Research article

Scavenging of reactive oxygen and nitrogen species by the prodrug sulfasalazine and its metabolites 5-aminosalicylic acid and sulfapyridine

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Sulfasalazine is a prodrug composed by a molecule of 5-aminosalicylic acid (5-ASA) and sulfapyridine (SP), linked by an azo bond, which has been shown to be effective in the therapy of inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease, as well as of rheumatic diseases, such as rheumatoid arthritis and ankylosing spondylitis. The precise mechanism of action of sulfasalazine and/or its metabolites has not been completely elucidated, though its antioxidant effects are well established and are probably due to its scavenging effects against reactive oxygen and nitrogen species (ROS and RNS), as well as metal chelating properties, in association to its inhibitory effects over neutrophil oxidative burst. The present work was focused on screening and comparing the potential scavenging activity for an array of ROS ($O_2^{\bullet-}$, H_2O_2, $^1O_2$, $ROO^\bullet$ and HOCl) and RNS ($^\bullet NO$ and $ONOO^-$), mediated by sulfasalazine and its metabolites 5-ASA and SP, using validated *in vitro* screening systems. The results showed that both 5-ASA and sulfasalazine were able to scavenge all the tested ROS while SP was practically ineffective in all the assays. For HOCl, $^1O_2$, and $ROO^\bullet$, 5-ASA showed the best scavenging effects. A new and important finding of the present study was the strong scavenging effect of 5-ASA against $^1O_2$. 5-ASA was shown to be a strong scavenger of $^\bullet NO$ and $ONOO^-$. Sulfasalazine was also able to scavenge these RNS, although with a much lower potency than 5-ASA. SP was unable to scavenge $^\bullet NO$ in the tested concentrations but was shown to scavenge $ONOO^-$, with a higher strength when the assay was performed in the presence of 25 mM bicarbonate, suggesting further scavenging of oxidizing carbonate radical. In conclusion, the ROS- and RNS-scavenging effects of sulfasalazine and its metabolites shown in this study may contribute to the anti-inflammatory effects mediated by sulfasalazine through the prevention of the oxidative/nitrative/nitrosative damages caused by these species.

Keywords: sulfasalazine, sulfapyridine, 5-aminosalicylic acid, reactive oxygen species, reactive nitrogen species, scavenging activity

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Introduction

Sulfasalazine is a prodrug composed by a molecule of 5-aminosalicylic acid (5-ASA) and sulfapyridine (SP;

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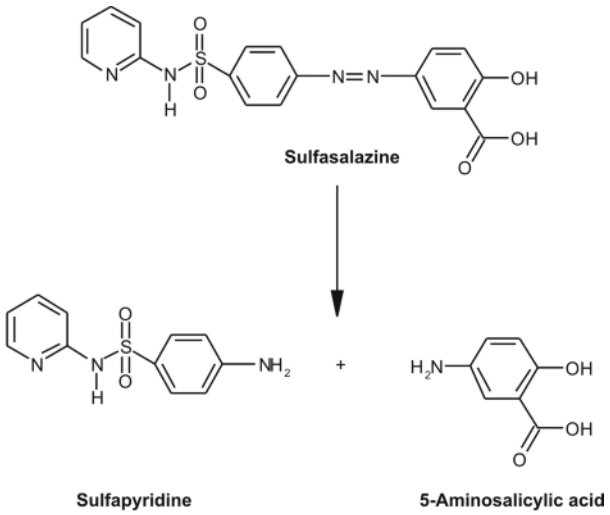

Figure 1 Chemical structures of sulfasalazine and its metabolites – sulfapyridine and 5-aminosalicylic acid

Fig. 1), linked by an azo bond, which has been shown to be effective in the therapy of inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn's disease,[1,2] and of rheumatic diseases, such as rheumatoid arthritis and ankylosing spondylitis.[3–5] Nearly all the prodrug taken orally reaches the colon intact, where it is converted into its two metabolites by colonic bacterial azoreductases.[3] The precise mechanism of action of sulfasalazine has not been completely elucidated, although a number of potential effects of sulfasalazine and its metabolites have been proposed: (i) inhibition of cyclooxygenase and 5-lipoxygenase enzymes;[6,7] (ii) inhibition of colonic production of chemo-attractant leukotrienes;[8] (iii) inhibition of cytokines release by various cell types, including those produced by T-cells, such as interleukin (IL)-2 and gamma interferon (IFN-γ), and those produced by monocytes or macrophages, like IL-1, IL-12 and tumour necrosis factor alpha (TNF-α) as reviewed by Barrera *et al.*;[9,13] (iv) inhibition of imunoglobulin (Ig) M and IgG production by B cells;[10] (v) interference with TNF-α binding;[11] (vi) inhibition of nuclear factor κB (NF-κB) activation;[12,13] (vii) increase in the intestinal epithelial cell heat shock protein response;[14] and (viii) antioxidant effects.[15]

