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Effect of sulphasalazine and its active metabolite, 5-amino-salicylic acid, on toxic oxygen metabolite production by neutrophils

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SUMMARY **The possibility that the mode of action of sulphasalazine and its active metabolite 5-amino-salicylic acid (5ASA) involves modification of toxic oxygen metabolite production by neutrophils has been investigated by measuring the effect of these drugs on luminol-dependent chemiluminescence, superoxide release and oxygen consumption by stimulated neutrophils *in vitro*. 5ASA, and to a lesser extent sulphasalazine, had profound inhibitory effects on the luminol dependent chemiluminescent response of neutrophils stimulated with formyl-methionyl-leucyl-phenylalanine (1 μM)+cytochalasin B (5 μg/ml). A concentration of 50 μM 5ASA or sulphasalazine produced 93·8 (2·3)% and 65·7 (3·7)% inhibition of control responses respectively. The concentration of 5ASA and sulphasalazine producing 50% inhibition of chemiluminescence were 3·6 (1·8) μM and 16·5 (6) μM respectively. Both drugs had little effect on the chemiluminescent response of neutrophils stimulated with phorbol myristate acetate (1 μg/ml), producing only 11·4 (3·9)% and 34 (7)% inhibition respectively, at a concentration of 50 μM. Superoxide release from fMLP+CB stimulated neutrophils was also inhibited slightly by 5ASA (50 μM) by 35·6% and by sulphasalazine (50 μM) by 7·9%. Similarly, there was little inhibition in the rate of oxygen consumption by fMLP+CB stimulated neutrophils by either 5ASA or sulphasalazine at concentrations which produced near total abolition of luminol dependent chemiluminescence. These results show that sulphasalazine and 5ASA inhibit the reaction of toxic metabolites produced by stimulated neutrophils with luminol, without inhibition of the oxidase system producing these metabolites. The site of action of these drugs on neutrophils *in vitro* is thus extracellular, by scavenging a released metabolite, probably hypochlorite. This has important implications for their mode of action *in vivo* in inflammatory bowel disease.**

The beneficial effects of sulphasalazine on ulcerative colitis were first noted in the 1940s.[1] Sulphasalazine is broken down in the colon by enteric bacteria into sulphapyridine and 5-amino-salicylic acid (5ASA).[2,3] The active metabolite of SAL is 5ASA[4,5] which has recently been used in the treatment of ulcerative colitis, being delivered to the colon in a pH sensitive coated capsule.[6]

The mode of action of sulphasalazine and its active metabolite, 5ASA, in inflammatory bowel disease remains uncertain. Both ulcerative colitis and Crohn's disease are characterised by infiltration of the mucosa with neutrophils and macrophages.[7] These cells produce highly reactive metabolites of oxygen including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\cdot$).[8,9] Further reactions with myeloperoxidase, released simultaneously, results in the formation of hypochlorite (OCl^-).[10] Extracellular release of these phagocyte generated metabolites of oxygen is thought to play a major role in the pathogenesis of tissue damage at inflammatory sites.[10,11] In inflammatory bowel disease, phagocytic cells retain the ability to produce toxic oxygen metabolites,[12] further-

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more, in inflamed tissue, these cells produce an enhanced response to stimulation.[13]

A drug which reduces reactive oxygen metabolite production by phagocytic cells, either by a direct inhibitory effect on the cells, or by scavenging released oxygen metabolites, may therefore reduce tissue damage at inflammatory sites.

In this paper, we investigate the possibility that sulphasalazine and 5ASA modify reactive oxygen metabolite production by neutrophils *in vitro*. By monitoring different aspects of oxygen metabolite production by stimulated neutrophils, we show that these drugs act at a specific extracellular site.

Methods

ISOLATION OF NEUTROPHILS

Neutrophils were isolated from freshly drawn whole blood from healthy volunteers, by dextran sedimentation[14] and density gradient centrifugation over Ficoll-Paque (Pharmacia, Sweden).[15] Neutrophils were washed twice in HEPES (N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid) buffered Krebs solution (120 mM NaCl, 4·8 mM KCl, 1·2 mM $MgSO_4$, 1·2 mM KH_2PO_4, 1·3 mM $CaCl_2$, 0·1% bovine serum albumin and 25 mM HEPES, pH was adjusted to 7·4 with NaOH). After washing, neutrophils were suspended in fresh Krebs solution (between 1 and 5×10^6 cells/ml) on melting ice until required. Neutrophil suspensions were >95% pure and >95% viable (assessed by exclusion of trypan blue).

