

5-AMINOSALICYLIC ACID (5-ASA) has been used for over 50 years in the treatment of inflammatory bowel disease in the pro-drug form sulphasalazine (SASP). SASP is also used to treat rheumatoid arthritis. However whether the therapeutic properties of SASP are due to the intact molecule, the 5-ASA or sulphapyridine components is unknown. Several mechanisms of action have been proposed for 5-ASA and SASP including interference in the metabolism of arachidonic acid to prostaglandins and leukotrienes, scavenging of reactive oxygen species, effects on leucocyte function and production of cytokines. However, it is unlikely that the anti-inflammatory properties of SASP and 5-ASA are due to several different properties but more likely that a single property of 5-ASA explains the therapeutic effects of 5-ASA and SASP. Reactive oxygen species (ROS) are involved in the metabolism of prostaglandins and leukotrienes and can act as second messengers, and so the scavenging of ROS may be the single mechanism of action of 5-ASA that gives rise to its anti-inflammatory effects in both inflammatory bowel disease and rheumatoid arthritis.

**Key words:** 5-Aminosalicylic acid, Inflammatory bowel disease, Reactive oxygen species, Rheumatoid arthritis, Sulphasalazine

## Mechanism of action of 5-aminosalicylic acid

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## Introduction

Although corticosteroids are the major anti-inflammatory agents used to treat the chronic inflammatory conditions rheumatoid arthritis (RA) and inflammatory bowel disease (IBD), another useful agent is the salicylate derivative 5-aminosalicylic acid (5-ASA). 5-ASA has been used for over half a century in the pro-drug form sulphasalazine (SASP) to treat ulcerative colitis (UC), but was originally designed for use in RA, in which, until relatively recently, it has not been widely used. More recently, SASP has also been used in the treatment of mild psoriasis.<sup>1</sup>

In the late 1930s Dr Nanna Svartz of the Karolinska Institute, Stockholm, became interested in treating RA, believed then to be due to an infectious agent, with the recently developed anti-bacterial agents, the sulphonamides, which were beneficial in the treatment of septic arthritis.<sup>2</sup> However, treatment with these agents either alone or in conjunction with salicylates, which were already used to treat RA, proved ineffective. As salicylate drugs reduced the swelling in inflamed joints but had a lesser effect in reducing inflammation elsewhere in the body it was possible that they became concentrated in the joints, and thus Dr Svartz had the concept that if the salicylate was bound to the sulphonamide then the salicylate

might carry it into the joint, where it would be antibacterial. Dr Svartz therefore tried to bond salicylic acid chemically to sulphapyridine, but without success.

However, as a result of a chance meeting she convinced Pharmacia in Stockholm to help<sup>2</sup> and in collaboration with their chemists, E. Askelof and Dr P. H. Willstaedt, a variety of different combinations of salicylate and sulphonamide were produced. One of these, salicylazosulphapyridine, more commonly referred to as sulphasalazine (SASP) and consisting of 5-aminosalicylic acid (5-ASA) and sulphapyridine (SP) joined together by a diazo bond, proved to be effective in treating RA.

In collaboration with Sture Helander, SASP was shown to have an affinity for connective and elastic tissue, such as in the joint capsules and in the bowel, to the extent of forming deposits that gradually broke down to produce 5-ASA and SP.<sup>3-5</sup> As in UC the inflammatory changes occurred in the sub-epithelial connective tissue and as SP had been used with moderate success by Dr Svartz in the treatment of UC, which was then also believed to be due to an infectious agent,<sup>6,7</sup> she treated UC patients with SASP and found that "some cases which did not become free of symptoms with sulphapyridine rapidly improved with salazopyrine".<sup>6</sup>

However, the Second World War and the development of more effective gold based drugs in

the treatment of RA<sup>8</sup> retarded the use of SASP in UC and RA. It was therefore not until the early 1950s that SASP was used in the USA<sup>9</sup> and the UK, and in 1963 the first controlled, double-blind trials showed SASP to be effective in the treatment of mild UC,<sup>10</sup> and later in maintaining remission of UC.<sup>11</sup> In the 1970s its use for RA was rediscovered<sup>12</sup> and confirmed in a series of studies.<sup>13</sup>

In 1977 direct application of the components of SASP to the colonic mucosa in patients with acute UC demonstrated that 5-ASA, but not SP, produced remission in UC similar to that achieved with SASP,<sup>14</sup> as confirmed in other studies,<sup>15,16</sup> and that SP was relatively inactive.<sup>17</sup> Thus it is now considered that in the treatment of UC the SASP acts as a pro-drug, with SP acting as a carrier, delivering the active moiety, 5-ASA, to the inflamed colon. The realization that 5-ASA is the active moiety and that the SP component is responsible for the side effects of SASP, such as infertility, haemolytic anaemia, photosensitization and agranulocytosis, has led to the development of new preparations of 5-ASA. These consist of 5-ASA alone, either covered by a pH sensitive coating or as slow release microgranules that break down in the colon, or of 5-ASA bound to an inactive carrier molecule or to another molecule of 5-ASA by an azo bond (azodisalicylic acid) that, as for SASP, is broken down by bacteria releasing the 5-ASA in the bowel. The main use of 5-ASA in IBD is in UC, although it has some benefit in the treatment of patients with the IBD Crohn's disease (CD).

In RA, although some studies have indicated that SP may be the active moiety<sup>18-21</sup> due to a bacteriostatic effect, others show that SP on its own is ineffective<sup>22</sup> and it has been suggested that the intact SASP molecule may possess anti-inflammatory properties.<sup>23-25</sup> The ability of SASP to accumulate in connective tissues of the joints where it is broken down to release 5-ASA, raises the possibility that SASP may act also as a pro-drug in RA, but delivering 5-ASA, the active component, to the inflamed joint where it is slowly released. The lack of effect of oral 5-ASA in RA can be explained on the basis of its rapid metabolism and excretion once absorbed from the gastrointestinal tract.

Despite its use for over 50 years, the mechanism of action of SASP and 5-ASA remains unclear. A large number of studies have led to a wide range of hypotheses. This review will attempt to relate the effects of SASP and 5-ASA on production of biological mediators to their effects on cells and tissues, and to those of nonsteroidal anti-inflammatory agents, but is mainly concerned with the proposed mechanisms of action in IBD, from which comes most of the information available.

## Arachidonic Acid Metabolism

Enzymatic oxidation of the essential fatty acid arachidonic acid gives rise to three main groups of compounds: the prostaglandins (PGs) and thromboxanes (TXs) produced by the cyclooxygenase pathway; the leukotrienes (LTs) and the intermediate hydroxyecosatetraenoic acids (HETEs) from the lipoxygenase pathway; and another group, the lipoxins. The lipoxins have not been studied with respect to SASP and 5-ASA. The products of arachidonic acid are rapidly turned over, locally acting mediators that have been implicated in a range of physiological procedures including reproduction, inflammation, immunological responses and cell growth, division, motility and transport processes.

*The cyclooxygenase pathway:* Early studies indicated that some PGs, for example prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) were proinflammatory. Thus the increased levels found at sites of inflammation or in samples derived from inflamed sites, and the increased PG production by leucocytes from patients with chronic inflammatory diseases all supported this. Raised concentrations of prostaglandins in the stools of patients with active UC were first described by Gould<sup>26</sup> and subsequently increased concentrations of PGE<sub>2</sub> were found in rectal mucosal biopsy specimens,<sup>27-31</sup> stools<sup>32,33</sup> and rectal dialysates of UC patients<sup>34-37</sup> with active disease, suggesting that the increased PGs were associated with generation of the inflammation in IBD. The non-steroidal anti-inflammatory agents (NSAIDs) are considered to act through inhibition of the cyclooxygenase pathway, and so increased PG production by the inflamed bowel on removal of SASP treatment or decreased production with SASP<sup>27,28,34,36</sup> or 5-ASA<sup>35,37</sup> suggested that the mechanism of action of SASP and 5-ASA was also through inhibition of the cyclooxygenase enzyme.