A possible key to an integrated understanding of the mechanism of action was recently suggested by Desreumaux and Ghosh[16] following the realization that the anti-inflammatory actions of 5-ASA produce effects similar to activation of the γ-form peroxisome proliferator-activated receptors (PPAR-γ), which results in the modulation of inflammatory cytokine production and modulation of RelA/p65 dephosphorylation, leading to decreased transcriptional activity of NF-κB, and reduced synthesis of prostaglandins and leukotrienes. However, that does not cover the sulfasalazine-related antioxidant effects. In that respect, sulfasalazine has been clearly shown to inhibit intestinal epithelial cell injury and apoptosis induced by oxidative stress.[17–19] In addition, sulfasalazine and 5-ASA, but not SP, also protected the bowel against both xanthine oxidoreductase (XOD) and bile acid-induced damage mediated by oxidative stress,[20] and 5-ASA was shown to prevent experimentally induced ischaemia/reperfusion gastric injury.[21]

The antioxidant effects are probably due to the scavenging effects against reactive oxygen and nitrogen species (ROS and RNS), as well as metal chelating properties of sulfasalazine and its metabolites, in association to its inhibitory effects over neutrophil oxidative burst.[9,15,22–28] On the other hand, these data were sparsely generated, through the use of different methodologies, which brings difficulties in the comparison of potencies with other antioxidants and anti-inflammatory drugs bearing ROS and RNS scavenging properties.[29–34] Moreover, the literature is still incomplete and sometimes contradictory, relatively to this type of activity, for sulfasalazine and its metabolites, probably due to flawed methodologies or inadequate probes used during the last two decades. Therefore, the present work was focused on screening and comparing the potential scavenging activity for an array of ROS ($O_2^{\bullet -}$, H_2O_2, $^1O_2$, $ROO^{\bullet}$ and HOCl) and RNS ($^{\bullet}NO$ and $ONOO^-$), mediated by the sulfasalazine and its metabolites 5-ASA and SP, using validated *in vitro* screening systems.

Materials and methods

Equipment

A microplate reader (Synergy HT, BIO-TEK), for fluorescence, absorbance in UV/Vis and luminescence measurements, plus temperature control capacity, was used for all the assays.

Chemicals

All the chemicals and reagents used were of analytical grade. 5-Aminosalicylic acid, sulfasalazine, sulfapyridine, dihydrorhodamine 123 (DHR), 4,5-diaminofluorescein (DAF-2), 30% hydrogen peroxide, ascorbic acid, sodium hypochlorite solution, with 4% available chlorine, lipoic acid, diethylenetriaminepentaacetic acid (DTPA), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), β-nicotinamide adenine

dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), rutin, lucigenin, tiron, and ebselen, were obtained from Sigma–Aldrich (St Louis, MO, USA). α,α′-Azodiisobutyramidine dihydrochloride (AAPH), histidine and Trolox were obtained from Fluka Chemie GmbH (Steinheim, Germany). Fluorescein sodium salt was obtained from Aldrich (Milwaukee, WI, USA).

ROS and RNS scavenging assays

Superoxide radical scavenging assay

Superoxide radical was generated by the NADH/PMS system, at pH 7.4. Though at lower pH values $O_2^{\bullet-}$ is partially converted in to the hydroperoxyl radical ($HO_2^{\bullet}$), at pH 7.4 only $O_2^{\bullet-}$ exists in the reaction media. $O_2^{\bullet-}$ is known to act as both oxidizing and reducing agent. The reaction of $O_2^{\bullet-}$ with diphenols is an example of an oxidation promoted by this species. In fact, phenolic compounds have previously shown to be effective $O_2^{\bullet-}$ scavengers at physiological pH. $O_2^{\bullet-}$ scavenging activity was determined by monitoring the effect of the tested compound on the $O_2^{\bullet-}$-induced reduction of NBT at 560 nm as previously described.[35] The antioxidant tiron was used as positive control. The results were expressed as the inhibition (in percentage) of the NBT reduction to diformazan. Each study corresponds to at least four experiments, performed in triplicate.