MEASUREMENT OF TOXIC OXYGEN METABOLITE PRODUCTION

Chemiluminescence

Luminol dependent chemiluminescence[16] was measured with a photomultiplier tube (Thorn EMI 30 mm bialkaline front surface, no 9924B. High voltage supply 940 V, discriminator set at 0·1V) in a specially constructed light tight chamber, thermostatically controlled at 37°C.[17] One millilitre of neutrophil suspension was placed in a plastic test tube, to which was added luminol dissolved in dimethyl sulphoxide (DMSO), final concentration luminol 11 μM, 0·1% v/v DMSO. Sulphasalazine and 5ASA were dissolved in DMSO and added to the cell suspensions before addition of the stimulus (final concentration of drugs ranged from 0·1 μM to 100 μM). A similar volume of DMSO (1·5 μl) was added to control samples.

Neutrophils were stimulated with either chemotactic peptide N formyl-methionyl-leucyl-phenylalanine (fMLP, final concentration 1 μM) plus cytochalasin B (CB, final concentration 5 μg/ml) or the C kinase activator 4 β phorbol 12 myristate 13 acetate PMA (final concentration 1 μg/ml). The luminol dependent chemiluminescent response in photon counts per second was recorded with a pen chart recorder.

SUPEROXIDE PRODUCTION

Superoxide production by stimulated neutrophils was measured by reduction of cytochrome C.[18] One millilitre aliquots of neutrophil suspension (1×10^6 neutrophils) were placed in plastic cuvettes (1 cm light path), to which was added cytochrome C type III (Sigma Chemical Co., Poole, Dorset) to a final concentration of 50 μM. Sulphasalazine or 5ASA, dissolved in DMSO, were added to the neutrophils (final concentration of sulphasalazine and 5ASA, 50 μM; DMSO 0·15% v/v). Cells were stimulated with fMLP (1 μM) plus CB (5 μg/ml).

Reduction of cytochrome C was measured by absorbance at 550 nm in a PYE SP30 UV spectrophotometer, against a blank cuvette containing a similar volume of neutrophil suspension, and similar concentrations of cytochrome C, fMLP+CB and 300 units superoxide dismutase (Sigma Chemical Co.). Superoxide production was calculated from the rate of change of absorbance using Beer-Lamberts law, with the extinction coefficient $E_{550}=2\cdot1\times10^4$/M/cm. Each experiment was performed in triplicate using neutrophils from five different subjects.

OXYGEN CONSUMPTION

Oxygen consumption was measured using a Clark electrode in a purpose built chamber, maintained at 37°C.[19] The polarising voltage of the electrode was maintained at 0·6 V and the output of the electrode was measured with a purpose built nano-ammeter and a chart recorder. The apparatus was calibrated by assuming that the concentration of oxygen dissolved in HEPES buffered Krebs medium at 37°C was 210 μM.[20] $1\cdot5\times10^7$ neutrophils, suspended in 2 ml HEPES buffered Krebs medium, were placed in the specimen chamber, and allowed to reach steady state resting oxygen consumption.

Neutrophils were stimulated by adding FMLP (final concentration, 1 μM) and CB (final concentration 5 μg/ml). Experiments were performed in triplicate on neutrophils from five different subjects.

Results

INHIBITION OF LUMINOL DEPENDENT CHEMILUMINESCENCE

Both sulphasalazine and 5ASA inhibited the burst of chemiluminescence produced by neutrophils when stimulated by fMLP+CB. This inhibition was characterised by a decrease in peak chemilumines-

