge $^{\cdot}OH$ and HOCl at biologically-significant rates. Del Soldato *et al.* [8] have already suggested that these molecules might be good scavengers of $^{\cdot}OH$ radicals.

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Table 1. Action of sulphasalazine, sulphapyridine and 5-aminosalicylate on elastase, its inhibition by α_1-antiprotease and on the inactivation of α_1-antiprotease by HOCl

Drug added	Final concentration in reaction system 1 (mM)	Elastase activity (% maximum rate)		
		A	B	C
None	—	95	100	4
Sulphasalazine	0.1	95	97	5
	0.5	97	96	4
	1.0	99	97	5
5-Aminosalicylate	0.025	102	—	—
	0.05	101	—	2
	0.1	102	—	—
	0.4	58	99	—
	0.6	31	—	1
	0.8	0	98	3
Sulphapyridine	0.025	100	—	—
	0.05	95	—	—
	0.1	98	—	—
	0.2	99	—	—
	0.8	106	104	2
	1.0	107	106	1

α_1-Antiprotease (final concentration 1.0 mg/ml) was mixed with the final concentration of drug stated plus HOCl (75 μM final concentration) in 35.1 μl of buffer (140 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4, 2.9 mM KH_2PO_4, pH 7.4) and incubated at 24° for 60 min (*reaction system* 1). 3 ml of the same buffer was then added, followed by elastase [22]. After further incubation for 30 min, the elastase activity remaining was assayed as a rise in absorbance at 410 nm after addition of substrate [22]. Results are expressed as a % of maximum elastase activity, where 100% was a ΔA_{412} of 0.053 min^{-1}, and are the means of duplicates that differed by 5% or less. Column A: experiment as above; column B: both HOCl and antiprotease omitted from reaction system 1, so that any effect of drug on elastase itself can be tested; column C: HOCl omitted from reaction system 1, so that any effect of drug on the ability of α_1-antiprotease to inhibit elastase can be tested.

MATERIALS AND METHODS

Reagents. α_1-Antiprotease (type A9024), deoxyribose, $FeCl_3$, sulphapyridine, 5-aminosalicylate and thiobarbituric acid were from Sigma. Porcine pancreatic elastase, NaOCl and other reagents used were from BDH Chemicals Ltd. Sulphasalazine was a gift from Pharmacia. Sulphasalazine, sulphapyridine and 5-aminosalicylate were dissolved in water containing the minimum quantity of KOH to ensure solution, or in 0.5% (w/v) Na_2CO_3. (Identical results were obtained in either case.) The pH of all reaction mixtures was adjusted, where necessary, to 7.4.

Assays. Elastase and α_1-antiprotease were assayed essentially as described in [22] and [31]; full details are given in the legend to Table 1. HOCl was produced immediately before use by adjusting sodium hypochlorite (Na^+OCl^-) to pH 6.2 with dilute H_2SO_4, and its concentration determined as in [21]. Deoxyribose degradation in the presence of ascorbate, H_2O_2 and $FeCl_3$-EDTA was measured as described in [32]. Pulse radiolysis was performed using the Paterson laboratories' linear accelerator facility.

RESULTS

Scavenging of the myeloperoxidase-derived oxidant HOCl

Perhaps the most important biological target that can be attacked by HOCl is the α_1-antiprotease protein, permitting uncontrolled protease (e.g. elastase) activities [5, 33]. If the ability of a drug to react with HOCl contributes to the drug's biological activity, then the drug ought to be able to protect α_1-antiprotease against inactivation by a physiological concentration of HOCl. Table 1 shows the results of experiments designed to test the protective ability of sulphasalazine, sulphapyridine and 5-aminosalicylate.

Table 1 (column B) shows that none of these three compounds had any effect on the activity of elastase over the concentration range tested. α_1-Antiprotease inhibits elastase, and a concentration sufficient to inhibit by approximately 95% was used in these experiments. None of the compounds interfered with the ability of α_1-antiprotease to inhibit elastase (Table 1, column C). If the antiprotease is treated with HOCl, its elastase-inhibitory capacity is lost (Table 1, column A, first line). The concentration of HOCl used is within the range of concentrations known to be produced adjacent to myeloperoxidase or stimulated neutrophils *in vivo* [4, 6, 34, 35].

