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Inhibition of the myeloperoxidase chlorinating activity by non-steroidal anti-inflammatory drugs investigated with a human recombinant enzyme

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) were investigated for their ability to affect the chlorinating activity of human myeloperoxidase and to scavenge HOCl, the main myeloperoxidase system product. Fourteen drugs representative of various NSAIDs families were tested with the chlorination of taurine used as a detection system. All were unable to inhibit taurine chlorination in a system without myeloperoxidase. In contrast, most of them induced a dose-dependent inhibition of the taurine chlorination mediated by a myeloperoxidase/H_2O_2/Cl^- system. This took place at variable drug concentrations and IC_{50} were calculated. The inhibitory effect was therefore due to a direct interaction with the enzyme rather than to HOCl scavenging. A spectroscopic method used to measure the myeloperoxidase compound II lifetime in presence of the different drugs showed that all the drugs, which inhibited chlorination activity were able to induce accumulation of compound II. The extent of chlorinating activity inhibition (IC_{50}) was inversely related to the duration of the block of enzyme in compound II form. This further demonstrates that myeloperoxidase is an interesting target for anti-inflammatory therapy. The recombinant myeloperoxidase used for the first time in this kind of study was as convenient for pharmacological purposes as the purified one.

Keywords: NSAID (non-steroidal anti-inflammatory drug); Myeloperoxidase; Compound II; Inflammation; Recombinant human enzyme; Reactive oxygen species

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a wide family of drugs used in the treatment of inflammatory diseases including chronic articular disorders. The latter are characterised by a progressive erosion of the articular cartilage leading to functional disorders of the joints. The mechanisms of development of articular diseases are not fully understood but proteases are recognised to play an important role. In previous studies, reactive oxygen species were identified as potent agents of cartilage and connective tissue destruction (Weiss, 1989; Shingu et al., 1994; Flugge et al., 1999). There are several sources of reactive oxygen species in inflamed articulations such as phagocytic cells, which infiltrate the inflammatory site and generate species such as the superoxide radical anion O_2^-, hydrogen peroxide (H_2O_2) and the hydroxyl radical $^{\cdot}OH$. In addition, some of them, namely neutrophils and to a lesser extent monocytes, produce hypochlorous acid (HOCl) through the activity of myeloperoxidase (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7), a green hemoprotein located in their azurophilic granules. Articular chondrocytes themselves are capable of producing reactive oxygen species (Rathakrisnan et al., 1992; Henrotin et al., 1993). Accumulation of transition metals at the inflammatory site as is the case in rheumatoid arthritis, could also lead to the formation of hydroxyl radicals and to further tissue damage (Kaur et al., 1996). The present study more specifically focussed on the reactions of the myeloperoxidase/halide system of phagocytic cells.

Myeloperoxidase catalytic activity is complex and involves two distinct pathways (Fig. 1). Beside its regular peroxidative activity (Fig. 1; reactions 1–3), myeloperoxidase has a chlorinating activity (Fig. 1; reactions 1 and 4) in contrast to most other peroxidases. The enzyme uses

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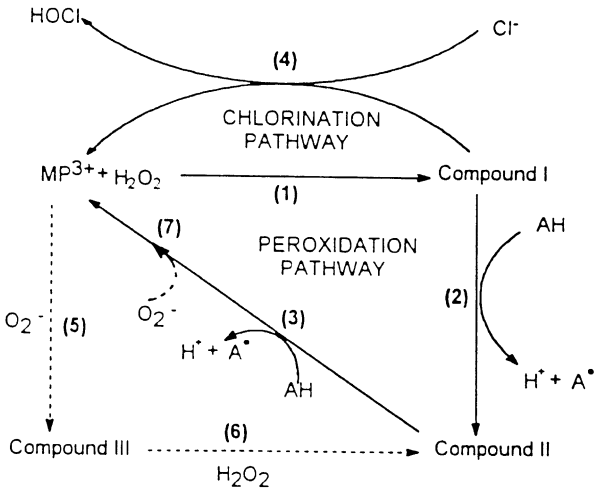

Fig. 1. Representation of myeloperoxidase redox transformations and catalytic pathways. MP^{3+}: Native enzyme; Compound I: MP^{3+} H_2O_2; Compound II: MP^{2+} H_2O_2; Compound III: MP^{3+} O_2. AH: reducing substrate; O_2^-: superoxide anion. The broken lines mean that the reaction is hypothetical under in vivo conditions (adapted from Kettle and Winterbourn, 1988).