Hydrogen peroxide scavenging assay

The H_2O_2 scavenging activity was measured by monitoring the H_2O_2-induced oxidation of lucigenin as previously described.[35] The endogenous antioxidant ascorbic acid was used as positive control. The results were expressed as the inhibition (in percentage) of the H_2O_2-induced oxidation of lucigenin. Each study corresponds to at least four experiments, performed in triplicate.

Singlet oxygen scavenging assay

The $^1O_2$ scavenging activity was measured by monitoring the oxidation of non-fluorescent DHR to fluorescent rhodamine 123 by this ROS, as previously described.[35] The chemical generation of $^1O_2$ was promoted using the thermodissociable endoperoxide disodium 3,3′-(1,4-naphthalene)bispropionate ($NDPO_2$). During the thermal decomposition of the water-soluble $NDPO_2$ to NDP and molecular oxygen, one half of the oxygen is in the $^1O_2$ excited state. Ascorbic acid was used as positive control. The results were expressed as the inhibition (in percentage) of $^1O_2$-induced oxidation of DHR. Each study corresponds to at least four experiments, performed in triplicate.

Hypochlorous acid scavenging assay

HOCl was prepared by adjusting the pH of a 1% (v/v) solution of NaOCl to 6.2 with dropwise addition of 10% H_2SO_4. The concentration of HOCl was further determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 $M^{-1}cm^{-1}$. For each assay, HOCl was appropriately diluted with 50 mM potassium phosphate buffer, pH 7.4. The HOCl scavenging activity was measured by monitoring the HOCl-induced oxidation of non-fluorescent DHR to fluorescent rhodamine.[35] Lipoic acid was used as positive control. The results were expressed as the inhibition (in percentage) of HOCl-induced oxidation of DHR. Each study corresponds to at least four experiments, performed in triplicate.

Peroxyl radical scavenging assay

The $ROO^{\bullet}$ scavenging activity was measured by monitoring the fluorescence decay resulting from $ROO^{\bullet}$-induced oxidation of fluorescein and expressed as the 'oxygen radical absorbance capacity' (ORAC), as previously described.[35] $ROO^{\bullet}$ was generated by thermal decomposition of AAPH, at 37ºC, which releases alkyl radical ($R^{\bullet}$) that, in turn, reacts with the O_2 present in the reaction media, originating $ROO^{\bullet}$. Trolox was used as the standard control in each assay. Ascorbic acid was used as positive control. The results were expressed as ORAC values. Each study corresponds to at least four experiments, performed in triplicate.

Nitric oxide scavenging assay

The $^{\bullet}NO$ scavenging activity was measured by monitoring the $^{\bullet}NO$-induced oxidation of non-fluorescent DAF-2 to the fluorescent triazolofluorescein (DAF-2T), as previously described.[35] Nitric oxide was generated by NOC-5, which spontaneously releases two equivalents of $^{\bullet}NO$, at physiological conditions. Rutin was used as positive control. The results were expressed as the inhibition (in percentage) of $^{\bullet}NO$-induced oxidation of DAF-2. Each study corresponds to at least four experiments, performed in triplicate.

Peroxynitrite scavenging assay

$ONOO^-$ was synthesized by mixing an acidic solution (0.7 M HCl) of 0.6 M H_2O_2 with 0.66 M $NaNO_2$ in a Y-junction and collecting the reaction mixture in ice-cold 3 M NaOH. The obtained $ONOO^-$ solution was frozen at –80ºC. Prior to each experiment, the concentration of peroxynitrite was determined spectrophotometrically (ε_{302nm} = 1670 $M^{-1}cm^{-1}$) and the solution was appropriately diluted in 0.05 M NaOH. The ONOO- scavenging activity was measured by monitoring the $ONOO^-$-induced oxidation of non-fluorescent DHR

Table 1 Scavenging activities for $O_2^{\bullet-}$, H_2O_2, HOCl and $^1O_2$ (IC_{50}, mean ± SEM) presented by the studied compounds and by the respective positive controls

Compound	$O_2^{\bullet-}$	H_2O_2	HOCl	$^1O_2$
		IC_{50} (μM)		
Sulfasalazine	173 ± 17	457 ± 50	298 ± 16.1	357 ± 33
Sulfapyridine	26.3%^{4mM*}	NA^{4mM}	**	37.6%^{4mM*}
5-Aminosalicylic acid	807 ± 154	1422 ± 128	1.85 ± 0.26	29.5 ± 5.7
Tiron	322 ± 10	–	–	–
Ascorbic acid	–	797 ± 107	–	5.2 ± 0.9
Lipoic acid	–	–	1.52 ± 0.26	–