Earlier *in vitro* studies supported this, but later ones do not (Tables 1 and 2). Although there are exceptions, we<sup>38</sup> and others<sup>39-46</sup> have demonstrated that relatively low concentrations of SASP and/or 5-ASA can enhance PG production with inhibition of PG production mainly only occurring at high concentrations. Even in some studies in which PG production was only inhibited, promotion can be observed either as a negative inhibition<sup>41</sup> or by a dose related, but not significant, enhancement.<sup>40</sup> Although SP enhanced production of one out of four PGs measured in one study,<sup>41</sup> in the others, either in cell free incubations,<sup>33,41,43,47,48</sup> or in incubations of gastrointestinal biopsies<sup>27,30</sup> or mononuclear (MN) cells,<sup>38</sup> SP either had no effect<sup>33,36,40,48</sup> or inhibited<sup>27,30,31,38,39,41,47,49</sup> PG production, dose dependently.<sup>38</sup>

Except in one study where 5-ASA actually

**Table 1.** *In vitro* studies of the effects of sulphasalazine on prostaglandin and thromboxane production

Study	Tissue studied	Concentration mol l <sup>-1</sup>	Effects on cyclooxygenase products
Collier <i>et al.</i> ; 1976 <sup>47</sup>	Bull seminal vesical homogenates	IC <sub>50</sub> = 4.7 × 10 <sup>-4</sup>	↓(E <sub>2</sub> + F <sub>2α</sub> ) <sup>#</sup>
Smith <i>et al.</i> ; 1978 <sup>27</sup>	Human normal and inflamed mucosal homogenates	0.2–50 × 10 <sup>-4</sup>	↓(I <sub>2</sub> + F <sub>2α</sub> )
Sharon <i>et al.</i> ; 1978 <sup>31</sup>	Human normal and inflamed rectal biopsies	2.5 × 10 <sup>-4</sup>	↓E <sub>2</sub>
Hoult & Moore; 1978 <sup>49</sup>	Rat colonic microsomes	10 <sup>-8</sup> –10 <sup>-3</sup>	↓E <sub>2</sub>
Hoult & Moore; 1980 <sup>48</sup>	Animal microsomal preparation	0.1 & 1 × 10 <sup>-3</sup>	↓(E <sub>2</sub> + F <sub>2α</sub> )
Ligumsky <i>et al.</i> ; 1981 <sup>30</sup>	Human normal and inflamed rectal biopsies	2.52 × 10 <sup>-4</sup>	↓I <sub>2</sub> , ↓E <sub>2</sub> , ↓TXA <sub>2</sub>
Gould <i>et al.</i> ; 1981 <sup>33</sup>	Rat fundus strip (indirect bioassay)	0.2–2 × 10 <sup>-3</sup>	↓PG
Schlenker & Peskar; 1981 <sup>52</sup>	Human colon mucosal microsomes	2.5 × 10 <sup>-4</sup>	↑↑E <sub>2</sub> <sup>d</sup>
Rachmilewitz <i>et al.</i> ; 1982 <sup>40</sup>	Unstimulated PBMC from healthy subjects	1.26 & 2.52 × 10 <sup>-4</sup> 5 × 10 <sup>-4</sup>	NA. §(↑ <sup>a</sup> ) E <sub>2</sub> , ↓TXA <sub>2</sub>
Hawkey & Trulove; 1983 <sup>41</sup>	Colitic rectal biopsy homogenates	10 <sup>-6</sup> & 10 <sup>-4</sup> , 10 <sup>-3</sup> –10 <sup>-2</sup>	↑E <sub>2</sub> <sup>c</sup> ↓E <sub>2</sub>
Stenson & Lobos; 1983 <sup>42</sup>	Platelets	0.1–4.0 × 10 <sup>-3</sup>	↑E <sub>2</sub> , ↑F <sub>2α</sub> , ↓TXA <sub>2</sub>
Hawkey <i>et al.</i> ; 1985 <sup>43</sup>	Human colonic mucosal homogenates	5 × 10 <sup>-5</sup> 10 <sup>-3</sup>	§I <sub>2</sub> , §E <sub>2</sub> , ↑F <sub>2α</sub> , ↓TXA <sub>2</sub> ↓I <sub>2</sub> , §E <sub>2</sub> , ↑F <sub>2α</sub> , ↓TXA <sub>2</sub>
Kolassa <i>et al.</i> ; 1985 <sup>45</sup>	Rabbit colonic microsomes	10 <sup>-3</sup>	NA on I <sub>2</sub> , E <sub>2</sub> , F <sub>2α</sub> , TXA <sub>2</sub>
Hillier <i>et al.</i> ; 1984 <sup>134</sup>	Stimulated PBMC	1.26 × 10 <sup>-4</sup>	↑E <sub>2</sub>
Keating <i>et al.</i> ; 1988 <sup>46</sup>	Stimulated PBMC from IBD patients	(not quoted)	↑E <sub>2</sub>
Punchard <i>et al.</i> ; 1992 <sup>38</sup>	PBMC from healthy subjects;		
	Unstimulated	10 <sup>-7</sup> –10 <sup>-5</sup> 10 <sup>-4</sup> 10 <sup>-3</sup>	NA ↑I <sub>2</sub> , ↑E <sub>2</sub> , ↑F <sub>2α</sub> §I <sub>2</sub> , ↑E <sub>2</sub> , F <sub>2α</sub>
	LPS-stimulated	10 <sup>-7</sup> & 10 <sup>-6</sup> 10 <sup>-5</sup> & 10 <sup>-4</sup> 10 <sup>-3</sup>	NA ↑I <sub>2</sub> , ↑E <sub>2</sub> , ↑F <sub>2α</sub> ↓I <sub>2</sub> , §E <sub>2</sub> , ↓F <sub>2α</sub>

E<sub>2</sub> = Prostaglandin PGE<sub>2</sub>; F<sub>2α</sub> = PGF<sub>2α</sub>; I<sub>2</sub> = PGI<sub>2</sub>; TXA<sub>2</sub> = Thromboxane A<sub>2</sub>; PBMC = peripheral blood mononuclear cells.

In these studies PGI<sub>2</sub> and TXA<sub>2</sub> were measured as their respective stable hydrolysis products 6KF<sub>1α</sub> and TXB<sub>2</sub>.

§ = no effect; ↑ = enhancement; ↓ = inhibition. NA = no effect on any PGs measured, as opposed to those PGs measured as stated which were effected.

<sup>#</sup> In this study the sum of both PGs was reported.

<sup>a</sup> Enhancement in this study was not significant but as it is large and increased with increasing concentration of drug we have included it.

<sup>c</sup> In this study results are presented as % inhibition and this enhancement is apparent as negative (<0%) inhibition which is reduced with increasing concentration to produce inhibition at high concentrations.

<sup>d</sup> In this study whether PG production was enhanced or inhibited depended on the concentration of substrate used.

All concentrations have been converted to mol l<sup>-1</sup> to allow direct comparison.

enhanced production, both SASP and 5-ASA inhibit production of thromboxane A<sub>2</sub> (TXA<sub>2</sub>), a potent vasoconstrictor that increases the adhesion to the endothelium and aggregation of platelets and polymorphonuclear cells (PMN). SP either inhibits<sup>30,40,41</sup> or has no effect<sup>42</sup> on TXA<sub>2</sub> production. Although inhibition of TXA<sub>2</sub> production by SASP is associated with increased PG production,<sup>42,50</sup> inhibition of TXA<sub>2</sub> synthesis by 5-ASA<sup>42</sup> and specific TXA<sub>2</sub> inhibitors<sup>42,50</sup> is not, suggesting that the promotion of PGs is not just a result of inhibition of TXA<sub>2</sub> synthesis increasing the availability of substrate. However, the converse, that increased synthesis of PGs removes substrate for TXA<sub>2</sub> synthesis, may be true. In a model of colitis 5-ASA and inhibitors of TXA<sub>2</sub> were equally effective anti-inflammatory agents, suggesting that

the anti-inflammatory properties might be through inhibition of TXA<sub>2</sub> production.<sup>51</sup>