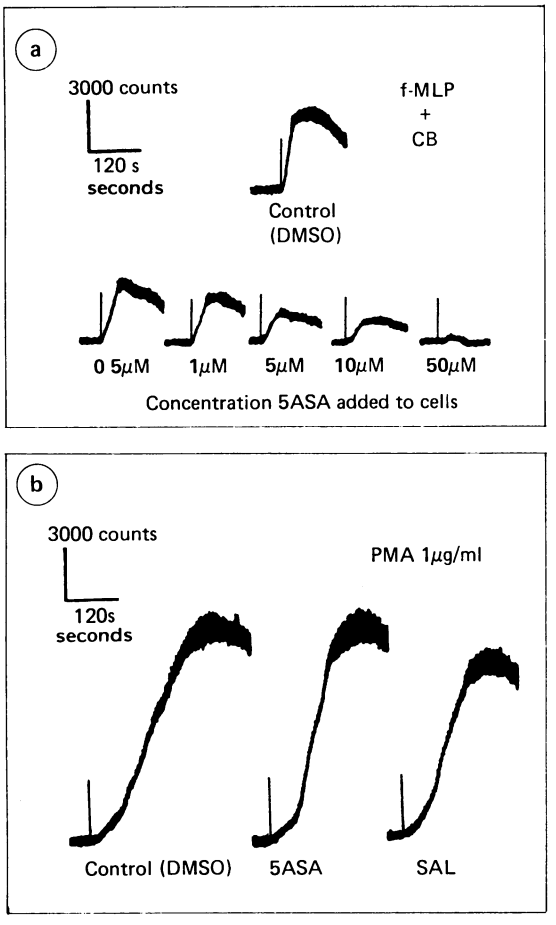

Fig. 1 *Luminol dependent chemiluminesence. Pen-chart recordings, showing;* (a) *the effect of increasing concentrations of 5ASA on the luminol-dependent chemiluminescent response of neutrophils stimulated with fMLP (1 μM) and CB (5* μg/ml), and (b) *the luminol-dependent chemiluminesent response of neutrophils stimulated with PMA (1* μg/*1ml), comparing the response of control cells with those cells pretreated with 50 μM sulphasalazine or 50 μM5 ASA. Neutrophils from the same subject, 2×10⁶ cells. Stimuls added at vertical mark. Drugs dissolved in DMSO, control samples contained a similar volume of DMSO (1·5 μl).*

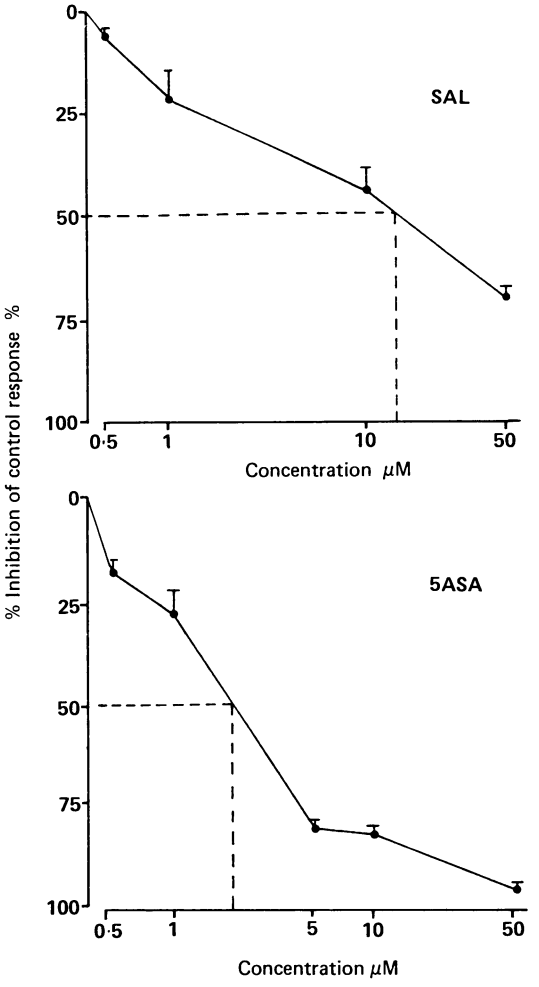

Fig. 2 *Luminol dependent chemiluminesence. Graphs showing the inhibitory effect of increasing concentrations of sulphasalazine (SAL) and 5-amino-salicylic acid (5ASA) expressed as percentage inhibition of control values. Each point represents the mean (SD) of observations performed in triplicate on neutrophils from six subjects for 5ASA and five for SAL. Dotted lines mark the concentration producing 50% inhibition (Ki_{50}). 5ASA-3·6 μM, SAL 16·5 μM.*

cence, without a decrease in the rate of rise, or increase in the lag time between stimulation and onset of response (Fig. 1a). 5ASA was the more potent, 50 μM producing 93·8 (2·3)% inhibition of the control chemiluminescent response (mean (SE), n=12, p=0·01 Wilcoxon's rank-sum test on paired data), whereas sulphasalazine at a similar concentration, produced 65·7 (3·7)% inhibition (n=9, p=0·01 Wilcoxon's rank-sum test). The concentration of sulphasalazine producing 50% inhibition (Ki_{50}) of the chemiluminescent response of neutrophils, stimulated with fMLP+CB was 16·5 (6) μM (mean (SE) n=5, Fig. 2). The Ki_{50} for 5ASA was 3·6 (1·8) μM (n=6, Fig. 2). Neutrophils stimulated with fMLP alone produced smaller chemiluminescent responses than in the presence of CB. This smaller chemiluminescent response, however, was also markedly inhibited by 50 μM 5ASA (88%, n=3) or 50 μM SAL (81·7%, n=3).