NA, no activity was found up to the highest tested concentration (in superscript).
*Scavenging effect (mean %) at the highest tested concentration (in superscript).
**Interference with the methodology.

to fluorescent rhodamine 123, as previously described.[35] Ebselen was used as positive control. In a parallel set of experiments, the assays were performed in the presence of 25 mM $NaHCO_3$ in order to simulate the physiological CO_2 concentrations. This evaluation is important because, under physiological conditions, the reaction between $ONOO^-$ and bicarbonate is predominant, with a very fast rate constant (k_2 = 3–5.8 × 10^4 $M^{-1}s^{-1}$).[36] This reaction results in the formation of the nitrosoperoxycarbonate anion ($ONOOCO_2^-$), whose decomposition leads to the formation of different species including the highly reactive $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ radicals. Thus, reaction with $ONOO^-$ in presence of HCO_3^- or CO_3^{2-} is indeed a reaction with $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ radicals. The results were expressed as the inhibition (in percentage) of $ONOO^-$-induced oxidation of DHR. Each study corresponds to at least four experiments, performed in triplicate.

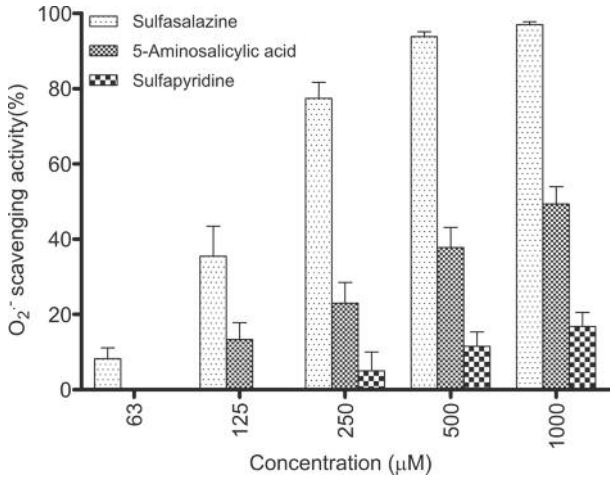

Figure 2 $O_2^{\bullet-}$ scavenging activity of sulfasalazine, sulfapyridine and 5-aminosalicylic acid. Each point represents the values obtained from at least four experiments, performed in triplicate (mean ± SEM)

Results

Superoxide radical scavenging activity

Sulfasalazine was shown to be a highly effective scavenger of $O_2^{\bullet-}$ in a concentration-dependent manner. The scavenging effects were observed at all concentrations tested, with full effectiveness observed at 500 μM. 5-ASA also had some scavenging activity, though with much lower potency, while SP only displayed residual activity (Fig. 2; IC_{50} values in Table 1).

Hydrogen peroxide scavenging activity

Hydrogen peroxide was also effectively scavenged by sulfasalazine, in a concentration-dependent manner, though higher concentrations were required to attain this effect than those required to scavenge $O_2^{\bullet-}$. 5-ASA also had some scavenging activity, though with much lower potency, while SP had no effect at the tested concentrations (Fig. 3; IC_{50} values in Table 1).

Singlet oxygen scavenging activity

5-ASA was shown to be the most potent, with scavenging effects already observed at the low micromolar range. Like the previous assays, sulfasalazine was an effective scavenger of $^1O_2$, in a concentration-dependent manner, at higher micromolar range (125–1000 μM). SP only displayed residual activity at the tested concentrations (Fig. 4; IC_{50} values in Table 1).

Hypochlorous acid scavenging activity

The pattern of HOCl scavenging assay results was similar to that observed for singlet oxygen and gave rise to distinct results comparatively to the $O_2^{\bullet-}$ and H_2O_2 assays. In fact, this time 5-ASA was shown to be the most potent, with scavenging effects already observed at the nanomolar range and almost full effectiveness observed at 10 μM. Sulfasalazine was also shown to be a scavenger of HOCl, but only at higher concentrations, within the range of those

