Sulphonamides as anti-inflammatory agents: old drugs for new therapeutic strategies in neutrophilic inflammation?

Luciano OTTONELLO, Patrizia DAPINO, Maria C. SCIROCCO, Alessandro BALBI*, Maurizio BEVILACQUA† and Franco DALLEGRI

First Medical Clinic, Department of Internal Medicine, University of Genova Medical School, Genova, Italy, †Institute of Pharmaceutical Sciences, University of Genova, Genova, Italy, and *Department of Endocrinology, L. Sacco Hospital – Vialba, Milan, Italy

(Received 29 June/27 October 1994; accepted 16 November 1994)

1. It is well known that neutrophils act as mediators of tissue injury in a variety of inflammatory diseases. Their histotoxic activity is presently thought to involve proteinases and oxidants, primarily hypochlorous acid (HOCl). This oxidant is also capable of inactivating the specific inhibitor of neutrophil elastase (α1-antitrypsin), thereby favouring digestion of the connective tissue.

2. In the present work, we found that sulphanilamide and some sulphanilamide-related anti-inflammatory drugs such as dapsone, nimesulide and sulphapyridine reduce the availability of HOCl in the extracellular microenvironment of activated neutrophils and prevent the inactivation of α1-antitrypsin by these cells in a dose-dependent manner. The ability of each drug to prevent α1-antitrypsin from inactivation by neutrophils correlates significantly with its capacity to reduce the recovery of HOCl from neutrophils. Five other non-steroidal anti-inflammatory drugs were completely ineffective.

3. Therefore, sulphanilamide-related drugs, i.e. dapsone, nimesulide and sulphapyridine, have the potential to reduce the bioavailability of neutrophil-derived HOCl and, in turn, to favour the α1-antitrypsin-dependent control of neutrophil elastolytic activity. These drugs appear as a well-defined group of agents which are particularly prone to attenuate neutrophil histotoxicity. They can also be viewed as a previously unrecognized starting point for the development of new compounds in order to plan rational therapeutic strategies for controlling tissue injury during neutrophilic inflammation.

INTRODUCTION

In the last decade, considerable interest was focused on the concept of human neutrophils as mediators of tissue injury during inflammatory diseases, ranging from chronic obstructive pulmonary disease, adult respiratory distress syndromes and autoimmune arthritides to neutrophilic dermatoses, inflammatory bowel diseases and immune vasculitides [1, 2]. The growing knowledge of neutrophil physiopathology has recently led to the identification of a major histotoxic pathway. Neutrophils, recruited at sites of inflammation, generate superoxide anion ($O_2^-$), which rapidly dismutates to hydrogen peroxide ($H_2O_2$) [3, 4]. In turn, $H_2O_2$ is transformed into hypochlorous acid (HOCl) by neutrophil myeloperoxidase (MPO) [3, 5]. As a consequence of its extremely high reactivity [5], HOCl represents the most toxic oxidant generated by neutrophils, also capable of reacting with various glycoproteins such as α1-antitrypsin (AT) [3, 5, 6]. This glycoprotein, synthesized by hepatocytes and macrophages [7], belongs to the system of acute-phase proteins [8], diffuses into inflamed tissue from the circulation easily [8] and represents the specific inhibitor of the major neutrophil proteolytic enzyme, elastase [7, 8]. Owing to the capacity of HOCl to react and oxidatively inactivate AT [3, 8], elastase can escape regulation by AT and digest important connective tissue components such as elastin, certain types of collagen, fibronectin, laminin and the proteic domains of proteoglycans [7, 8]. Therefore, oxidative attack and elastolytic injury of inflamed tissue by neutrophils appear to be interlinked events, HOCl playing a pivotal role in these processes.

At present, non-steroidal anti-inflammatory drugs (NSAIDs) represent the major candidates for controlling neutrophil-mediated tissue injury. Although numerous studies have tested the effects of NSAIDs on various neutrophil functions (reviewed in [9]), the possibility that they can interfere with the aforementioned histotoxic pathway has not been experimentally tested. Recently, we found that the NSAID nimesulide [10, 11] is particularly efficient in reducing HOCl availability in the neutrophil surroundings, in turn preventing AT inactivation [12]. Consequently, we planned the present study to...
test the effects of various NSAIDs on the neutrophil HOCl-dependent AT inactivation. Nimesulide, sodium salicylate, acetylsalicylic acid, ibuprofen, naproxen and tenoxicam were tested in parallel assays. We also studied dapsone and sulphapyridine, reported to have beneficial effects in diseases characterized by neutrophilic inflammation [13].

