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# In Vitro Study of the Antioxidant Properties of Non Steroidal Anti-Inflammatory Drugs by Chemiluminescence and Electron Spin Resonance (ESR)

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*Objectives.* To determine the antioxidant activities of nonsteroidal anti-inflammatory drugs (NSAIDS), we examined by chemiluminescence (CL) and electron spin resonance (ESR) their scavenging properties towards lipid peroxides, hypochlorous acid and peroxynitrite.

*Methods.* The antioxidant properties of nimesulide (NIM), 4-hydroxynimesulide (4-HONIM), aceclofenac (ACLO), 4-hydroxyaceclofenac (4-HOA-CLO), diclofenac (DICLO) and indomethacin (INDO) were tested on four different reactive oxygen species (ROS) generating systems: (I) phorbol-myristate acetate (PMA)-activated neutrophils, (II) $Fe^{2+}$/ascorbate-induced lipid peroxidation, (III) HOCl-induced light emission, (IV) the kinetics of $ONOO^-$ decomposition followed by spectrophotometry. ROS production was monitored by luminol-enhanced CL or by ESR using two different spin traps.

*Results.* At 10 μM, ACLO, NIM, 4-HONIM, 4-HOA-CLO, and DICLO decreased luminol-enhanced CL generated by PMA-activated neutrophils. Inversely, INDO increased the luminol enhanced CL. Interestingly, hydroxylated metabolites were more potent antioxidants than the parent drugs. Furthermore, all drugs tested, excepted ACLO, lowered lipid peroxidation induced by $Fe^{2+}$/ascorbate system. ACLO and DICLO, even at the highest concentration tested (100 μM), did not significantly lower HOCl induced CL, whereas the other drugs were potent scavengers. Finally, all the NSAIDS accelerated decomposition of $ONOO^-$, suggesting a potential capacity of the molecules to scavenge peroxynitrite.

*Conclusion.* The NSAIDs possess variable degrees of antioxidant activities, linked to their ability to react with HOCl, lipid peroxides or $ONOO^-$. These antioxidant activities could offer interesting targeted side-effects in the treatment of joint inflammatory diseases.

*Keywords:* Antioxidants; NSAIDs; neutrophils; reactive oxygen species; lipoperoxidation; peroxynitrite

## INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used in the treatment of arthritis. They act by inhibiting prostaglandins, plasminogen activator, leukotrienes and pro-inflammatory cytokines (IL-1, IL-6, IL-8) synthesis by

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infiltrated and resident cells[1–6]. Furthermore, some NSAIDs have been demonstrated to be potent scavengers of reactive oxygen species (ROS) and inhibitors of the intracellular oxidase activity[7–16]. Since we know that ROS produced in excess at the inflammatory site play key roles in the progression of the rheumatoid inflammation, they constitute potential targets for drugs used in rheumatoid arthritis (RA) treatment. Some studies have evidenced the therapeutic effects of antioxidant agents in the treatment of RA. Therapeutic remission of RA was obtained following intraarticular administration of the copper-zinc-containing enzyme superoxide dismutase (SOD)[17]. A significant alleviation of articular pain and morning stiffness was obtained following selenium and vitamin E supplementation in a double-blind study on RA patients[18].

More recently, researchers attention was focused on peroxynitrite anion, a newly identified strong oxidant resulting from the reaction of nitric oxide with superoxide anion[19]. It was demonstrated to be formed in rheumatoid joint where it can oxidize essential biomolecules implicated in the homeostasis of cells and tissues[20,21]. Peroxynitrite can also inactivate $\alpha_1$-antiproteinase, degrade glycosaminoglycans and induce nitration of tyrosine[22,23,24]. At this time, the reactivity of NSAIDs with $ONOO^-$ has not yet been investigated.