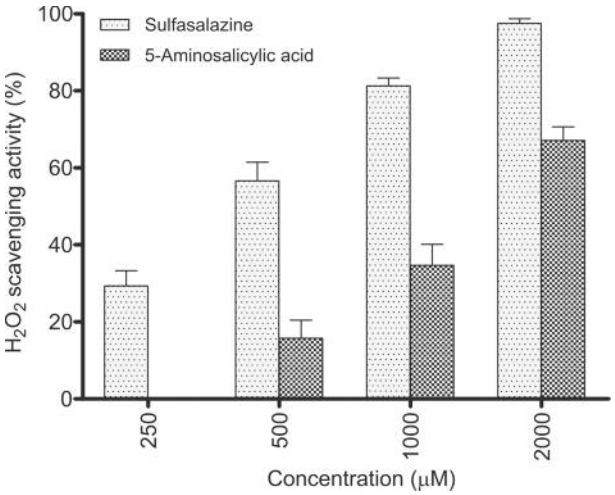

Figure 3 **H_2O_2 scavenging activity of sulfasalazine and 5-aminosalicylic acid. Each point represents the values obtained from at least four experiments, performed in triplicate (mean ± SEM)**

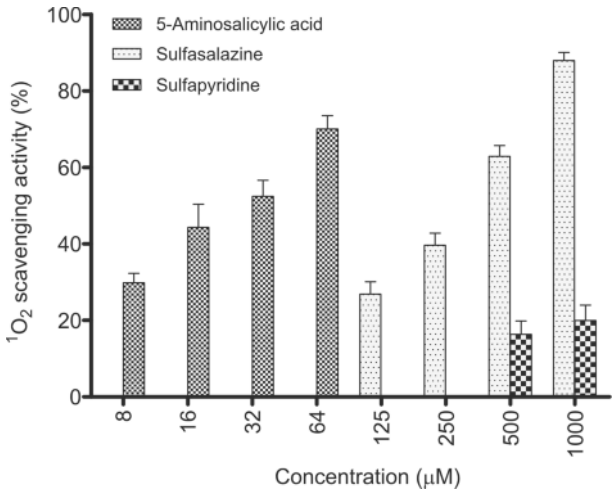

Figure 4 **$^1O_2$ scavenging activity of sulfasalazine, sulfapyridine and 5-aminosalicylic acid. Each point represents the values obtained from at least four experiments, performed in triplicate (mean ± SEM)**

required to scavenge $O_2^{\bullet-}$. It was not possible to test SP within the present assay conditions, because it interfered with the methodology (Fig. 5; IC_{50} values in Table 1).

Peroxyl radical scavenging activity

The results from the ORAC assay are listed in Table 2. All the tested compounds were able to delay the ROO•-dependent oxidation of fluorescein, 5-ASA being the most potent and SP the one with lower potency. Noteworthy, all were considerably more active than ascorbic acid.

Table 2 **ROO• scavenging activity of the tested sulfasalazine and metabolites and ascorbic acid expressed as ORAC values (mean ± SEM)**

Compound	$ORAC_{ROO•}$ ± SEM (μM Trolox equiv/μM compound)
Sulfasalazine	0.61 ± 0.04
Sulfapyridine	0.32 ± 0.05
5-Aminosalicylic acid	2.77 ± 0.35
Ascorbic acid	0.19 ± 0.04

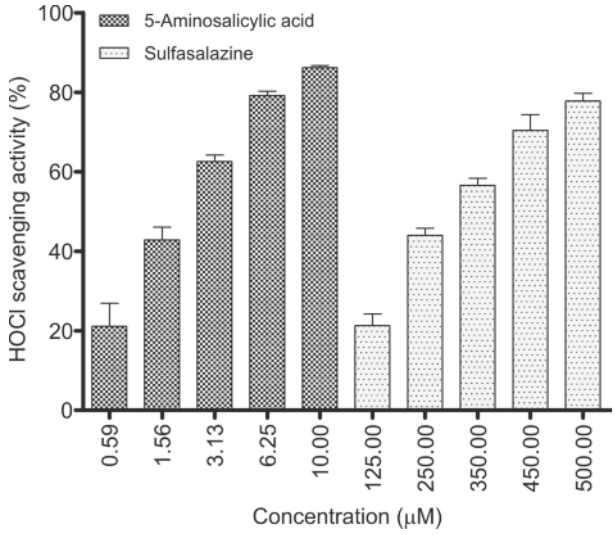

Figure 5 **HOCl scavenging activity of sulfasalazine and 5-aminosalicylic acid. Each point represents the values obtained from at least four experiments, performed in triplicate (mean ± SEM)**

Nitric oxide scavenging activity

The results obtained with the •NO-scavenging assay were very similar to those obtained at the HOCl assay. In fact, 5-ASA was shown to be the most potent, with scavenging effects already observed at the nanomolar range and almost full effectiveness observed at 10 μM. Sulfasalazine was also shown to be a scavenger of HOCl, but only at higher concentrations (125–2000 μM). SP had no effect at the tested concentrations (Fig. 6; IC_{50} values in Table 3).