MATERIALS AND METHODS

Media and reagents

Hanks’ balanced saline solution containing 1 mg/ml glucose and without Phenol Red (HBSS; ICN Biomed, Milan, Italy) was used as incubation medium. Taurine, L-methionine, sodium benzoate, sodium azide, o-dianisidine, mannitol, superoxide dismutase type I (bovine blood) and ferricytochrome c type VI (horse heart) were purchased from Sigma (St Louis, MO, U.S.A.). α1-Antitrypsin (AT; lot 286029), porcine pancreatic elastase (PPE, lot 801800) and N-succinyl-(1-alanyl)-p-nitroanilide (lot 510008) were purchased from Calbiochem (San Diego, CA, U.S.A.). Heparin (Liquemine) was purchased from Roche (Milan, Italy) and Ficoll-Hypaque from Nyegaard (Oslo, Norway). Phorbol 12-myristate 13-acetate (PMA; Sigma), stored at −20°C as stock solution of 2 mg/ml in dimethyl sulfoxide (DMSO; C. Erba, Milan, Italy) was diluted in medium and used at the final concentration of 10 ng/ml. The reagent 5-mercapto-2-nitrobenzoic acid was prepared by reducing 3,3′-dithiobis [6-nitrobenzoic] acid (Sigma) [14]. Hypochlorous acid (HOCl) was generated by dissolving sodium hypochlorite (BDH, Poole, U.K.) into solution buffered at pH 7.4 [12]. Sodium salicylate, acetylsalicylic acid, ibuprofen, naproxen, tenoxicam, dapsone, sulphapyridine, sulphanilamide and sulphamethoxazole were purchased from Sigma. Nimesulide was a gift from LPB Institute (Milan, Italy). Other reagent-grade compounds were used as obtained from commercial suppliers.

Neutrophils

Heparinized (heparin 10 units/ml) venous blood was obtained from healthy male volunteers after informed consent was obtained. Neutrophils were isolated by dextran sedimentation and subsequent centrifugation on a Ficoll-Hypaque density gradient, as described previously [14]. Contaminating erythrocytes were removed by hypotonic lysis [14]. Neutrophils were washed three times with HBSS and resuspended in HBSS. Final cell suspensions contained 97% or more neutrophils and more than 98% viable cells, as evaluated by the ethidium bromide–fluorescein diacetate test [14].

Inactivation of AT by neutrophils

The inactivation of AT by neutrophils was accomplished using 2.5 × 10⁶ neutrophils, 125 µg of AT and 10 ng/ml PMA in a final volume of 0.25 ml. The experiments were carried out in the absence and presence of acetylsalicylic acid, sodium salicylate, ibuprofen, naproxen, tenoxicam, dapsone, sulphapyridine, sulphanilamide and sulphamethoxazole at final concentrations of 10, 20, 50, 100 µmol/l. Moreover, other experiments were carried out in the presence of 1 mmol/l azide, 20 mmol/l methionine or taurine and 20 mmol/l mannitol or benzoate. The incubations (30 min, 37°C) were in Falcon Plastic tubes (10 × 100 mm, Falcon Plastic, Oxnard, CA, U.S.A.). At the end of the incubation period, methionine (500 nmol) was added to each tube to quench residual oxidants and the cell-free supernatants were isolated by centrifugation (500 g for 5 min at 4°C). The AT activity was then determined in the supernatants.

Assay of AT activity

The capacity of AT to inhibit PPE, i.e. the elastase inhibitory capacity (EIC) was used as a measure of active AT. The method was a modification of the standard spectrophotometric assay [12]. Briefly, 40 µl of supernatant containing AT and 20 µl of 37 units/ml PPE were mixed with 100 µl of 0.2 mol/l Tris–HCl buffer (pH 8.0) in a microtitre tray (Titertek microtitration equipment, Flow). The tests were carried out in triplicate. The microplate was agitated for 30 min (37°C) in a microplate spectrophotometer reader (Titertek Twinreader Plus, Flow). Then 20 µl of the reaction mixture was transferred into the wells of another microtitre tray containing 230 µl of the PPE substrate [6.6 mmol/l N-succinyl-(1-alanyl)-p-nitroanilide in 0.05 mol/l Tris–HCl, pH 8.0, containing 0.3% DMSO]. The tray was agitated for 10 s (25°C), the absorbance at a wavelength of 405 nm was read immediately and again every minute for 5 min, and the change in absorbance was calculated. The percentage loss of EIC was calculated by comparing the ability of equal amounts of control and test samples of AT to suppress PPE activity. The reproducibility of the assay, expressed as coefficient of variation, was 11.02% (n = 10).