Differences in the incubation conditions used may alter the responses of the tissues to the drugs and this may be why 5-ASA and SASP inhibit PG production in some studies and, at the same concentration, enhance production in others. For example, in one study the concentration of the exogenous substrate (arachidonic acid) determined whether SASP enhanced or inhibited PGE<sub>2</sub> production,<sup>52</sup> while in another addition of substrate reversed the inhibition of MN cell PG production by SASP.<sup>53</sup> We have also found that stimulation of MN cells increases their sensitivity to the effects of SASP, 5-ASA and SP, which may result from increased concentrations of free arachidonic acid released by phospholipases activated by stimulation

**Table 2.** *In vitro* studies of the effects of 5-aminosalicylic acid on prostaglandin and thromboxane production

Study	Tissue studied	Concentration mol l <sup>-1</sup>	Effects on cyclooxygenase products
Collier <i>et al.</i> ; 1976 <sup>47</sup>	Bull seminal vesical homogenates	IC <sub>50</sub> = 7.1 × 10 <sup>-3</sup>	↓(E <sub>2</sub> + F <sub>2x</sub> ) <sup>#</sup>
Sharon <i>et al.</i> ; 1978 <sup>31</sup>	Human normal and inflamed rectal biopsies	3.2 × 10 <sup>-4</sup>	↓E <sub>2</sub>
Hoult & Moore; 1980 <sup>48</sup>	Animal microsomal preparation	0.1 & 1 × 10 <sup>-3</sup>	↓(E <sub>2</sub> + F <sub>2x</sub> )
Hoult & Page; 1981 <sup>39</sup>	Human normal colonic fragments	5 & 50 × 10 <sup>-5</sup>	↑I <sub>2</sub> , ↑F <sub>2x</sub>
	Rat caecal fragments	5 × 10 <sup>-3</sup>	NA
		5 × 10 <sup>-5</sup>	NA
		5 × 10 <sup>-4</sup>	↑I <sub>2</sub> , ↑F <sub>2x</sub>
		5 × 10 <sup>-3</sup>	↓I <sub>2</sub> , ↓F <sub>2x</sub>
Ligumsky <i>et al.</i> ; 1981 <sup>30</sup>	Human normal and inflamed rectal biopsies	3.2 × 10 <sup>-3</sup>	↓I <sub>2</sub> , ↓E <sub>2</sub> , ↓TXA <sub>2</sub>
		6.5 × 10 <sup>-4</sup>	§I <sub>2</sub> , ↓E <sub>2</sub> , ↓TXA <sub>2</sub>
		1.3 × 10 <sup>-3</sup>	↓I <sub>2</sub> , ↓E <sub>2</sub> , ↓TXA <sub>2</sub>
Gould <i>et al.</i> ; 1981 <sup>33</sup>	Rat fundus strip (indirect bioassay)	0.1–10 × 10 <sup>-3</sup>	↓PG
Rachmilewitz <i>et al.</i> ; 1982 <sup>40</sup>	Unstimulated PBMNC from healthy subjects	3.3 × 10 <sup>-5</sup>	NA
		3.3 × 10 <sup>-4</sup>	↓E <sub>2</sub> , §TXA <sub>2</sub>
		4.9 & 32.7 × 10 <sup>-4</sup>	↓E <sub>2</sub> , ↓TXA <sub>2</sub>
Hawkey & Trulove; 1983 <sup>41</sup>	Colitic rectal biopsy homogenates	10 <sup>-5</sup> –10 <sup>-3</sup>	§E <sub>2</sub>
		10 <sup>-3</sup> –10 <sup>-1</sup>	↓E <sub>2</sub>
Stenson & Lobos; 1983 <sup>42</sup>	Platelets	0.1–4.0 × 10 <sup>-3</sup>	§E <sub>2</sub> , §F <sub>2x</sub> , ↓TXA <sub>2</sub>
Hawkey <i>et al.</i> ; 1985 <sup>43</sup>	Human colonic mucosal homogenates	5 × 10 <sup>-5</sup>	NA
		1 × 10 <sup>-3</sup>	§I <sub>2</sub> , ↓E <sub>2</sub> , §F <sub>2x</sub> , §TXA <sub>2</sub>
Berry & Hoult; 1984 <sup>44</sup>	Rat caecal fragments	5 × 10 <sup>-4</sup>	↑I <sub>2</sub>
		>5 × 10 <sup>-4</sup>	↓I <sub>2</sub>
Kolassa <i>et al.</i> ; 1985 <sup>45</sup>	Rabbit colonic microsomes	10 <sup>-5</sup>	↑I <sub>2</sub> , §E <sub>2</sub> , §F <sub>2x</sub> , ↑TXA <sub>2</sub>
		10 <sup>-4</sup>	↑I <sub>2</sub> , ↑E <sub>2</sub> , §F <sub>2x</sub> , ↑TXA <sub>2</sub>
		10 <sup>-3</sup>	↑I <sub>2</sub> , ↑E <sub>2</sub> , ↑F <sub>2x</sub> , ↑TXA <sub>2</sub>
Keating <i>et al.</i> ; 1988 <sup>49</sup>	Stimulated PBMNC from IBD patients	(not quoted)	↑E <sub>2</sub>
Mahida <i>et al.</i> ; 1991 <sup>138</sup>	Human, inflamed biopsy	3.3 & 6.6 × 10 <sup>-4</sup>	§TXA <sub>2</sub>
Panchard <i>et al.</i> ; 1992 <sup>38</sup>	PBMNC from healthy subjects:		
	Unstimulated	10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup> , 10 <sup>-4</sup>	NA
		10 <sup>-3</sup>	↓I <sub>2</sub> , ↓E <sub>2</sub> , ↓F <sub>2x</sub>
	LPS-stimulated	10 <sup>-7</sup>	NA
		10 <sup>-6</sup>	§I <sub>2</sub> , ↑E <sub>2</sub> , ↑F <sub>2x</sub>
		10 <sup>-5</sup>	§I <sub>2</sub> , §E <sub>2</sub> , ↑F <sub>2x</sub>
		10 <sup>-4</sup>	NA
		10 <sup>-3</sup>	↓I <sub>2</sub> , ↓E <sub>2</sub> , ↓F <sub>2x</sub>

E<sub>2</sub> = Prostaglandin PGE<sub>2</sub>; F<sub>2x</sub> = PGF<sub>2x</sub>; I<sub>2</sub> = PGI<sub>2</sub>; TXA<sub>2</sub> = Thromboxane A<sub>2</sub>; PBMNC = peripheral blood mononuclear cells.

In these studies PGI<sub>2</sub> and TXA<sub>2</sub> were measured as their respective stable hydrolysis products 6KF<sub>1α</sub> and TXB<sub>2</sub>.

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\* In this study the sum of both PGs was reported.

<sup>a</sup> Enhancement in this study was not significant but as it is large and increased with increasing concentration of drug we have included it.

<sup>c</sup> In this study results are presented as % inhibition and this enhancement is apparent as negative (<0%) inhibition which is reduced with increasing concentration to produce inhibition at high concentrations.

<sup>d</sup> In this study whether PG production was enhanced or inhibited depended on the concentration of substrate used.

All concentrations have been converted to mol l<sup>-1</sup> to allow direct comparison.

with LPS. The increase in production of PG by MN cells from healthy subjects with 5-ASA<sup>38,54</sup> is much less than that found using MN cells from IBD patients.<sup>46</sup> This may indicate that cells from IBD patients are more sensitive to 5-ASA, possibly due to prior activation by the inflammatory process *in vivo*. Some *in vitro* incubations may thus have used relative proportions of SASP, substrate and enzyme that only favoured inhibition. The conversion of added radiolabelled arachidonic acid to PGs, as used in some studies, may not have mimicked the unlabelled endogenous substrate and a low

conversion substrate could even result from increased competition and conversion of endogenous substrate.