When they were included in the reaction mixture with HOCl and α_1-antiprotease, neither sulphasalazine nor sulphapyridine, tested at concentrations up to 1.0 mM, were able to protect α_1-antiprotease against inactivation by HOCl. However, 5-aminosalicylate did exert a significant protective action at concentrations of 400 μM or greater. This could be biologically significant, since greater concentrations of 5-aminosalicylate than this are present in the bowel after therapeutic doses of sulphasalazine [28].

Scavenging of hydroxyl radical

Radiolysis of a dilute aqueous phosphate-buffered

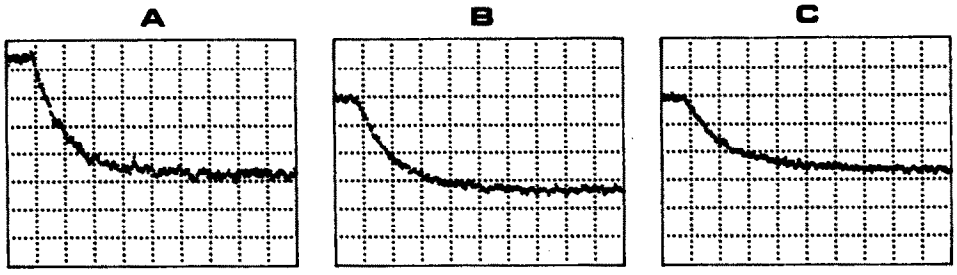

Fig. 1. All solutions contained 10 mM KH_2PO_4–KOH buffer pH 7.4 and were purged with N_2O before irradiation. The radiation dose delivered was equivalent to [$^{\cdot}OH$] = 1.9 μM and the optical path length was 2.5 cm. The traces show typical examples of the growth of optical absorption, at a suitable wavelength for each drug, upon reaction of the drug with $^{\cdot}OH$. Sensitivity was 1.6% transmission per division (trace A), 0.9% (trace B) or 1.1% (trace C). Trace A, sulphasalazine (0.5 mM), 250 nsec/division, 480 nm; trace B, sulphapyridine (0.5 mM), 200 nsec/division, 390 nm; trace C, 5-aminosalicylate (0.5 mM), 250 nsec/division, 390 nm. Drugs were tested in the concentration range 30 μM–1.0 mM.

solution, pH 7.4, produces hydroxyl radical, $^{\cdot}OH$

$$H_2O \rightarrow {}^{\cdot}OH, e^-_{(aq)}, H^{\cdot}, H_2O_2, H_2 \quad (1)$$

$$e^-_{(aq)} + N_2O + H_2O \rightarrow {}^{\cdot}OH + OH^- + N_2 \quad (2)$$

If sulphasalazine, sulphapyridine and 5-aminosalicylate were included in the reaction mixture, they were observed to react with the $^{\cdot}OH$ with the formation of characteristic absorption spectra (data not shown). Figure 1 shows the rise in absorbance at an appropriate wavelength for each compound at various times after generation of $^{\cdot}OH$. Second-order rate constants were calculated from this data and were (as mean ± 1 SD)

sulphasalazine $(6.6 \pm 0.7) \times 10^9\ M^{-1}\ sec^{-1}$

sulphapyridine $(8.0 \pm 0.8) \times 10^9\ M^{-1}\ sec^{-1}$

5-aminosalicylate $(5.6 \pm 0.6) \times 10^9\ M^{-1}\ sec^{-1}$

It was observed that the initial products of $^{\cdot}OH$ attack on 5-aminosalicylate and sulphasalazine underwent further reactions over the time scale 3–10 μsec/division, possibly due to loss of water from the initial product of attack of $^{\cdot}OH$ upon the phenolic ring [36, 37].