Hypochlorous acid assay

The generation of HOCl by neutrophils, incubated in the absence or presence of the aforementioned drugs (10, 20, 50, 100 µmol/l), was measured by the taurine trapping technique [15], as previously described [14]. The incubations were carried out using 5 × 10⁵ neutrophils and 10 ng/ml PMA in a final volume of 1 ml containing 20 mmol/l taurine. At the end of the incubation period (60 min, 37°C) the amount of HOCl trapped by taurine (yielding taurine monochloramine) in the cell-free supernatants was determined by measuring spectrophotometrically (absorbance = 412 nm, ε = 1.36 ×
Sulphonamides and neutrophilic inflammation

Other assay

To test the capacity of dapsone, nimesulide, sulphanilamide and sulphapyridine to compete with AT for reagent HOCl, each drug (1 mmol/l) was mixed with AT (33.3 µg) before the addition of reagent HOCl (30 µmol/l). The final volume of the mixture was 200 µl. After incubation (15 min), the percentage EIC of AT was measured as described above. Experiments were carried out in triplicate. The generation of hydroxyl ·OH radicals by neutrophils was studied by a deoxyribose oxidation spectrophotometric assay [16]. The production of superoxide anion (O₂⁻) by neutrophils was studied by using a modification of the method of Babior et al. [17], as previously described [16]. The MPO activity released by neutrophils was determined by an o-dianisidine method, as described previously [14].

Statistical analysis

The results were expressed as means ± 1 SD. The effect of drugs on the EIC of AT incubated with neutrophils, and their ability to reduce the recovery of HOCl from neutrophils, were analysed by calculating the inhibitory dose 50% (ID₅₀) for each drug. In this regard, differences among drugs were tested by the non-parametric Kruskal–Wallis test. As summarized in Table 1, negative trends were found between the capacity of each drug to preserve the EIC of neutrophil-exposed AT and the HOCl recovery from neutrophils in the presence of drugs. The slope obtained with sulphanilamide was significantly lower than that calculated for the other drugs (Table 1). With the exception of sulphamethoxazole, the remaining drugs incapable of preventing AT inactivation by neutrophils (Fig. 1) did not affect the HOCl recovery from neutrophils (data not shown). Although incapable of preventing AT inactivation, sulphamethoxazole reduced HOCl

RESULTS

When exposed to 2.5 × 10⁶ stimulated neutrophils, 125 µg of AT was completely inactivated during a 30-min incubation period. Conversely, resting neutrophils, i.e. cells incubated in absence of PMA, were completely ineffective. As summarized in Fig. 1, 4 of 10 drugs prevented the AT inactivation by neutrophils (dapsone, nimesulide, sulphanilamide and sulphapyridine) in the presence of these compounds (>92%). Inactivators of ·OH radicals such as mannitol and benzoate (20 mmol/l) were ineffective (EIC of AT <2%), consistent with the incapacity of neutrophils to generate these oxidants (·OH radicals could not be detected, using the deoxyribose oxidation assay). Since these data point towards the intervention of HOCl, a set of experiments was planned to test in parallel assays both the ability of drugs to prevent AT inactivation by neutrophils and the ability to reduce the recovery of HOCl from neutrophils, as measured by the taurine-trapping technique. The four drugs inhibiting the inactivation of AT by neutrophils (Fig. 1) were found to exert their action in a dose-dependent manner (Fig. 2). The activity of sulphanilamide was significantly lower than that of the other three drugs (legend to Fig. 2). Moreover, these drugs were found to reduce the HOCl recovery from neutrophils in a dose-dependent manner (Fig. 3). In this regard, differences among drugs were not significant (legend to Fig. 3). As summarized in Table 1, negative trends were found between the capacity of each drug to preserve the EIC of neutrophil-exposed AT and the HOCl recovery from neutrophils in the presence of drugs. The slope obtained with sulphanilamide was significantly lower than that calculated for the other drugs (Table 1). With the exception of sulphamethoxazole, the remaining drugs incapable of preventing AT inactivation by neutrophils (Fig. 1) did not affect the HOCl recovery from neutrophils (data not shown). Although incapable of preventing AT inactivation, sulphamethoxazole reduced HOCl

10⁴ mol⁻¹ cm⁻¹) the oxidation of 5-mercapto-2-nitrobenzoic acid [15].