There is indeed little evidence that SASP and 5-ASA inhibit PG synthesis in patients. In some studies PG production *in vivo* in patients who relapsed during maintenance therapy with SASP increased despite treatment<sup>34</sup> and in patients treated with 5-ASA but who failed to respond to therapy PG levels increased,<sup>37</sup> suggesting that disease activity rather than treatment produces the changes in PG production. Removal of SASP therapy in

patients with inactive disease did not affect production.<sup>55</sup> The *in vivo* and *ex vivo* changes observed in patients associated with SASP or 5-ASA therapy may equally have been due to a reduction in the numbers of PG-producing cells in the bowel, specifically the mononuclear cells (MN) since polymorphonuclear cells produce little or no PG.<sup>56</sup> The degree of inflammatory cell infiltrate in animals<sup>55,57</sup> and the increased levels of the substrate arachidonic acid in the mucosa in IBD<sup>58</sup> have been correlated with the cell density of the inflammatory infiltrate<sup>59</sup> and thus changes in PG production may be the secondary consequence of the invasion of the inflamed bowel by MN cells.<sup>28</sup> Changes in permeability with inflammation could also lead to increased leakage of PGs into the bowel. Thus it is likely that the decrease in PG production associated with SASP reflects only a decrease in the number of PG-producing MN cells present in the colonic mucosa during the healing process.

Indomethacin and other (NSAIDs) are far more potent inhibitors of PG production than either SASP or 5-ASA and are not effective in the treatment of, and may even exacerbate, UC.<sup>60-64</sup> Moreover, not only can PGs and their analogues protect the gastric and small intestinal mucosa against injury (cytoprotection) but they are also protective in the colon.<sup>65-69</sup> Production of PGs may therefore be beneficial rather than damaging. A failure to produce protective PGs in response to stimulation in the inflamed bowel may exacerbate the disease and be important in maintaining the inflammatory process. However, in RA NSAIDs are beneficial. This would suggest that either there are different mechanisms involved in the inflammation in RA, that the site of the inflammation modifies the action of the drugs, or that the effects of NSAIDs are not directly related to their actions on the cyclooxygenase enzyme.

The promotion of PG production with low concentrations of 5-ASA may be because 5-ASA, acting as an antioxidant and reducing co-factor, prevents free radical mediated inactivation of cyclooxygenase.<sup>39,44</sup> Similarly, SASP (see below) can also act as an antioxidant and could thus also act as a reducing co-factor in PG synthesis. Conversely, the inhibition of PG production at high concentrations of SASP and 5-ASA may be due to inactivation of cyclooxygenase by free radical intermediates of the drug.<sup>70</sup> Other antioxidants can also affect PG synthesis,<sup>71-76</sup> and thus some of the inconsistencies between previous *in vitro* studies may be due either to the addition to the incubation media of the antioxidants adrenaline and reduced glutathione,<sup>27,41,47</sup> both of which affect PG production,<sup>41,42,49,72</sup> or to losses of endogenous antioxidants during preparation of the homogenates.<sup>77</sup>

SASP inhibits the NAD-dependent 15-hydroxy

PG dehydrogenases (NAD-PGDH), which are enzymes that catabolize PGs to their 15-keto-13,14 dihydro derivatives, in cell free systems,<sup>48,49,78-80</sup> the isolated perfused rat lung<sup>48</sup> and when perfused into rats.<sup>48</sup> NSAIDs such as indomethacin, which are ineffective or deleterious in IBD, also inhibit NAD-PGDH non-competitively,<sup>48,78,80-82</sup> although unlike SASP, they are more active as inhibitors of PG synthesis than breakdown.<sup>49,80</sup> However, NAD-PGDH is unstable under conditions similar to those used in most of the above *in vitro* studies<sup>81</sup> and the inhibition of PGDH by indomethacin is through formation of an unreactive complex and denaturation of the NADH enzyme *in vitro* rather than a phenomenon of physiological importance.<sup>81,82</sup> It is recommended that inhibition of PGDH observed in aqueous solutions should be confirmed under conditions such that denaturation or inactivation would not occur.<sup>81,82</sup> Such studies have not been performed with SASP and thus it is unclear whether SASP inhibits or simply inactivates NAD-PGDH. Other types of PGDH with different specificities for the different PGs, higher reactivity with cofactors other than NAD,<sup>83</sup> and broad substrate specificities<sup>84</sup> have been described and it is unclear whether SASP has the same effects on all of these.

The human lung is less sensitive to SASP than the rat<sup>85</sup> and the high concentrations of SASP used and removal of the effects of SASP upon the addition of albumin to the perfusate suggests that SASP in the circulation will have little effect upon PG degradation *in vivo*. It is possible that SASP, rather than acting inside tissues, blocks PG uptake.<sup>85,86</sup> The unphysiologically high concentrations of PGs and SASP used, and lack of information on the changes in endogenous, unlabelled pools in some of these studies also raises doubts about the interpretation of the results. When PG synthesis and catabolism have been measured together, inhibition of PG catabolism by SASP is not associated with increased production<sup>45,49</sup> or production of both the PG and its metabolite are enhanced simultaneously.<sup>52</sup> Furthermore 5-ASA, and also SP, were not active as inhibitors of the NAD-PGDH.<sup>48,78,79</sup>

Whether SASP and 5-ASA inhibit or enhance MN cell PG production *in vitro* also depends on the compound, its concentration, which PG is being measured, and whether PG production is stimulated or not. The species of animal used also alters the inhibition of PG production by SASP and 5-ASA.<sup>48</sup> The incubation conditions affect the results seen, and so whether the conditions in the mucosa are accurately reproduced in cell and tissue incubations is unknown. The results from such *in vitro* studies should therefore be extrapolated to the therapeutic mechanisms *in vivo* with caution.

Whereas the concentrations of these drugs in the bowel lumen could favour either enhancement or inhibition of PG production, they are probably much lower in the mucosa where they are likely to enhance PG production. The enhancement of PG production by SASP and 5-ASA, with mainly inhibition by the clinically ineffective SP, and the cytoprotective effects of PGs all suggest that enhancement is a more likely mechanism of action of SASP and 5-ASA than inhibition. Thus SASP and 5-ASA could act therapeutically in IBD by enhancing protective PG production either alone, or together with inhibition of pro-inflammatory products of arachidonic acid.

SASP is poorly absorbed in the colon, and is unlikely to have systemic effects on PG degradation. 5-ASA has little, if any, effect on PGDH<sup>48,78,79</sup> and thus inhibition of PG degradation is unlikely to be the mechanism of action of 5-ASA in IBD. SP also inhibits PG production and the PG-inhibiting NSAIDs are ineffective in IBD, thus inhibition of PG production also seems unlikely as a mechanism of action in IBD. That SP also inhibits TXA<sub>2</sub> production and yet is ineffective in IBD may suggest that this is also an unlikely mechanism. Whether this is also true in RA depends upon which component of SASP is the active ingredient and whether NSAIDs possess other anti-inflammatory properties besides inhibition of PG production. Whether the mechanism by which 5-ASA and SASP promote PG production *in vitro* is the same or different is not certain, but given that PGs are protective to the colon, promotion of PG production by 5-ASA could explain the therapeutic properties of 5-ASA and the lack of effectiveness of SP in IBD. Whether promotion of PGs could also be protective in RA is unknown and it is possible that SASP and 5-ASA possess other properties that explain their anti-inflammatory effects in RA.

*Lipoxygenase products:* Much of the cellular damage at sites of inflammation is due to the local actions of activated leucocytes at the inflamed site. The increased recruitment of leucocytes from the peripheral blood into the inflamed site is in part a result of increased production of potent chemotactic lipoxygenase products, for example 5-HETE and LTB<sub>4</sub>. The anti-inflammatory effects of SASP and 5-ASA could thus be through reducing leucocyte recruitment into the inflamed bowel by inhibiting production of these chemotactic agents.

In cell free systems 5-ASA,<sup>87,88</sup> but not SP,<sup>86</sup> inhibits soyabean lipoxygenase activity, but there is disagreement as to whether SASP does<sup>88</sup> or does not,<sup>89</sup> probably a result of different analytical methods being used in the two studies. In cells both SASP and 5-ASA inhibit the production of HETEs by PMN<sup>90-93</sup> and also sulphidopeptide leukotrienes

by rat peritoneal cells,<sup>94</sup> but in platelets both these drugs enhance 12-HETE production.<sup>42</sup> SP has either a weak<sup>93</sup> or no effect on the lipoxygenase pathway.<sup>42,87,94</sup> SASP inhibits production of HETEs by inflamed mucosa *in vitro*,<sup>43,94</sup> and both SASP and 5-ASA inhibit synthesis of LTB<sub>4</sub> and sulphidopeptide leukotrienes by both normal and inflamed mucosa<sup>96-98</sup> and in rat basophil leukaemia cells,<sup>99</sup> probably through inhibition of glutathione transferase.<sup>99</sup> Although 5-ASA inhibited production of HETEs by inflamed mucosa *in vitro* in one study,<sup>33</sup> it did not in another.<sup>43</sup> Similarly, although acetyl 5-ASA was as good an inhibitor of soyabean lipoxygenase as 5-ASA,<sup>87</sup> other studies show acetyl 5-ASA to have no effect on PMN lipoxygenase activity.<sup>91,92</sup> It is possible that the methods used for determining the activity of the lipoxygenase pathway or, as for PG production, differences in the incubation conditions may alter the result obtained, suggesting that these *in vitro* results should be correlated to the *in vivo* situation with caution.