At sites of inflammation, any $^{\cdot}OH$ generated probably arises by reactions dependent upon transition metal ions, especially iron [3, 9]. The ability of the three drugs to scavenge $^{\cdot}OH$ was therefore tested in a system in which $^{\cdot}OH$ generation is iron-dependent. Hydroxyl radicals were generated by a mixture of $FeCl_3$, EDTA, ascorbic acid and H_2O_2 [38] and detected by their ability to degrade the sugar deoxyribose [39, 40]. Any scavenger of $^{\cdot}OH$ added to the reaction mixture competes with deoxyribose for the $^{\cdot}OH$ generated and so decreases the observed extent of deoxyribose degradation [32, 39]. Table 2 shows that sulphasalazine, sulphapyridine and 5-aminosalicylate all inhibited deoxyribose degradation by $^{\cdot}OH$. The order of effectiveness was sulphapyridine > sulphasalazine ≥ 5-aminosalicylate, consistent with the order of the rate constants firmly established by pulse radiolysis (see above).

DISCUSSION

Neither sulphasalazine nor sulphapyridine could protect α_1-antiprotease, at a concentration similar to that present in human plasma (1.2–1.3 mg ml^{-1} [41]), against attack by a physiological concentration of HOCl. This suggests that they do not react with HOCl sufficiently quickly. Another possibility is that reaction of HOCl with sulphasalazine or sulphapyridine generates a different oxidant that can also inactivate α_1-antiprotease, as has been demonstrated for the product of reaction of taurine with

Table 2. Inhibition of hydroxyl radical-dependent deoxyribose degradation by sulphasalazine and its metabolites

Drug added	Final concentration of drug in reaction mixture (mM)	% Inhibition of deoxyribose degradation
None	—	0
Sulphasalazine	0.125	17
	0.25	19
	0.5	39
	1.0	53
	1.5	62
	2.0	67
	2.5	75
5-Aminosalicylate	0.125	11
	0.25	23
	0.5	40
	1.0	54
	1.5	63
	2.0	67
	2.5	72
Sulphapyridine	0.125	21
	0.25	40
	0.5	60
	1.0	72
	1.5	92
	2.0	96
	2.5	97

Hydroxyl radicals were generated by a mixture of $FeCl_3$, EDTA, ascorbic acid and H_2O_2, and detected by their ability to degrade deoxyribose [32, 39]. Reaction mixtures were as described in [32]. An added "scavenger" of $^{\cdot}OH$ decreases the rate of deoxyribose degradation. The results of a typical experiment are shown. Percentage inhibitions are calculated from the means of duplicate experiments that differed by 10% or less. The absorbance corresponding to 0% inhibition (no drug added) was 0.687.

HOCl [5]. In either case, the results mean that protection of α_1-antiprotease against attack by HOCl is unlikely to contribute to the therapeutic effects of sulphasalazine and sulphapyridine. On the other hand, 5-aminosalicylate, at concentrations well within those present in the bowel during therapy with sulphasalazine [28], was able to protect α_1-antiprotease against HOCl attack. This raises the possibility that its anti-inflammatory action in ulcerative colitis might, to an unknown extent, be due to protection of α_1-antiprotease, thereby limiting proteolytic damage to the inflamed tissue [5].

Both sulphasalazine and its products (sulphapyridine, 5-aminosalicylate) are good scavengers of $^{\cdot}OH$ radicals, with rate constants at or near the diffusion-controlled limit [42], supporting the proposals of Del Soldato [8]. The fact that all three compounds react well with $^{\cdot}OH$ whereas only 5-aminosalicylate is the active agent in ulcerative colitis, might suggest that $^{\cdot}OH$ scavenging does not account for the therapeutic actions of 5-aminosalicylate in this disease. However, since $^{\cdot}OH$ radicals are thought to be responsible for some of the tissue damage occurring in the inflamed rheumatoid joint [7, 9], it is possible that $^{\cdot}OH$ scavenging may contribute to the anti-rheumatic activity of sulphasalazine. It should be noted, however, that carbon-centred and peroxy radicals formed by attack of $^{\cdot}OH$ on certain drugs can themselves cause biological damage [10].

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