H_2O_2 produced by the respiratory burst of neutrophils to oxidise halides into the corresponding hypohalous acid, mainly Cl^- into HOCl which can subsequently react with endogenous amine-containing compounds to form long-lived nitrogen-chloramines. *N*-chlorotaurine was demonstrated to account for 40–50% of the nitrogen-chloramine derivatives and to be the major chloramine produced by neutrophils (Harrison and Schultz, 1976; Test and Weiss, 1986). Both chloramines and HOCl are potent oxidants implicated in the bactericidal activity of the neutrophils and also in the cytotoxicity mediated by these cells. They can oxidise a number of cellular targets and promote proteolytic damage at the inflammatory site via the indirect activation of elastase and collagenase, two major proteolytic enzymes released from the lysosomal granules of these cells (Test and Weiss, 1986; Saari et al., 1990; Desrochers et al., 1992). This makes HOCl and myeloperoxidase privileged targets for antioxidant and antirheumatic therapy.

NSAIDs had already been reported to interfere in the myeloperoxidase system. Most of them are able to scavenge HOCl in water systems but few seem able to do it in the presence of biological targets (Wasil et al., 1987; Shacter et al., 1991; Zuurbier et al., 1990). Tenoxicam reacts with leukocyte peroxidase extracts containing mainly myeloperoxidase (Ichihara et al., 1985, 1989). In previous studies, some NSAIDs were able to inhibit the halogenation activity of myeloperoxidase (Shacter et al., 1991; Zuurbier et al., 1990; Theron et al., 1979) presumably by promoting the accumulation of myeloperoxidase compound II (see Fig. 1) which diverts the enzyme from its chlorination pathway (Zuurbier et al., 1990; Kettle and Winterbourn, 1991).

The present study was conducted with a large panel of NSAIDs currently used in the treatment of inflammatory pathologies. Our purpose was to quantify the effect of drugs on the enzymatic system by measuring pharmacological parameters such as IC_{50}. The study also aimed to confirm the mechanism, previously proposed by other authors, responsible for the interaction of NSAIDs with the myeloperoxidase system. A taurine chlorination assay was used to investigate HOCl scavenging and myeloperoxidase inhibition. We further tried to quantify, using an original method, the accumulation of compound II, which is the proposed way by which the indirect inhibition of the chlorinating activity of myeloperoxidase occurs. A recombinant human myeloperoxidase produced by a Chinese hamster ovary cell line (Moguilevsky et al., 1991) was used for the first time in place of the conventional enzyme purified from leukocytes. This recombinant form was previously shown to behave physicochemically very similarly to the mature purified enzyme and could therefore be useful in pharmacological studies (Jacquet et al., 1991).

2. Materials and methods

2.1. Reagents and drugs

Taurine, 5,5′-dithiobis(2-nitrobenzoic acid), sodium hypochlorite 5% stock solution (NaOCl), potassium iodide (KI), catalase type C 9322 (EC 1.11.1.6, from bovine liver) and sodium borohydride ($NaBH_4$) were from Sigma. H_2O_2 30% solution, KH_2PO_4, KOH and NaCl used for the buffers were from Merck Belgolabo. UV–visible measurements were performed with a UV160 Shimadzu spectrophotometer. Compound II spectra were recorded with a Hewlett-Packard 8451 diode array spectrophotometer. Drugs were generally purchased from their respective manufacturers, as follows: flurbiprofen and ibuprofen (Boots Pharmaceuticals), ketoprofen and acemetacin (Rhône-Poulenc Rorer), naproxen (Sarva Syntex), diclofenac Na (Ciba Geigy), flufenamic acid (Trenker), indomethacin and sulindac (Merck Sharp and Dohme), niflumic acid (Upsa Medica), tolmetin Na (Cilag), piroxicam (Pfizer), tenoxicam (Roche), and acetylsalicylic acid (Bayer).

2.2. Preparation of recombinant myeloperoxidase

Recombinant myeloperoxidase was prepared, as previously described, in the Department of Applied Genetics of the Free University of Brussels, Belgium (Moguilevsky et al., 1991). Each batch solution is characterised by its activity (U/ml), protein concentration (mg/ml) and specific activity (U/mg). The peroxidative activity was determined with *o*-dianisidine as substrate (Krawisz et al., 1984). The protein concentration was measured by the Lowry assay, using ovalbumin as standard.