The promotion of PG production, for example as observed in our MN cell incubations,<sup>38</sup> could have been due to inhibition of the lipoxygenase pathway and redirecting of substrate into prostaglandin synthesis. However, 5-ASA or SASP did not affect MN cell LTB<sub>4</sub> production when PG synthesis was enhanced<sup>46</sup> and inhibition of the lipoxygenase pathway was not associated with increased cyclooxygenase activity.<sup>95,97</sup>

How closely these *in vitro* studies are related to the clinical mechanism of 5-ASA and SASP is unclear. Acetyl 5-ASA does not affect IBD, and yet in one study was as good an inhibitor of soyabean lipoxygenase as 5-ASA.<sup>87</sup> Conversely, 4-ASA, as effective as 5-ASA in the treatment of UC, does not inhibit PMN lipoxygenase activity *in vitro*.<sup>90</sup> A 5-lipoxygenase inhibitor was as effective as 5-ASA in a rat model of colitis<sup>100</sup> and yet benoxaprofen, a putative lipoxygenase inhibitor, was ineffective in UC.<sup>101</sup> Although increased levels of LTB<sub>4</sub> occur in the inflamed bowel,<sup>95</sup> the concentrations of LTB<sub>4</sub> in the colonic lumen were unchanged during successful 5-ASA treatment of patients with IBD.<sup>37</sup> As for PG production, the changes that occur in LTB<sub>4</sub> production with remission induced either by a lipoxygenase inhibitor or 5-ASA in an animal model of colitis were concluded to be due more to a reduction in the numbers of PMN, the major source of LTB<sub>4</sub>, infiltrating the bowel than due to a direct effect on the lipoxygenase pathway.<sup>100</sup>

An action of 5-ASA and SASP as lipoxygenase inhibitors would explain the clinical effects of these drugs in IBD and RA and the ineffectiveness of SP. However, although lipoxygenase inhibitors reduce LTB<sub>4</sub> production in UC<sup>102</sup> they still have to be shown to benefit IBD and RA. Many of the

products of the lipoxygenase pathway resemble those generated through non-enzymic attack of arachidonic acid by reactive oxygen species (ROS), while ROS are generated in the lipoxygenase pathway. Thus it may be difficult to differentiate between an effect of SASP and 5-ASA as ROS scavengers and an effect as inhibitors of the lipoxygenase pathway.

## Reactive Oxygen Species

Reactive oxygen species (ROS) are forms of oxygen that are more strongly oxidizing than oxygen itself, and include hydrogen peroxide ( $H_2O_2$ ), lipid peroxides, hypochlorous acid (HOCl) and oxygen-free radical species such as the superoxide and hydroxyl free radicals. Their ability to react with cellular constituents, resulting in damage to proteins, nucleic acids and cell membranes, means that ROS are highly toxic to living systems. Their reactive nature also means that ROS react with molecules close to their origin, the distance migrated being directly proportional to their reactivity. The ability of antioxidants to prevent cellular damage at sites of inflammation and ischaemia suggest that the ROS produced in these situations play an important role in generating the tissue injury.

At sites of inflammation the major source of the ROS are activated PMN, although MN cells also produce ROS. Activated PMN produce antimicrobial and cytotoxic ROS, namely the superoxide radical and  $H_2O_2$  and, through the action of the enzyme myeloperoxidase (MPO) on  $H_2O_2$  and the chloride ion, release HOCl. The ROS released may also react with suitable transition metals to produce the highly reactive hydroxyl radical. In addition, HOCl inactivates alpha-1-protease, which protects tissues against proteolysis at inflamed sites, and activates collagenase released from PMN. Some of the damage in IBD and RA is also suggested to arise from ischaemia. During periods of ischaemia ATP is broken down to hypoxanthine, and the enzyme xanthine dehydrogenase is converted to xanthine oxidase (XOD), which, upon reperfusion of the tissues, utilizes the oxygen to metabolize the hypoxanthine and in so doing generates the superoxide radical. Furthermore, changes in the endothelium promote PMN endothelial interactions, leading to activation of the PMN and a subsequent burst of ROS production from the PMN.

In cell free systems SASP, 5-ASA and SP all scavenge the hydroxyl radical equally, with rate constants close to the diffusion controlled limits.<sup>102</sup> The products of the attack of 5-ASA by ROS from stimulated leucocytes are similar to those produced by the iron-mediated Fenton reaction and are

indicative of a reaction between 5-ASA and the hydroxyl radical.<sup>104</sup> However, neither 5-ASA, SASP nor SP affected hydroxyl radical production in a xanthine oxidase cell free system,<sup>105</sup> and only 5-ASA, out of the three, inhibited the effects of hydroxyl radical production by PMN suggesting that under some conditions the effects of SASP and its metabolites may be negligible. Although the ability of 5-ASA to scavenge the hydroxyl radical *in vitro* may not reflect an ability to act as a hydroxyl radical scavenger in IBD,<sup>106</sup> 5-ASA is as effective as the hydroxyl radical scavenger dimethylsulphoxide (DMSO) in preventing experimentally induced ischaemia/reperfusion gastric injury.<sup>107</sup>

The effects of 5-ASA on the hydroxyl radical may be less important in IBD than the effects on leucocyte MPO-derived HOCl.<sup>108</sup> In a cell free system 5-ASA and SP both inhibited MPO-generated HOCl and had a direct inhibitory effect on the MPO enzyme.<sup>109</sup> SASP inhibits the metabolic burst of PMN that fuels the MPO,<sup>110</sup> and SASP and 5-ASA could act as inhibitors of the MPO enzyme rather than as scavengers of the ROS produced by MPO. Certainly the methods used for detecting ROS in cells often do not distinguish between inhibition of ROS production and scavenging effects. However, others have found inhibition of PMN ROS by SASP and 5-ASA not to be associated with decreased oxygen consumption<sup>111,112</sup> and the products of ROS attack of 5-ASA are found in leucocyte incubations,<sup>104</sup> indicating that inhibition of ROS activity by scavenging does occur in some incubations.

When formyl-methionyl-leucyl-phenylalanine (f-mlp) is used to stimulate PMN, 5-ASA<sup>111</sup> and SASP<sup>111,113</sup> and SP<sup>113</sup> all inhibit MPO dependent chemiluminescence. However, when phorbil myristate acetate (PMA) has been used to stimulate PMN neither 5-ASA<sup>111</sup> SASP<sup>111,113</sup> nor SP<sup>111</sup> affected, chemiluminescence. This lack of effect may either be because the intracellular site of ROS generated by PMA stimulation is inaccessible to the drugs<sup>111</sup> or to a lack of effect of the drugs against ROS produced by mechanisms that are independent of intracellular calcium release, suggesting that the effects of SASP and 5-ASA are calcium dependent.<sup>113</sup> Thus the effects of SASP and 5-ASA on PMN ROS generation depend on the stimulant used.

However, in other studies 5-ASA was an effective inhibitor of MPO activity in PMA-stimulated PMN<sup>112</sup> and in zymosan-stimulated PMN 5-ASA, but not SP, inhibited chemiluminescence.<sup>105</sup> However, SP was a better inhibitor of MPO induced iodination than 5-ASA.<sup>114</sup> SASP was inactive in both studies.<sup>105,114</sup> In cell free systems SP and 5-ASA inhibited MPO by different mechanisms.<sup>114</sup> Although in one study 5-ASA, but not

SASP nor SP, effectively inhibited production of the superoxide radical by PMN,<sup>105</sup> yet conversely, SASP and SP, but not 5-ASA inhibited the activity in another.<sup>114</sup> Differences in incubation conditions and methodology may modify the effect of 5-ASA and SASP on ROS *in vitro* and account for the differences between these studies.