Peroxynitrite scavenging activity

Peroxynitrite was shown to be the most effectively scavenged reactive species by the tested compounds. As for •NO, 5-ASA was shown to be the most potent, with scavenging effects already observed at the low nanomolar range and almost full effectiveness observed at 5 μM. Sulfasalazine was also shown to be a potent scavenger of $ONOO^-$, at higher concentrations, but still lower than those required for the other reactive species tested (7.8–125 μM). SP only displayed residual activity at the tested concentrations (Fig. 7; Table 3).

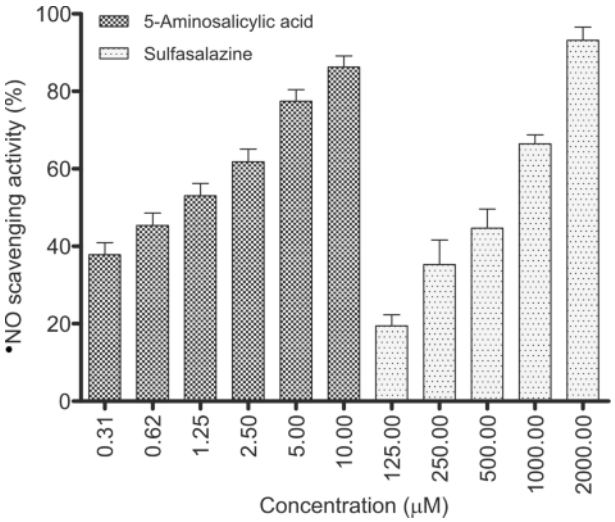

Figure 6 **•NO scavenging activity of sulfasalazine and 5-aminosalicylic acid. Each point represents the values obtained from at least four experiments, performed in triplicate (mean ± SEM)**

This assay needs to be performed also in the presence of $NaHCO_3$, to simulate physiological conditions. In the presence of $NaHCO_3$, the effectiveness of sulfasalazine and 5-ASA was lowered, while that of SP was increased (Fig. 7; IC_{50} values in Table 3).

Discussion

The scavenging effects of sulfasalazine against ROS were first suggested by Del Soldato and collaborators, specifically for hydroxyl radicals (HO•).[22] This effect was later confirmed by Aruoma *et al.*,[25] who found that sulfasalazine and its metabolites 5-ASA and SP scavenged HO•, while 5-ASA, but not sulfasalazine or SP, was able to scavenge HOCl at biologically-