2.3. Preparation of solutions for the different assays

Drug solutions were prepared daily in deoxygenated water by addition to their aqueous suspension of the minimum volume of a solution of sodium carbonate (0.4 M). The pH was then rapidly adjusted to 7.4. Stock 2-nitro-5-thiobenzoate (TNB) solutions were prepared as previously described (Ching et al., 1994) and then diluted to 0.45 mM. Concentration of the stock solution was determined daily, using an extinction coefficient of 13,600 M^{-1} cm^{-1} at 412 nm. HOCl solutions were prepared just before use: the NaOCl stock solution was diluted (0.5/25 ml water) and adjusted to pH 6.2 with dilute H_2SO_4. The concentration of this solution was determined iodometrically, using 0.2 mM KI and I_2 formed was measured using an extinction coefficient of 22,900 M^{-1} cm^{-1} at 350 nm (Gressier et al., 1995). H_2O_2 solutions were made daily by appropriate dilution of a 30% solution, and the concentration was measured using an extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm (Beers and Sizer, 1952).

2.4. Scavenging of hypochlorous acid (HOCl)

HOCl was measured with the taurine chlorination assay adapted from Gressier et al. (1995). The reaction mixture contained the following reagents at the concentrations indicated between brackets in a final volume of 1 ml: 100 μl taurine 150 mM (15 mM), 100 μl of a solution of drugs of increasing concentrations, 700 μl of a pH 7.4 phosphate buffer KH_2PO_4/KOH 10 mM containing 140 mM NaCl. The assay was initiated by the addition of 100 μl of HOCl 600 μM (60 μM). The mixture was incubated 10 min at 37°C. After the addition of 2-nitro-5-thiobenzoate 0.45 mM (750 μl) and 2250 μl of water, absorbance was measured at 412 nm. Results were expressed as % inhibition of taurine chlorination at each drug concentration.

2.5. Inhibition of myeloperoxidase-chlorinating activity

Meloperoxidase chlorinating activity was determined by measuring the chlorination of taurine with a myeloperoxidase/H_2O_2/Cl^- system. Myeloperoxidase concentration was determined in order to produce about 60 μM of HOCl during the 5 min of incubation. The reaction mixture contained the following reagents, at the concentrations stated between brackets, in a final volume of 1 ml: 15 μl of recombinant myeloperoxidase (74 nM), 585 μl of a pH 7.4 KH_2PO_4/KOH phosphate buffer 10 mM containing 300 mM NaCl, 100 μl of taurine 150 mM (15 mM), 200 μl of a drug solution of increasing concentration. The assay was initiated by the addition of 100 μl H_2O_2 1 mM (100 μM). The mixture was incubated 5 min at 37 °C and the reaction was stopped by addition of 100 μl of catalase (4 U/μl). After addition of 750 μl of 2-nitro-5-thiobenzoate 0.45 mM and 2150 μl water, absorbance was measured at 412 nm. In this assay, a myeloperoxidase batch with the following characteristics was used: 102 U/ml, 0.412 mg/ml, 248 U/mg.

2.6. Accumulation of compound II

In a 1.5-ml quartz cell, the following reagents were introduced, at the final concentrations stated between brackets, for a final volume of 1 ml: 100 μl of myeloperoxidase (1655 nM), 400 μl of pH 7.4 KH_2PO_4/KOH phosphate buffer 10 mM containing 300 mM NaCl, 400 μl of a 10 mM drug solution (4000 μM). The spectrum of the native enzyme was then recorded and the reaction was initiated by addition of 100 μl of a 300 μM H_2O_2 solution (30 μM). Absorbances were monitored at the same time at the wavelengths which were typical with respect to compound II (456 and 625 nm) and native enzyme (430 and 675 nm). Compound II lifetime measured in the presence of all the drugs inducing inhibition of myeloperoxidase chlorinating activity was measured as the time of intersection between curves at 430 and 456 nm. This time correlates experimentally with the complete conversion of compound II to native enzyme, which is shown by a shift in the enzyme spectrum. In this assay, a myeloperoxidase batch with the following characteristics was used: 488 U/ml, 1.39 mg/ml, 351 U/mg.

2.7. Statistics

A Friedman non-parametric two-way analysis was used to find the significance of the mean inhibitory curve for the inhibition in the myeloperoxidase-chlorinating activity assay. Results were considered statistically significant at $P < 0.05$. The concentrations of drugs that inhibited HOCl production by 50% (IC_{50}) were measured directly from the dose–effect curves; mean and S.E.M. were determined for each set of n separate experiments.