The ability of SASP and diazosalicylic acid to inhibit the activity of the superoxide radical produced by PMN and to inhibit oxygen uptake by the cells, whereas SP was relatively inactive,<sup>110</sup> suggest that this results from direct inhibition of the enzymes.<sup>110</sup> In the same study SASP and diazosalicylic acid inhibited the activity of XOD-generated superoxide and urate production suggesting that again they acted directly on the XOD enzyme and not on the ROS produced.<sup>110</sup> This may be due to the diazo bond, rather than the 5-ASA structure, found in both molecules, since in a cell free system SASP, but not SP nor 5-ASA, inhibited XOD-generated superoxide activity.<sup>105</sup> Conversely, in another cell free study both 5-ASA and SASP reduced the activity of XOD generated superoxide radical and thus might equally be due to the 5-ASA component.<sup>115</sup> However, in this latter study uric acid production was not altered suggesting that the result was due to a scavenging effect rather than inhibition of the XOD enzyme. It is thus unclear whether in addition to scavenging PMN ROS, SASP and 5-ASA may also inhibit the enzymes involved in their production *in vitro*.

5-ASA and SASP also inhibit the action of peroxides. 5-ASA, and to a lesser extent SASP but not SP, reduce the activity of H<sub>2</sub>O<sub>2</sub> produced by PMN and xanthine oxidase.<sup>105</sup> Furthermore, we have shown that 5-ASA, and to a lesser extent SASP, but SP only marginally, inhibit production of lipid peroxides in ROS stressed erythrocytes,<sup>54,110</sup> and others that 5-ASA is more effective than SASP, diazosalicylic acid or SP in inhibiting haemoglobin catalysed lipid peroxidation.<sup>117</sup> In other studies 5-ASA, but not SASP nor SP, scavenged (reduced) the organic radical 1,1-diphenyl-2-picrylhydrazyl,<sup>92,118</sup> and thus the anti-inflammatory effects of 5-ASA may be directed at other, organic radical species. 5-ASA, but not SASP nor SP, also protects the protein, alpha-1-antiprotease against inactivation by HOCl.<sup>103</sup>

The ability of 5-ASA and SASP to react with ROS with sufficient affinity enables them to protect cellular constituents against ROS-mediated injury as illustrated by the ability of 5-ASA to protect cells in culture against the superoxide radical and H<sub>2</sub>O<sub>2</sub><sup>104</sup> and also stimulated PMN.<sup>112</sup> Moreover, in a cellular system 5-ASA and SASP, but not SP, also reduced ROS-induced chemiluminescence in colonic mucosal scrapings, and reduced cytochrome C reduction by colonic crypt cells.<sup>115</sup> This suggests that

scavenging of ROS may be more important than any effect on the enzymes involved in their generation.

The ROS scavenging ability of 5-ASA and SASP may be the basis of their anti-inflammatory properties. SASP reduced inflammation in acetic acid induced colitis in an animal model as effectively as did superoxide scavengers.<sup>119</sup> The iron chelator desferrioxamine was without effect<sup>119</sup> and thus it is unlikely that the action of SASP and 5-ASA is through binding to ROS generating transition metals.<sup>106</sup> As both XOD inhibitors and DMSO have limited effectiveness in this model, HOCl rather than the hydroxyl or XOD, was the probable target of the scavenging properties of SASP.<sup>119</sup> However, both 5-ASA and DMSO were anti-inflammatory in a model of ischaemia/reperfusion induced gastric injury, suggesting that 5-ASA was an effective hydroxyl radical scavenger.<sup>107</sup> 5-ASA and SASP, but not SP, protected the bowel against both XOD and bile acid induced ROS damage<sup>115</sup> and also in an experimental model of acute ileitis in rats induced by perfusion of f-mlp where PMN-generated ROS give rise to cellular damage.<sup>109</sup> It would thus appear that the ability of SASP and 5-ASA to react with a wide range of ROS allows them to protect against the damage due to the different ROS produced in different circumstances.

SASP, but not 5-ASA, prevent NSAID generated inflammation,<sup>119</sup> which is also reduced or abolished by free radical scavengers in animals. The lack of effect of 5-ASA in this model was suggested to be due to rapid inactivation of 5-ASA when given orally.<sup>120</sup> Moreover, whereas SASP and 5-ASA, but not SP, directly infused into the colon prevents the bile acid induced loss of DNA and increased cellular turnover, the NSAID indomethacin increased it.<sup>115</sup> Although NSAIDs also scavenge ROS,<sup>121,122</sup> which may account for some of their anti-inflammatory properties, the reaction with ROS may also generate toxic free radical derivatives of the NSAIDs,<sup>123</sup> hence the ability of other antioxidants to suppress the toxicity of NSAIDs *in vivo*. However, there is no evidence that the products of the reaction between ROS and 5-ASA and SASP are toxic, and SASP, like other antioxidants, protects against NSAID damage *in vivo*.

The above studies suggest a role for SASP and 5-ASA as scavengers of ROS. Although *in vitro* they may also have a direct effect on the enzymes producing the ROS in the inflamed bowel it is unlikely that, *in vivo*, they will be absorbed in sufficiently high enough concentrations for this effect to exceed their scavenging properties. Thus the antioxidant effects of SASP and 5-ASA are a more likely anti-inflammatory mechanism of action. The lack of effect of SP as a ROS scavenger in most



systems is consistent with its lack of therapeutic properties in IBD. Differences observed between some studies may be a result of methodological differences altering the effects of SASP and 5-ASA, for example in one study 5-ASA could not be tested because it directly reacted with the cytochrome C.<sup>110</sup> Although the products of the reaction between 5-ASA and ROS have been found in faeces of patients with IBD, supporting the concept that 5-ASA reacts with ROS in IBD,<sup>123</sup> convincing evidence that the ability of 5-ASA and SASP to act as scavengers of ROS is the basis of their anti-inflammatory properties is still lacking. Attempts to correlate changes in ROS production at sites of inflammation with antioxidant effects will be difficult for, as for PG and leukotriene production, changes seen may be more a reflection of changes in numbers of leucocytes present.<sup>123</sup> Nevertheless, if SASP and 5-ASA can be shown to act as scavengers of ROS in IBD and RA, then other drugs that are potential antioxidants may also be beneficial in RA and IBD.<sup>124</sup>

## Cellular and Tissue Effects

**Natural killer (NK) cell activity:** A population of the MN cells that is toxic to epithelial cells has been identified with the same phenotype as natural killer (NK) cells,<sup>125</sup> suggesting that cell mediated cytotoxicity by NK cells contributes to the cellular injury in IBD.<sup>126</sup> Thus a possible mechanism of action of SASP and 5-ASA could be through inhibition of NK cell activity.

SASP and SP inhibited *in vitro* cell mediated cytotoxicity by peripheral blood MN cells and SASP by intestinal MN cells,<sup>126</sup> while SASP therapy reduced *ex vivo* NK cell activity in IBD patients.<sup>127</sup> However, 5-ASA in *in vitro* incubations,<sup>125,126,128</sup> or *in vivo* when given to patients,<sup>127</sup> has little effect on NK activity, and thus the effects of SASP may be due to the SP component.<sup>126</sup> Alternatively, they may be due to the diazo bond of SASP since azodisalicylic, that shares with SASP the possession of a diazo bond, also inhibits NK activity,<sup>125</sup> and may thus be a property of the intact SASP molecule. The effects of SASP on NK cell activity may be indirect, through effects on production of cytokines, but are unlikely to involve the cyclooxygenase pathway as indomethacin had no effect on NK activity at concentrations required to inhibit cyclooxygenase.<sup>125,126</sup> SP accounts for the toxic effect of SASP *in vivo* and has toxic effects *in vivo* and *in vitro* on MN cells (as discussed below). It is thus possible that the effects on NK cell activity are a toxic action and the low NK activity reported in IBD a result of drug treatment rather than the disease process.<sup>129</sup>

Whether NK activity is lower<sup>127</sup> or higher<sup>125</sup> in IBD is unclear and in one study neither SASP nor SP inhibited NK activity *in vitro*. The disparity between the therapeutic efficacy of SASP and its metabolites and their effects on NK activity *in vitro* suggests that NK activity is not a major pathogenic mechanism in UC<sup>125</sup> for 5-ASA is the active molecule in IBD and yet has no effect on NK activity. Although the effects of SASP on NK activity are not relevant to IBD, whether they are relevant in RA depends on whether the intact SASP molecule is the active therapeutic moiety, and on the role of NK activity in RA.