In contrast with the marked inhibition of fMLP+CB stimulated chemiluminescence by sulphasalazine

and 5ASA, these drugs in similar concentrations only weakly inhibited chemiluminesence by neutrophils stimulated with PMA (Fig. 1b). Fifty micromolar sulphasalazine produced 34 (7)% inhibition of control responses (mean (SE), n=6, p=0·01 Wilcoxon's rank-sum on paired data). 5ASA, at a similar concentration did not have a significant inhibitory effect (11·4 (3·9)%, n=10).

EFFECT ON SUPEROXIDE PRODUCTION BY NEUTROPHILS

At a concentration producing abolition of the chemiluminesent response (50 μM), 5ASA produced only 35·6% inhibition of the rate of production of superoxide (1·65 (0·39) nmoles/min/10^6 cells, p=0·05 Wilcoxon's rank-sum test). Sulphasalazine did not significantly inhibit superoxide production by stimulated neutrophils (2·36 (0·19) nmol/min/10^6 neutrophils, 7·9%; Table).

EFFECT ON OXYGEN CONSUMPTION BY NEUTROPHILS

5-amino-salicylic acid had no significant inhibitory effect on the rate of oxygen consumption by neutrophils stimulated with fMLP+CB (Fig. 3). The uninhibited stimulated rate was 0·0381 (0·025) nmol/sec/10^6 neutrophils, and in cells pretreated with 5ASA the rate was 0·0349 (0·02) nmol/sec/10^6 neutrophils (mean (SD), mean inhibition 8·4%).

Sulphasalazine also failed to significantly inhibit oxygen consumption by stimulated neutrophils (Fig. 3). The uninhibited and SAL pre-treated cells consuming oxygen at 0·0363 (0·022) nmol/sec/10^6 neutrophils, and 0·032 (0·021) nmol/sec/10^6 neutrophils (mean inhibition 11·9%) respectively.

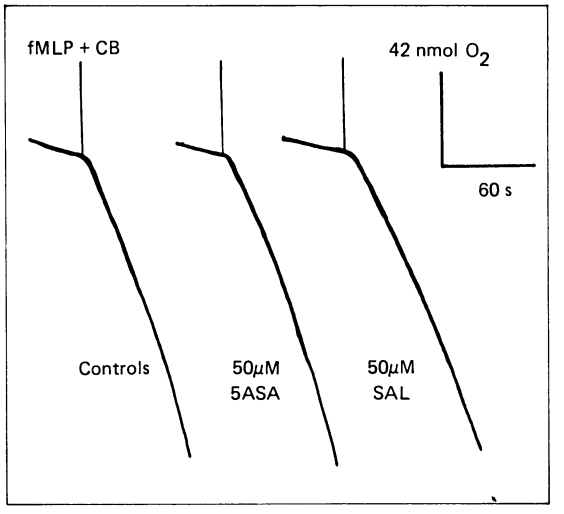

Fig. 3 *Oxygen consumption by stimulated neutrophils. Typical pen chart-recording tracings of oxygen consumption by neutrophils measured with a Clark electrode. 1·5×10^7 neutrophils in the chamber. Traces show resting oxygen consumption and 'respiratory burst' produced by adding fMLP (1 μM) and CB (5 μg/ml) at vertical bar on trace. Control rate of oxygen consumption 0·036 nmoles/sec/10^6 neutrophils, + 50 μM 5ASA −0·034 nmoles/sec/10^6 neutrophils and 50 μM sulphasalazine −0·032 nmol/sec/10^6 neutrophils.*

Discussion

In this paper, we have shown that sulphasalazine and its active metabolite 5ASA, have a profound inhibitory effect on the luminol dependent chemiluminescent response of neutrophils after chemotactic stimulation, which was not mirrored by similar inhibition of superoxide production or oxygen consumption.