3. Results

3.1. Scavenging of hypochlorous acid (HOCl)

In a system without myeloperoxidase, most NSAIDs, namely flufenamic and niflumic acids, sulindac, naproxen, ibuprofen, ketoprofen, flurbiprofen, diclofenac, tolmetin, acetylsalicylic acid, indomethacin, acemetacine and diflunisal, tested at concentrations ranging from 0 to 2000 μM, were unable to react directly with HOCl and to protect taurine against chlorination. Nevertheless, two drugs, tenoxicam and piroxicam, showed a weak inhibitory effect in this system. As both drugs absorbed at 412 nm, it was difficult to determine a correct IC_{50} value. Tenoxicam gave about 30% of inhibition of taurine chlorination at 800 μM. Piroxicam, which absorbs less at this wavelength, had

an IC_{50} of 1156 ± 152 μM ($n = 4$). Both drugs therefore seemed to have roughly the same order of potency for HOCl scavenging.

3.2. Inhibition of the myeloperoxidase-chlorination activity

Most NSAIDs, namely flufenamic and niflumic acids, sulindac, naproxen, diclofenac, tolmetin, indomethacin and acemetacine, inhibit taurine chlorination mediated by a myeloperoxidase/H_2O_2/Cl^- system in a dose-dependent manner. All the dose–inhibition curves were found to be significant ($P < 0.01$) and IC_{50} values could be calculated (Table 1). Acetylsalicylic acid was found to be only a weak inhibitor with about 17% taurine chlorination inhibition at 2000 μM. Other NSAIDs, particularly ibuprofen, ketoprofen and flurbiprofen, tested between 0 and 2000 μM, had no inhibitory effect on taurine chlorination in this system.

3.3. Accumulation of compound II

The drugs which inhibited taurine chlorination by the myeloperoxidase/H_2O_2/Cl^- system were able to initiate and promote the accumulation of compound II. Fig. 2 illustrates a typical set of spectra obtained with niflumic acid. The formation and accumulation of compound II in the presence of myeloperoxidase, H_2O_2, Cl^- and niflumic acid was first observed; after a period of time which depended on the efficiency of inhibition, compound II returned to the native form, causing a shift in the enzyme spectrum (from 456 to 430 nm for the main peak). The time necessary for the return of compound II to the native enzyme was measured as mentioned in Section 2. The results are summarised in Table 1. The formation of oxidised products absorbing between 350 and 450 nm was observed for three drugs, diclofenac, niflumic and flufenamic acids. However, these by-products did not interfere with the measurement of compound II lifetime as the shift in the enzyme spectra could be observed without difficulty.