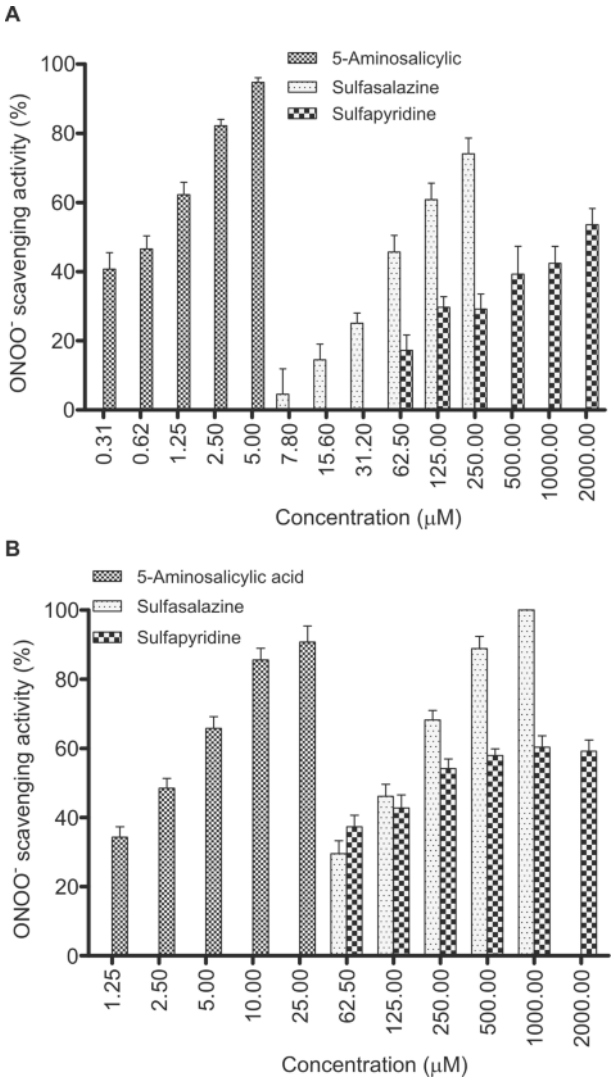

Figure 7 **$ONOO^-$ scavenging activity of sulfasalazine, sulfapyridine and 5-aminosalicylic acid without $NaHCO_3$ (A) and in the presence of 25 mM $NaHCO_3$ (B). Each point represents the values obtained from at least four experiments, performed in triplicate (mean ± SEM)**

Table 3 **Scavenging activities for •NO and $ONOO^-$ (with and without 25 mM $NaHCO_3$) (IC_{50}, mean ± SEM) presented by the studied compounds and by the respective positive controls**

Compound	•NO	$ONOO^-$ (without $NaHCO_3$	$ONOO^-$ (with $NaHCO_3$)
		IC_{50} (μM)	
Sulfasalazine	536 ± 89	91 ± 22	148 ± 16
Sulfapyridine	NA^{2mM}	1676 ± 35	257 ± 41
5-Aminosalicylic acid	1.06 ± 0.21	0.68 ± 0.09	2.88 ± 0.43
Rutin	0.71 ± 0.04	–	–
Ebselen	–	0.29 ± 0.03	2.94 ± 0.28

NA, no activity was found up to the highest tested concentration (in superscript).

significant rates. The results obtained in the present study showed that both 5-ASA and sulfasalazine were able to scavenge all the tested ROS while SP was practically ineffective in all the assays. For HOCl, $^1O_2$, and ROO$^\bullet$, 5-ASA showed the best scavenging effects, with IC_{50} values of 1.85 ± 0.26 μM and 29.5 ± 5.7 μM, and an ORAC of 2.77 ± 0.35, respectively. Sulfasalazine was about 200 times less potent than 5-ASA concerning the HOCl scavenging effect, which is in accordance with the mentioned previous results.[25] On the other hand, the IC_{50} value obtained for sulfasalazine (298 ± 16 μM) is still comparable to obtained for sulindac sulphide, which is an non-steroidal anti-inflammatory drug (NSAID) with well-known antioxidant properties,[29] while many other NSAIDs with antioxidant properties had no effect against HOCl,[30,33] as measured by the same methodology. Interestingly, adding to the HOCl scavenging effects, it was also previously demonstrated that myeloperoxidase (MPO) is inhibited by sulfasalazine (and by 5-ASA, though at much lower potency).[37] MPO is a green heme enzyme stored in azurophilic granules of neutrophils and monocytes.[38] MPO is released into the phagosome during phagocytosis and uses H_2O_2 and Cl^- as substrates to catalyze the formation of HOCl. Considering the primary role of neutrophils in the course of IBD and rheumatic diseases, the reaction of 5-ASA with HOCl is probably an important factor in sulfasalazine therapeutic effect. On the other hand, it is known that the reaction of 5-ASA with HOCl produces two reactive intermediates, an iminoquinone that rapidly hydrolyzes to a quinone, and 2,5-dihydroxybenzoic acid (gentisic acid) as a final product.[39] Since both the reactive iminoquinone and the quinone intermediates react with other nucleophiles, such as GSH, their formation may also result in deleterious effects to the patient. Thus, the balance between the beneficial versus detrimental effects resulting from the reaction of 5-ASA with HOCl still needs to be equated.

A new and important finding of the present study was the strong scavenging effect of 5-ASA against $^1O_2$ (IC_{50} = 29.5 ± 5.7). When compared to several other NSAIDs with antioxidant properties, only aminopyrine and dipyrone showed such high potencies, as measured by the same methodology.[34] Again, this scavenging effect will be critical for lessening the deleterious effects resulting from the overstimulation of neutrophils. In fact, given that H_2O_2 and HOCl are present in high concentrations within the phagosome, there exists the prospect for a chemical production of high levels of the highly reactive $^1O_2$ according to the following reaction:[40]