Table 1 *Superoxide production by neutrophils*

Control	*50 μM 5ASA*	*50 μM SAL*
1 2·42 (0·33)	1·45 (0·28) [39·9]	2·59 (0·09) [+7·2]
2 2·25 (0·51)	1·99 (0·59) [11·8]	2·48 (0·48) [+10·2]
3 2·66 (0·08)	1·34 (0·28) [49·5]	2·11 (0·31) [20·4]
4 2·67 (0·25)	2·15 (0·26) [19·4]	2·41 (0·36) [9·7]
5 2·79 (0·48)	1·33 (0·35) [52·5]	2·21 (0·21) [20·9]
Mean		
2·56 (0·22)	1·65 (0·39) [35·6]	2·36 (0·19) [7·9]

Effect of 50 μM 5ASA and 50 μM SAL on superoxide (O_2^-) production (nmol/min/10^6 neutrophils) by neutrophils from five healthy subjects, stimulated with fMLP (1 μM) and CB (5 μg/ml). Each value is the mean of triplicate measurements (SD). Figures in square parenthesis indicate the % inhibition of control values. +ve values=enhancement of response.

Inhibition may result from interference at one or more sites in the chain of events which result in the production of reactive oxygen metabolites by stimulated neutrophils (Fig. 4). These include: (a) blocking of the stimulus receptor on the cell membrane, (b) direct, blanket inhibition of the cell, (c) inhibition of the oxidase system, or (d) extracellularly, by scavenging released oxygen metabolites.

Sulphasalazine and 5ASA have both been shown to inhibit the chemotactic activity of neutrophils.[21] It has been suggested that these drugs inhibit neutrophil activity by blocking binding of fMLP to its receptor.[22] The inhibition of luminol dependent chemiluminesence by SAL and 5ASA showed here, however, is unlikely to be a consequence of blocking the fMLP receptor, because the drugs produced little inhibition of oxygen consumption during the respiratory burst of neutrophils stimulated with fMLP+CB.

A direct non-specific effect of the drugs on the neutrophil was also excluded as there was little inhibition of PMA stimulated luminol-dependent