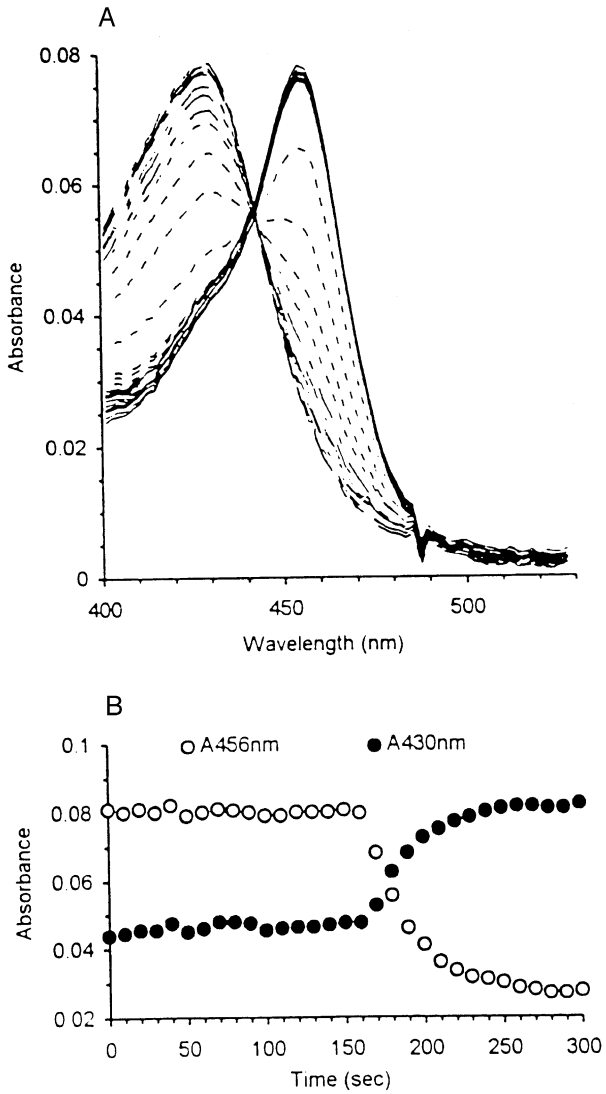

Fig. 2. (A) Accumulation of compound II of myeloperoxidase and return to the native enzyme in presence of niflumic acid; (B) Monitoring of the absorbances at the characteristic wavelengths of compound II (λ_{max}: 456 and 625 nm) and native enzyme (λ_{max}: 430 and 575 nm).

Table 1
Concentration of NSAIDs giving 50% inhibition of the myeloperoxidase mediated chlorination of taurine (IC_{50}) compared to compound II lifetime IC_{50} and compound II lifetime are mean values from *n* separate experiments. Individual values were obtained as described in Section 2. All the compound II lifetime determinations were performed at 4 mM drug concentration.

Drug	$IC_{50} \pm$ S.E.M. (μM)	*n*	Lifetime ± S.E.M. (s)	*n*
Flufenamic acid	3.3 ± 0.3	3	8 ± 2	3
Diclofenac	12 ± 1	5	57 ± 5	3
Niflumic acid	28 ± 2	3	160 ± 17	3
Tenoxicam	36 ± 4	3	258 ± 29	3
Piroxicam	42 ± 2	3	210 ± 14	3
Indomethacin	95 ± 17	3	146 ± 10	3
Acemetacin	163 ± 18	3	(n.d.)	
Sulindac	307 ± 9	4	(n.d.)	
Tolmetin	455 ± 10	3	65 ± 14	3
Naproxen	777 ± 62	3	36 ± 11	3

(n.d.) means that the exact value was not determined.

4. Discussion

Most of the NSAIDs tested being able to inhibit the myeloperoxidase chlorinating activity, we tried to further investigate the mechanism of inhibition. Indeed, the results showed that drugs can act at different levels in the myeloperoxidase reactions cycle to induce this inhibition (Fig. 1; reactions 1–4). For example, derivates such salicyl and benzylhydroxamic acids have been reported as potent inhibitors of the enzyme that block compound I formation

by linkage to the active site in place of H_2O_2 (Davey and Fenna, 1996). Drugs, or their oxidised derivates, can also inhibit chlorinating activity either by direct scavenging of H_2O_2 or HOCl produced by the myeloperoxidase system, or by interference with the enzymatic cycle by reacting with compound I or II. Finally, they can promote reactions 2 and/or 3, an effect which diverts myeloperoxidase from its chlorination pathway and leads to its indirect inhibition, which is a function of their relative rates.

The first part of the study demonstrated that NSAIDs did not directly scavenge HOCl. Only piroxicam and tenoxicam showed a very weak HOCl scavenging activity ($IC_{50} \approx 1000$ μM), but at concentrations far above those required to inhibit chlorinating activity ($IC_{50} \approx 35–40$ μM). These results are in agreement with those of other studies in which several NSAIDs were unable to scavenge HOCl in the presence of biological targets such as taurine or α_1-antiprotease (Desrochers et al., 1992; Shacter et al., 1991). As NSAIDs also do not directly react with H_2O_2 (Parij and Nève, 1996), it seems that the inhibiting activity of NSAIDs on myeloperoxidase-mediated taurine chlorination is the consequence of interference with the enzymatic system rather than of scavenging activity. Our study later established that the drugs do not block compound I formation. Others authors had already proposed that some NSAIDs inhibit the myeloperoxidase chlorinating activity by an indirect mechanism, namely through a reaction with compound I, which promotes compound II formation (Fig. 1; reaction 2). It was additionally reported that diclofenac could also react with the compound II formed in the first reaction and reduce it back to the native enzyme. Using this route, the drug can enter the peroxidation pathway and behaves as a reducing substrate (Zuurbier et al., 1990; Kettle and Winterbourn, 1991). The spectra we investigated for compound II showed that all the drugs which have an inhibitory effect on myeloperoxidase taurine chlorinating activity are able to induce the accumulation of compound II (Fig. 2). The intensity of the effect could be related to compound II lifetime measured in the presence of most drugs (Table 1). When compound II lifetimes were compared to IC_{50} values measured in the myeloperoxidase chlorinating assay, an inverse relation could be established between the two parameters for the drugs listed in the lower half of Table 1. However, this relation could not be clearly established for diclofenac, flufenamic and niflumic acids, which are listed in the upper half of Table 1. The absence of a significant relationship could be linked to the formation of oxidised by-products of drugs, which absorb around 400 nm as reported in Section 3. This could lead to an artefactual increase of the absorbance at 412 nm, which may explain why the inhibition of chlorinating activity is not related to accumulation of compound II. The formation of these strongly absorbing by-products has already been observed with diclofenac by Zuurbier et al. (1990) who reported the formation of an oxidised orange product identified as dihydroxyazobenzene. More recently, Miyamoto et al. (1997) identified other oxidised metabolites of diclofenac which also absorb in the 400–450 nm area. Orange oxidised species were actually observed by us under some experimental conditions and it could not be excluded that they act as HOCl scavengers or react with compound I. On the other hand, the structure of the oxidised metabolites of flufenamic and niflumic acids are unknown and we were not able to find any confirmation of their formation in the literature.