**Suppressor cell activity:** Inflammation is a balance between pro-inflammatory and anti-inflammatory aspects of the immune system. The anti-inflammatory properties of SASP and 5-ASA could result from an effect on MN cells, such as inhibition of antibody synthesis or modification of lymphocyte suppressor or helper cell function leading to down-regulation and suppression of the pro-inflammatory aspects of the immune system.<sup>23-25</sup>

This is supported by inhibition of mitogen-stimulated MN cell antibody secretion by SASP and 5-ASA,<sup>129</sup> and proliferation by SASP<sup>22,23,125,129</sup> *in vitro*. 5-ASA also inhibited mitogen-induced expression of cell surface activation markers,<sup>130</sup> but although 5-ASA inhibited mitogen-induced proliferation in some studies<sup>129,131</sup> but in others it was inactive.<sup>23,25,132</sup> SP also had no effect on MN cell antibody production<sup>133</sup> or proliferation,<sup>23,25,129,132</sup> even when added with 5-ASA suggesting that the intact SASP molecule is the active moiety.<sup>25</sup> Conflictingly, in one study SASP, 5-ASA and SP all inhibited mitogen-induced activation of MN cells from healthy subjects and RA patients *in vitro* suggesting that the metabolites of SASP could also be active under some conditions.<sup>24</sup> The effects seen differed with the mitogen used,<sup>24</sup> and it is possible that differences in the studies above are a result of different mitogens being used.

The effects of SASP on induction of a suppressor cell function might be through effects on PGs, which have been implicated in the regulation of suppressor cell activity. Although co-administration of indomethacin with SASP caused a reduction in the effects of SASP on mitogen induced activation in one study,<sup>129</sup> it did not in others.<sup>129,132,133</sup> Thus it is unclear whether PGs are involved. Other cytokines could also be involved.

SASP and 5-ASA inhibited mitogen activation of cells from IBD patients more than those from healthy subjects,<sup>129</sup> suggesting that such raised pro-immunological responses are susceptible to inhibition. In RA, SASP treatment reduced circulating levels of activated lymphocytes and

abnormal *ex vivo* lymphocyte responses to mitogens.<sup>24,135</sup> However, in the latter study the change occurred only in those who responded to treatment<sup>135</sup> and thus may have been due to a general reduction in disease activity rather than a direct effect of SASP.<sup>135</sup> Neither SASP, 5-ASA nor SP had any effect on suppressor cell activity when measured directly<sup>136</sup> or on MN cell subpopulations *in vitro* or *in vivo*,<sup>128</sup> and the ability of SASP to inhibit mitogen stimulation of MN cells activation may be due to a toxic effect.<sup>133</sup>

SASP could act therapeutically in IBD and RA by affecting suppressor cell function, with SP being inactive due to its lack of effect. However, 5-ASA is largely inactive in this system and yet is the active component in IBD. Thus if this effect of SASP occurs in patients, then the therapeutic mechanism of action of SASP and 5-ASA may be different. Alternatively, SASP may be toxic to MN cells, as indicated below and the effects seen *in vitro* irrelevant to either IBD or RA.

**Toxic effects:** The few clinical side effects that SASP possesses are associated with the SP component, rather than 5-ASA, although recent studies have suggested that 5-ASA may be nephrotoxic. However, *in vitro* SASP is toxic to MN cells<sup>38,125,133</sup> and other cells,<sup>125</sup> and 5-ASA is toxic to MN cells<sup>24,38</sup> and we have shown the toxicity of SASP and 5-ASA to be dose-dependent.<sup>38</sup> However, other studies have not found SASP<sup>23-25,137</sup> or 5-ASA<sup>125,133,137,138</sup> to be toxic to MN cells, and neither SASP nor 5-ASA to be toxic to PMN,<sup>93</sup> erythrocytes<sup>137</sup> or mouse spleen cells<sup>25</sup> *in vitro*. Additionally 5-ASA was not found to be toxic to other cells in culture.<sup>139</sup>

SASP produces chromosomal damage, namely sister chromatid exchange and micronuclei, to MN cells *in vivo* and *in vitro*, an effect caused by the SP component.<sup>140</sup> However, when the toxicity of SP has been studied neither we<sup>38</sup> nor others have found SP to be toxic in *in vitro* incubations of MN cells,<sup>23,24,133,138</sup> PMN<sup>93</sup> or mouse spleen cells.<sup>25</sup> The toxic side effect of SP *in vivo* may be due to a toxic metabolite rather than SP itself.<sup>137</sup> SASP and 5-ASA may not be toxic *in vivo* due to the rapid acetylation of these compounds once absorbed. Some of the *in vitro* toxic side effects of SASP in MN cells could arise through interference with folate metabolism.<sup>141</sup>

The reason why SASP and 5-ASA are toxic in some studies, but not in others could be due to differences in the incubation conditions, for example the length of time the cells are exposed to the drug. Cell death, leading to lysis and disintegration of cells, is not detected by techniques measuring membrane permeability, such as trypan blue exclusion. The toxic effect at high concentra-

tions of SASP and 5-ASA may indicate more subtle effects on cellular metabolism at lower concentration. If the ability of SASP to inhibit mitogen-stimulated MN cell activation is due to a toxic effect,<sup>129</sup> then some of the other reported effects of SASP and 5-ASA on cells in culture may also be a result of their toxicity.

Thus, SASP and 5-ASA can affect the viability of cells in culture, suggesting that 5-ASA and SASP may possess toxic properties *in vitro*, although there are inconsistencies between these studies. Reliable and sensitive measurement of cell viability may confirm that some of the reported effects of these drugs are due to their toxicity, rather than being physiologically relevant.

### Other Anti-inflammatory Properties

In addition to those discussed above SASP and 5-ASA possess other properties that might explain their anti-inflammatory effects in RA and IBD. These include effects of both 5-ASA and SASP, and not SP, such as the inhibition of synthesis of pro-inflammatory platelet activating factor (PAF),<sup>142</sup> or the inhibition of monocyte-dependent increases in synthesis of  $\alpha_1$ -acid-glycoprotein,<sup>143</sup> an acute phase protein with anti-inflammatory properties. Others are properties possessed only by 5-ASA, such as inhibition of IL-1 $\beta$  production<sup>138</sup> or the reduction of interferon- $\gamma$  induced expression of the HLA-DR major histocompatibility complex on cells.<sup>139</sup> An immunomodulatory function of SASP is also suggested by its beneficial effects in an experimental model of autoimmune disease,<sup>144</sup> its ability to prolong rat cardiac allografts<sup>145</sup> and suppress rejection of intestinal tumours,<sup>146</sup> mediated by suppression of antibody synthesis.<sup>146</sup>

SASP,<sup>114,147,148</sup> and 5-ASA<sup>147,148</sup> and SP<sup>114</sup> all inhibit leucocyte motility, although in some studies 5-ASA<sup>114</sup> and SP<sup>147</sup> had no effect. SASP, but not SP, also inhibits release of leucocyte granules<sup>110,114</sup> that contain various pro-inflammatory agents. Azodisalicylic acid also inhibited granule release,<sup>110</sup> whereas 5-ASA is relatively inactive,<sup>110,112,114</sup> suggesting that this may be a property of the diazo bond possessed by the intact SASP. Thus it is unlikely that this is relevant to clinical effects of both 5-ASA and SASP.