$$H_2O_2 + HOCl \rightarrow {}^1O_2 + HCl + H_2O \qquad \text{Eq. 1}$$

It is also known that $O_2^{\bullet-}$ production resulting from neutrophil oxidative burst, as well as chemotaxis, induced by different activating agents, was prevented by sulfasalazine.[26,27,41–43] More recently, Joshi and collaborators[28] applied the pulse radiolysis technique coupled with transient spectrophotometry for *in situ* generation of free radicals, to follow their reaction pathways with sulfasalazine, 5-ASA and SP. Using this technique, these authors demonstrated that sulfasalazine, 5-ASA and SP efficiently scavenged e^-_{aq}, $CO_2^{\bullet-}$, HO$^\bullet$, $^\bullet N_3$ and $^\bullet CCl_3O_2$ radicals. 5-ASA further scavenged glutathiyl, tryptophanyl and lipid peroxyl radicals, while sulfasalazine further scavenged lipid peroxyl and $O_2^{\bullet-}$. On the other hand, 5-ASA and SP were not found to scavenge $O_2^{\bullet-}$. In the antioxidant activity of sulfasalazine, the phenoxyl group of 5-ASA acted as electron (or H-atom) donor. This electron in oxidized sulfasalazine, SP and 5-ASA is then scavenged and thus regenerated by ascorbate,[28] which corresponds to a more friendly pathway, as compared to the production of reactive intermediates following the reaction of HOCl and 5-ASA, as mentioned above. In the present study, we corroborate the previous findings related to scavenging of $O_2^{\bullet-}$ and H_2O_2 by sulfasalazine. In fact, this drug was the most active, achieving an IC_{50} of 173 ± 17 and an IC_{50} of 457 ± 50, respectively. This activity certainly contributes to the observed inhibition of the neutrophils oxidative burst, mainly in IBD, in which sulfasalazine may reach high intestinal concentrations after oral intake. Worth mentioning, sulfasalazine was the most potent NSAID among those tested with the same methodology used in the present study for the scavenging of $O_2^{\bullet-}$ and H_2O_2.[29–31] 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 the intestine of these patients.[15]

Concerning RNS, it was previously reported that 5-ASA does not interact with $^\bullet NO$, but reacts with $ONOO^-$ and prevents $ONOO^-$-induced apoptosis in human colonic epithelial cells and the resultant barrier dysfunction, at concentrations that are well within the expected range achieved with current clinical dosing regimens.[19] Our results, showed that 5-ASA is a strong scavenger of $^\bullet NO$, generating an IC_{50} at the low micromolar range (1.06 ± 0.21 μM). The apparent contradictory results are probably due to the lack of a

positive control in the work of Sandoval and collaborators,[19] which is mandatory for the quality control of the applied methodology. On the other hand, we confirmed that 5-ASA is a strong scavenger of $ONOO^-$ (IC_{50} = 0.68 ± 0.09 μM), and extended the present knowledge by showing that this compound is still very active in the presence of bicarbonate (IC_{50} = 2.88 ± 0.43 μM). Sulfasalazine was also shown to be a scavenger of $^{\bullet}NO$ and $ONOO^-$ (IC_{50} values of 536 ± 89 and 91 ± 22 and 148 ± 16 μM, respectively), although with a much lower potency, as compared to 5-ASA. SP was unable to scavenge $^{\bullet}NO$ in the tested concentrations but was shown to scavenge $ONOO^-$, with a higher strength when the assay was performed in the presence of 25 mM of bicarbonate (IC_{50} values of 257 ± 41 μM in the presence and 1676 ± 35 μM in the absence of bicarbonate). The observed potentiation in the presence of bicarbonate indicates the ability of SP to react with the reactive species generated through the extremely fast reaction between $ONOO^-$ and CO_2 ($K = 3–5.8 \times 10^4\ M^{-1}s^{-1}$).[36,44,45] This reaction results in the formation of the nitrosoperoxycarbonate anion ($ONOOCO_2^-$), whose decomposition leads to the formation of different species including the highly reactive $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ radicals. Thus, reaction with $ONOO^-$ in presence of HCO_3^- or CO_3^{2-} is indeed a reaction with $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ radicals.[46] Again, sulfasalazine and 5-ASA were much more potent than other NSAIDs tested with the same methodologies used in the present study for the scavenging of $^{\bullet}NO$ and $ONOO^-$,[29–31] with only aminopyrine and dipyrone showing such high potencies.[33]

Conclusions

The ROS- and RNS-scavenging effects of sulfasalazine and its metabolites shown in this study may contribute to the anti-inflammatory effects mediated by sulfasalazine through the prevention of the oxidative/nitrative/nitrosative damages caused by these species.

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