![Graph illustrating the inactivation of AT by neutrophils in the presence of various anti-inflammatory drugs.](image-url)
Fig. 2. Dose-dependent inhibition of neutrophil-mediated AT inactivation by dapsone, nimesulide, sulphanilamide and sulphapyridine. The percentage EIC of AT was measured as reported in Materials and methods and in the legend to Fig. 1. Results are expressed as means of seven experiments. Difference among drugs’ ID_{50} was tested by non-parametric analysis (Kruskal-Wallis) as reported in Materials and methods. Dapsone versus sulphanilamide, P<0.05; nimesulide versus sulphanilamide, P<0.005; sulphapyridine versus sulphanilamide, P<0.005. Dapsone versus sulphapyridine, dapsone versus nimesulide, nimesulide versus sulphapyridine = not significant.

Fig. 3. Effect of dapsone, nimesulide, sulphanilamide and sulphapyridine on HOCl recovery from neutrophils. HOCl recovery (nmol/10^6 neutrophils/60 min) was measured as reported in Materials and methods and expressed as mean ± SD of seven experiments. Difference among drugs’ ID_{50} tested by non-parametric analysis (Kruskal-Wallis) as reported in Materials and methods, was not significant.
Table 1. Monotonic trend between the effect of drugs on the EIC of AT incubated with neutrophils and the effect of drugs on the HOCl recovery from neutrophils. Data presented in Figs. 2 and 3 were analysed using the non-parametric Theil procedure. *P<0.001 when compared with the other drugs (sulphanilamide versus dapsone, sulphanilamide versus nimesulide, sulphanilamide versus sulphapyridine). Dapsone versus nimesulide, dapsone versus sulphapyridine and sulphapyridine versus nimesulide = not significant.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Kendall's ( \tau )</th>
<th>( P )</th>
<th>Slope</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dapsone</td>
<td>-0.674603</td>
<td>&lt;0.001</td>
<td>-0.857544</td>
<td>96.669425</td>
</tr>
<tr>
<td>Nimesulide</td>
<td>-0.495767</td>
<td>&lt;0.001</td>
<td>-0.718515</td>
<td>95.587849</td>
</tr>
<tr>
<td>Sulphanilamide</td>
<td>-0.809524</td>
<td>&lt;0.001</td>
<td>-1.336170*</td>
<td>90.233971</td>
</tr>
<tr>
<td>Sulphapyridine</td>
<td>-0.613757</td>
<td>&lt;0.001</td>
<td>-0.709525</td>
<td>95.186298</td>
</tr>
</tbody>
</table>

Fig. 5. Structure of dapsone, nimesulide, sulphapyridine and related compounds, sulphanilamide and sulphamethoxazole.

Fig. 4. Effect of dapsone, nimesulide, sulphapyridine and sulphanilamide on the inactivation of AT by reagent HOCl. Tests were carried out as reported in Materials and methods. The percentage EIC of AT was calculated by comparison with the activity of control samples of AT incubated in the absence of HOCl. The percentage EIC of AT incubated with HOCl in absence of drugs was 0 (zero). Results are expressed as means±1 SD of four experiments. Difference among drugs (Kruskal-Wallis): nimesulide versus dapsone, \( P<0.05 \); nimesulide versus sulphanilamide, \( P<0.01 \).

recovery from neutrophils in a dose-dependent manner up to approximately 40% inhibition at 100 \( \mu \)mol/l (data not shown). Under the present setting, drugs did not affect oxidant production by neutrophils, as judged by measuring \( \text{O}_2^- \) generation (\( \text{O}_2^- \) generation 131.6 ± 11.7 nmol 30 min \( ^{-1} \) 10 \( ^{-6} \) cells, mean ± 1 SD, \( n=6 \)). Moreover, they did not inhibit the release of MPO by neutrophils (MPO release 1.388 ± 0.121 m-units 60 min \( ^{-1} \) 10 \( ^{-6} \) cells, mean ± 1 SD, \( n=5 \)). Finally, they did not interfere with the activity of MPO (data not shown). In contrast, drugs inhibiting the inactivation of AT by neutrophils were found to prevent AT inactivation by reagent HOCl as well (Fig. 4).