We have shown SASP to inhibit TNF-induced adhesion molecule expression on leucocytes,<sup>149</sup> although this may be due to effects on the receptor for TNF as SASP inhibits the binding of TNF to its receptor.<sup>150</sup> However, neither 5-ASA nor SP affects binding of TNF. Further studies on the effects of 5-ASA and SP on adhesion molecule are required to determine the mechanism of action of SASP on adhesion molecule expression. SASP also inhibits binding of f-mlp to its receptor on

leucocytes<sup>151</sup> and thus many of the effects of SASP *in vitro* may be through inhibiting activation of cells by preventing binding of the activating agent. SASP and 5-ASA are highly soluble in ionic and hydrophobic environments and it is possible that they bind to proteins on the surface of cells in a nonspecific fashion. If this is so then it is unlikely to be the basis of their action *in vivo*, given the large number of possible binding sites and proteins with which they will come into contact.

SASP may affect other cells besides the leucocytes. SASP inhibits histamine release from mast cells,<sup>152</sup> and this is suggested to be the basis of its anti-ulcer effects.<sup>153</sup> Although the effects of SASP and 5-ASA on platelet TXA<sub>2</sub><sup>42</sup> and PAF<sup>142</sup> production may be due to an effect on platelet function, 5-ASA had no effect on platelet aggregation and fibrinolytic activity either *in vitro* or *in vivo*.<sup>154</sup>

There is as yet insufficient information on the above properties of SASP and 5-ASA to suggest which might be a likely mechanism of action. The effects of the drugs above might be indirect and mediated through effects on production of cytokines such as PGs or leukotrienes, or antioxidant effects increasing the stability of cell membranes.

## Summary

The results of the many *in vitro* and *in vivo* studies performed suggest that there are several possible mechanisms that may explain the anti-inflammatory properties of SASP and 5-ASA. However, it is more probable that 5-ASA and SASP act in IBD by a single mechanism, rather than by several different mechanisms, and that some of the observations are erroneous due to artefactual effects, or misinterpretation of the results. If 5-ASA is the active moiety in IBD and RA then the effects seen with intact SASP molecule are likely to be irrelevant unless they are due to the 5-ASA component within the intact molecule. However, if the intact molecule is the active moiety in RA then SASP and 5-ASA could act by two different anti-inflammatory mechanisms, for although 5-ASA is as good as SASP in the treatment of IBD it has not been shown to be better. If SP acted as an anti-inflammatory agent in RA then it should also do so in the inflamed bowel, where the concentrations are higher. That it does not suggest that the only possible mechanism for SP is as an antibacterial agent in RA. This may suggest that effects seen with SASP or 5-ASA that are also seen with SP may be artefactual. The conflicting findings in some of the studies suggest that the methodology employed in *in vitro* studies can affect the results seen.

The result of studies of the effects of SASP and

5-ASA on the inflammatory changes in patients, either measured *ex vivo* in studies of cells and tissues or *in vivo*, are compounded by the heterogeneous nature of the patient population, such as drug treatments and the site and severity of the inflammation. It is also difficult to determine whether the measured parameter is due to a direct action of the drug or secondary to a change in the activity of the inflammation. As discussed above with respect to PGs the contribution of the leucocyte infiltrate in samples of inflamed tissue used for *ex vivo* and *in vivo* studies is often overlooked, as are the traumatic effects of isolation and preparation procedures on activation of such cells. Animal models are useful in studying the mechanism of action of compounds like 5-ASA, but they do not mimic chronic IBD. A protective effect of SASP and 5-ASA in animal models may be mediated by intervention at one of several points in the inflammatory process, and an effectiveness equal to that of other agents does not necessarily imply a common mechanism. Studies of 5-ASA and SASP in models of RA are still required to dissect out the active component of SASP. If 5-ASA and other compounds act upon mechanisms that are unique to the origin of IBD then the use of such models to determine the mechanism of action of SASP and 5-ASA, and for the development of new anti-inflammatory agents, may be severely limited.

Another problem in studying the mechanism of action of 5-ASA and SASP using *in vitro* models is the relevant concentration of drug that should be used. This is important for, as demonstrated with PG synthesis, opposite results can be obtained using low concentrations compared to those obtained using high concentrations. Furthermore the effective concentrations of 5-ASA and SASP in one anti-inflammatory mechanism are often different to those required for another. For example, it has been suggested that the concentrations achieved of 5-ASA *in vivo* are not high enough to compete with biological material for the ROS, the hydroxyl radical.<sup>106</sup> Which is the relevant concentration is unknown. There is a rapid gradient of drug concentration from the bowel lumen to the perfusing blood, which, due to its poor absorption, will be much steeper for SASP than 5-ASA. Moreover, once absorbed the drugs are rapidly inactivated through acetylation. The high concentrations of 5-ASA and SASP that exist in the bowel lumen are often quoted as justification of the use of similar high concentrations *in vitro* but there is no evidence that 5-ASA and SASP exert their therapeutic effect in the bowel lumen. In IBD although the majority of evidence points to a local action of 5-ASA this is likely to be sited within the colonic mucosa where concentrations will be lower. The effective concentration in RA will be much

lower due to rapid metabolism of SASP and 5-ASA, thus implying that high concentrations are not required for the mechanism of action.

The most relevant concentrations will be those at the active site and if this is in the interstitial fluid then it could be relatively high. If it is within a specific cell type, and at a specific organelle then it could be much lower. The ability of SASP to become concentrated in connective tissue where it is then broken down will also produce relatively high, but highly localized concentrations of 5-ASA. The effective concentration will also depend on the activity of the mechanism against which it is acting. Relative concentrations and incubation conditions used in *in vitro* models are designed more with regard to the detection system used to measure the products formed than to reproducing accurately the *in vivo* levels. Thus a high concentration of drug required to have an effect *in vitro* does not necessarily indicate that a similarly high concentration will be required *in vivo* where the activity of the system against which the drug is acting may be much lower. It may thus be more important to consider the relative concentration of the drug compared to the activity of potential target system *in vitro* to that *in vivo*, rather than just drug concentrations.

It is possible that a single property of SASP and 5-ASA explains many of their observed actions. For instance, SASP and 5-ASA can act as antioxidants and prevent lipid peroxidation. The production of PGs also involves ROS and PG synthesis is activated by low levels of peroxides, but is inhibited by high levels. Thus 5-ASA and SASP may affect PG production either by reacting with free radical intermediates in the cyclooxygenase enzyme or by altering levels of lipid peroxides. Similarly, the lipoxygenase pathway involves ROS and the antioxidant glutathione while many of the products resemble those produced by inorganic ROS generated lipid peroxidation. Thus 5-ASA and SASP could also affect this pathway through their scavenging properties. ROS have also been suggested to act as chemical messengers<sup>148</sup> and thus other effects of SASP and 5-ASA could also be due to the effects on ROS. The mechanism by which 5-ASA inhibited the myeloperoxidase activity through scavenging the haemoprotein-associated radical by acting as an alternative substrate<sup>108</sup> is similar to the effects of 5-ASA in PG synthesis and thus may be a general property of these compounds in haem based proteins. This could also explain the effects of SASP and 5-ASA in haemoglobin-induced lipid peroxidation.

Lower levels of von Willebrand factor in patients on SASP<sup>156</sup> suggest that SASP and/or 5-ASA have effects on endothelial cell function in IBD. The early studies by Dr Svartz and her colleagues showed

SASP to be able to bind to vascular tissues. The endothelium is also a potent producer of PGs and ROS and has been demonstrated to affect vascular PG production and function. The endothelium is also important in controlling the cellular damage seen in inflammation and ischaemia/reperfusion injury. Thus the protective effects of 5-ASA as an antioxidant might be mediated by the protection of the endothelium and maintenance of production of vascular prostacyclin and other protective compounds. In addition 5-ASA and SASP both have effect on leukotrienes and adhesion molecules which are both important in the regulation of the recruitment of leukocytes through the vascular endothelium and into inflamed sites. Thus whatever the mechanism the vascular endothelium may be the possible site of the anti-inflammatory properties of 5-ASA and SASP.

An understanding of the mechanisms of action of 5-ASA and SASP, when eventually obtained, will be a great asset in the development of new anti-inflammatory agents and may even lead to a deeper understanding of the pathogenesis of RA and IBD. More research is required into the mechanism of action of SASP and 5-ASA in IBD and RA before this can be achieved.

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