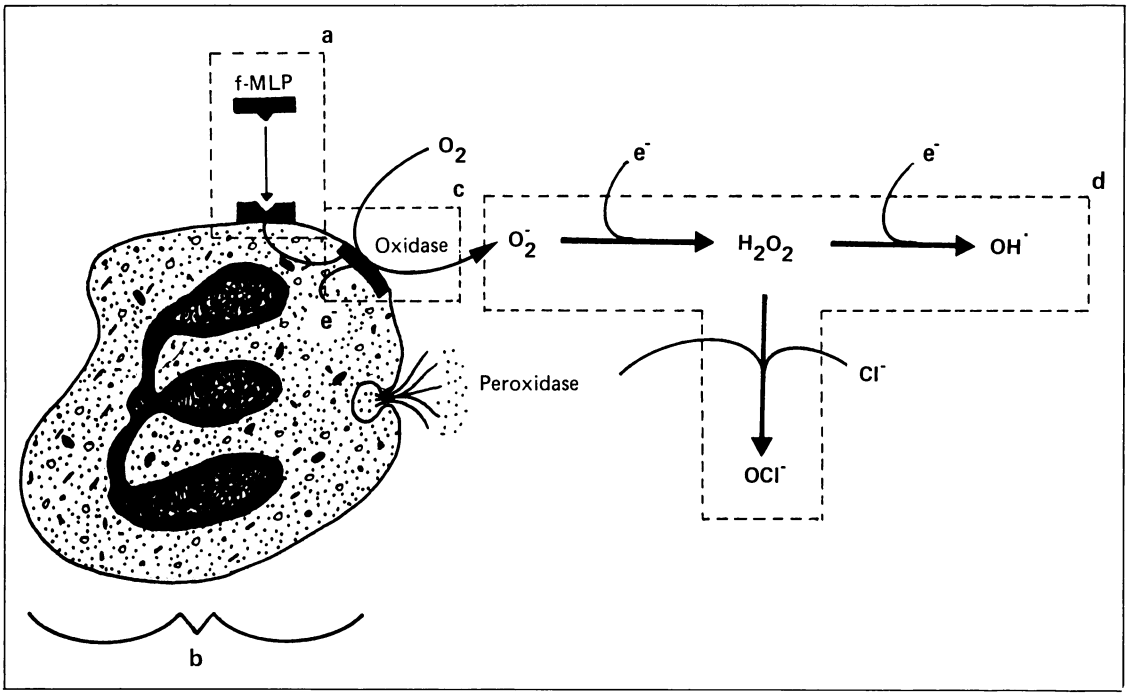

Fig. 4 *Toxic oxygen metabolite production by neutrophils. Schematic representation of the mechanism of production of toxic metabolites of oxygen by stimulated neutrophils. When activated, in this situation by the chemotactic peptide fMLP, the oxidase system converts oxygen into superoxide (O^-_2) by addition of an electron. Addition of further electrons results in the formation of hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH). Myeloperoxidase, released simultaneously with oxidase activation, reacts with H_2O_2 and chloride to form hypochlorite (OCl^-). Possible sites of action of 5ASA and SAL in inhibiting luminol-dependent chemiluminesence are shown.* (a) *Binding of fMLP to its receptor,* (b) *Direct general inhibition of cellular function,* (c) *Inhibition of the oxidase system,* (d) *Extracellularly by scavenging released radicals.*

chemiluminesence and oxygen consumption was not affected by sulphasalazine and 5ASA. This also excludes the possibility that the drugs acted by inhibition of the oxidase enzyme system (site c, Fig. 4).

An extracellular site of action (d, Fig. 4) therefore remains the only possibility. Previous authors have shown that sulphasalazine and its metabolites react variably with superoxide, hydrogen peroxide and hydroxyl radicals in cell-free systems.[23-26] Our results suggest that superoxide is not the major metabolite being scavenged, as 5ASA only inhibited superoxide production by neutrophils by 35% and sulphasalazine by 7·9%. Luminol dependent chemiluminesence was almost totally inhibited by similar concentrations of 5ASA, which would imply that a secondary radical, formed from superoxide, is being scavenged.

Triggering of luminol dependent chemiluminesence by neutrophils depends on release of myeloperoxidase and hydrogen peroxide, conditions which generate hypochlorite.[27,28] Sulphasalazine and 5ASA do not inhibit myeloperoxidase (unpublished observations) therefore, the inhibition of luminol-dependent chemiluminesence, may be the result of hypochlorite scavenging by sulphasalazine and 5ASA. Auroma *et al*[25] showed that 5ASA scavenges hypochlorite in a cell free system and our similar conclusion, based on experiments on neutrophils, strengthens the hypothesis that 5ASA acts by scavenging hypochlorite in inflamed tissue.

Oxygen radical production by fMLP+CB stimulated neutrophils involves exocytosis of myeloperoxidase, whereas little myeloperoxidase is released after stimulation with PMA.[29] Thus hypochlorite formation occurs extracellularly after fMLP stimulation and intracellularly after PMA. The differential effect of 5ASA and SAL on luminol dependent chemiluminesence stimulated by fMLP and PMA may reflect a difference in the availability of these drugs at extracellular and intracellular sites, or differences in the generation of the peroxidase-dependent metabolite.

After oral ingestion, one third of the dose of 5ASA is recovered in urine, mostly as the acetylated

metabolite. Half the dose remains in the faeces, mostly as unchanged 5ASA.[30] Plasma concentration of 5ASA are low, ranging from 1·3 μM[30] to 13 μM,[3] similar to the concentrations used in these experiments. Concentrations of acetyl 5ASA are roughly double this and the concentration of 5ASA in the faecal stream is much greater (10 mM).[2] The concentration of 5ASA in the bowel wall in ulcerative colitis is unknown. A higher concentration than the plasma concentration can be postulated, however, as a result of the close proximity of the ulcerated mucosa to 'high' concentrations of 5ASA in the colonic lumen.

The work presented here, provides strong evidence that 5ASA, and to a lesser extent sulphasalazine are able to modify toxic oxygen metabolite production by neutrophils *in vitro*, by scavenging a luminol reactive metabolite, probably hypochlorite. Hypochlorite ions play an important part in the pathogenesis of tissue damage in inflammatory sites. As well as being a powerful oxidant, hypochlorite inactivates alpha-1-proteinase inhibitor[31,32] and also activates released neutrophil collagenase.[33] Thus, a drug which rapidly removes hypochlorite, generated by neutrophils *in vivo*, would ameliorate tissue damage in the acute phase, and have a prophylactic effect against the initiation of tissue damage during longterm use.

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