It is generally assumed that accumulation of compound II leads to some inhibition of chlorinating activity, as it is thought to be inactive in the chlorination pathway. Nevertheless, this assumption has been criticised by some authors who suggested that compound II could participate indirectly in the chlorination pathway in vivo through reactions 5–7 (Fig. 1) (Kettle and Winterbourn, 1988, 1990). In this pathway, O_2^- anions produced by the same phagocytic cells as those that produce HOCl act as antagonists of the inhibitory activity of NSAIDs by reducing compound II back to the native enzyme. These authors suggested that drugs, which inhibit myeloperoxidase by converting it to compound II are unlikely to be effective against HOCl-mediated tissue damage (Kettle et al., 1993). We do not completely agree with these conclusions for several reasons. Firstly, there is presently no direct evidence that reactions 5–7 operate in vivo in the neutrophils even if they occur under some in vitro conditions. Secondly, most of the experimental observations reported by Kettle and Winterbourn were derived from the monochlorodimedon assay, which is questionable due to the ability of myeloperoxidase to react directly with monochlorodimedon (Kettle and Winterbourn, 1988, 1990; Kooter et al., 1999). Thirdly, we have recently demonstrated that NSAIDs generally inhibited the luminol amplified chemiluminescence of human neutrophils stimulated with opsonised yeast, which is mainly attributed to the reactions of the myeloperoxidase/halide system (Parij et al., 1998). These results therefore suggest that, if these reactions take place in vivo, they only have a limited impact on the inhibitory effects of NSAIDs on the myeloperoxidase system, as the global effect is inhibition of the luminol-amplified chemiluminescent response.

The present study, which is the first of this type to use a recombinant enzyme instead of the purified one, demonstrated that recombinant myeloperoxidase is very convenient for pharmacological purposes. During development of the assay, we found that the enzyme activity and the protein content of myeloperoxidase preparations have a direct influence on IC_{50} values (Parij et al., unpublished observations). It is therefore important to perform all experiments with myeloperoxidase solutions normalised in terms of specific activity or to use the same batch, in order to be able to compare the results for all the drugs tested. The commercial myeloperoxidase preparations contain an approximate number of units per mg of protein; this constitutes a critical point, which renders difficult any

comparison between results of different studies. For pharmacological purposes, each batch of myeloperoxidase should therefore be assessed for its specific activity and future papers should mention this as a part of the experimental results.

In conclusion, the NSAIDs tested here were generally good inhibitors of myeloperoxidase chlorinating activity. During the respiratory burst of human neutrophils, large amounts of reactive oxygen species are produced, including HOCl through the specific action of myeloperoxidase, an important neutrophilic enzyme. The present study clearly established that the conversion of myeloperoxidase compound I into compound II is definitely the way in which NSAIDs inhibit the chlorinating activity of the enzyme. Myeloperoxidase inhibition is probably the main mechanism by which they inhibit the respiratory burst of neutrophils. Even if the presence of relatively large quantities of O_2^- can reduce the efficiency of this inhibition in vivo, the myeloperoxidase system remains a very interesting target for antirheumatic therapy. In this context, it could be of interest both to study the contribution of myeloperoxidase to the development of oxidative damage by using developed selective inhibitors of the enzyme, such as salicylhydroxamic acid and to design new inhibitors as potential drugs. Human recombinant myeloperoxidase represents a powerful new tool to study the inhibitory mechanism and could be used in combination with site-directed mutagenesis to design structure–activity relationship studies.

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