**DISCUSSION**

The present results show that certain drugs (dapsone, nimesulide, sulphapyridine, sulphanilamide), used at concentrations achievable in vivo [10, 20], are capable of protecting AT from inactivation by neutrophils. These drugs act primarily by trapping neutrophil-derived HOCl before its collision with AT molecules. Several findings are consistent with this view. First, well-known inhibitors of the HOCl-generating MPO system prevented AT inactivation by neutrophils. This suggests that neutrophils preferentially use HOCl for suppressing AT function. This conclusion is in agreement with the results obtained by other authors [5, 21]. Second, dapsone, nimesulide, sulphapyridine and sulphanilamide caused a dose-dependent inhibition of HOCl recovery from neutrophils. As these drugs did not affect the oxidative burst and the MPO release or function, they appear to act by direct scavenging of released HOCl rather than by inhibition of HOCl production. Nevertheless, when tested with neutrophils challenged with opsonized zymosan particles, nimesulide at high concentrations (100 \( \mu \)mol/l) can also reduce oxygen consumption and \( \text{O}_2^- \) generation, and thus production of HOCl [22]. Third, for each of the aforementioned drugs, the ability to lower the HOCl recovery from neutrophils correlates significantly with the capacity to prevent AT inactivation. Although the four drugs were equally effective in inhibiting HOCl recovery, the capacity of sulphanilamide to protect AT from neutrophil-mediated inactivation was significantly lower than that of the other three drugs. This probably reflects a relatively low efficiency of sulphanilamide in competing with AT for neutrophil-derived HOCl. Consistent with this possibility, another drug, i.e. sulphamethoxazole, was found to be incapable of protecting AT from neutrophil-mediated inactivation at a concentration causing approximately 40% inhibition of HOCl recovery. Fourth, the four drugs inhibiting AT inactivation by neutrophils prevented AT inactivation by reagent HOCl as well. This is consistent with the possibility that they can actually act by directly inactivating neutrophil-derived HOCl. The different efficiency of the four drugs in protecting AT in the neutrophil system compared with the reagent HOCl system is likely to reflect different kinetics of HOCl delivery, i.e. neutrophil generation of HOCl as a flux versus direct bolus of HOCl in the cell-free system.
Owing to the potent oxidative properties of HOCl [5], and taking into account the fact that AT is the natural inhibitor of neutrophil elastase [7, 8], the present results suggest that these drugs have the potential to limit both the oxidative and the elastolytic histotoxicity of activated neutrophils. In other words, these drugs can counteract the oxidant–antioxidant and the protease–antiprotease imbalances which are crucial for the pathogenesis of tissue injury at sites of neutrophilic inflammation [3, 6]. On the other hand, the control of elastase activity by rescued AT can be expected to substantially contribute to the known anti-inflammatory properties of these drugs. In fact, neutrophil elastase promotes microvascular permeability changes leading to plasma exudation [23], favours the generation of kinins and the activation of complement by digesting the Cl-inhibitor [8] and induces the production of the neutrophil chemotaxin interleukin 8 by certain tissue cells [24]. It is, however, of note that these drugs also exert other anti-inflammatory effects upon neutrophil function, such as inhibition of adhesion and platelet-activating factor (PAF) production [25, 26].

The properties discussed above characterize a minority of the drugs studied. These properties are likely to reflect a particular affinity of these compounds for HOCl, at least in part independently on the presence of –NH₂ groups able to react easily with HOCl [5]. In fact, although the five compounds shown in Fig. 5 are endowed with –NH₂ groups, they have different abilities to prevent AT inactivation. Sulphamethoxazole is ineffective, and sulphanalimide is significantly less efficient than dapsone, nimesulide and sulphapyridine, which are equally active. In conclusion, the bulk of our data suggest some general and pertinent comments. First, the drugs proved to be active in the present systems (dapsone, nimesulide and sulphapyridine) have structural similarities and/or are derivatives of sulphanalimide [20] (Fig. 5). Second, these drugs are particularly prone to react with neutrophil-derived HOCl, preventing its toxicity. Third, they have been reported to have beneficial effects in conditions characterized by neutrophil inflammation [9, 11, 27]. Therefore, on the basis of these chemical, pharmacodynamic and clinical criteria, sulphanalimide-related (i.e. sulphonamide) anti-inflammatory agents [28] appear as a well-defined group of drugs to be considered for planning rational therapeutic strategies to control tissue injury during neutrophilic inflammation. Also, this group of drugs may be a starting point for the development of new compounds to counteract deleterious effects of activated neuphils.

ACKNOWLEDGMENTS

This work was supported by a grant from the Italian CNR (No. 93.04360.CT04) to F.D. We are grateful to Dr Giuseppe Mela, Department of Internal Medicine, University of Genova, Genova, for statistical analysis and helpful contributions.

REFERENCES