For instance, few informations are available on the antioxidant properties of NSAIDs. Nimesulide (NIM), indomethacin (INDO) and diclofenac (DICLO) were reported to exhibit an antioxidant profile on the oxidation of liposomes taken as membrane model[7]. In addition, Capecchi et al showed that NIM was able to reduce free cytosolic calcium intake induced by N-formyl-methionyl-leucyl-phenylalanine and ionophore ionomycin[8]. It also exhibited similar inhibiting effects on the lysis of red blood cells photosensitized by tiaprofenic acid[9]. Moreover, NIM reduced the generation of $O_2^{\bullet -}$ by stimulated polymorphonuclear leukocytes through the inhibition of protein kinase translocation and phosphodiesterase type IV activity, the principal enzyme responsible for the degradation of leukocyte cAMP[10]. NIM is also a potent scavenger of hypochlorous acid, which is responsible for a large part of tissue injury and inflammation[11]. Using an in vitro model of erythrocyte membrane lipoperoxidation induced by cumene hydroperoxide (CuOOH), 4-HONIM, the main metabolite of NIM, was demonstrated to protect erythrocyte membrane by directly quenching peroxyl and alkoxyl radical species[12]. On the other hand, 4-HONIM was identified as a chelating and antiradical agent on the model of peroxidation of phosphatidylcholin-liposomes induced by $Fe^{2+}$ via the Fenton reaction[13]. Iron excesses are found in vivo in the synovial fluid of rheumatoid arthritis patients and are capable of exacerbating the arthritic inflammation, probably throught their pro-oxidant potentials[14,15,16]. Therefore, iron chelation could be a limiting factor of the inflammation reaction. Nevertheless, the link between antioxidant profile of NSAIDs and a potential chelating effect is not yet clearly established.

The work presented herein was designed to investigate the mechanisms of action of some NSAIDs and their metabolites towards diverse oxidant species that are susceptible to be produced at the level of joint inflammation. The effects of NSAIDs were tested in 4 types of experiments: (i) production of ROS by phorbol-myristate acetate-activated neutrophils, monitored by chemiluminescence (CL) and electron spin resonance (ESR) techniques, (ii) $Fe^{2+}$/ascorbate-induced lipid peroxidation of linoleic acid monitored by ESR, (iii) HOCl-induced light emission monitored by CL, (iiii) the kinetics of $ONOO^-$decomposition followed by spectrophotometry. NIM and its metabolite 4-HONIM were compared to INDO, DICLO, aceclofenac (ACLO) and its metabolite 4-hydroxyaceclofenac (4-HOACLO).

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## MATERIAL AND METHODS

### Reagents

All experiments were carried out in phosphate buffer (0.066 M $Na_2HPO_4$; 0.066 M $KH_2PO_4$; pH 7.4) or in Hanks' Balanced Salt Solution (HBSS, pH 7.4). Ethylenediamine tetraacetic acid (EDTA), $FeSO_4.7H_2O$, hydrogen peroxide ($H_2O_2$), sodium nitrite ($NaNO_2$), sodium chloride (NaCl), sodium hydrogenophosphate ($Na_2HPO_4$), sodium hydrogenocarbonate ($NaHCO_3$) and potassium dihydrogenophosphate ($KH_2PO_4$), ammonium chloride ($NH_4Cl$) and manganese dioxide ($MnO_2$) were analytical grade and purchased from Merck (Bornem, Belgium). 5,5-Dimethyl-1-pyrroline-N-Oxide (DMPO), α-(4-pyridyl-N-oxide)-tert-butylnitrone (4-POBN), ascorbic acid (vitamin C) and 3-[(Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and INDO were from Aldrich (Bornem, Belgium). DMPO was purified with activated charcoal as previously described[25]. NIM and 4-HONIM were gifts from Helsinn Healthcare (Lugano, Switzerland). Sodium hypochlorite (NaOCl) was obtained from BDH Laboratory (Poole, England). Linoleic acid, phorbol 12-myristate 13-acetate (PMA), superoxide dismutase (SOD), mannitol, Ficoll solution and luminol were purchased from Sigma (Bornem, Belgium).

### Neutrophil isolation and activation

Buffy coats were obtained from healthy volunteers (Transfusion Center-CHU, Liège) and diluted (2:1) with Ficoll solution[26]. After 20 min of centrifugation at 450 g (20°C), the supernatant was diluted (2:1) with 0.9% NaCl and further centrifuged for 20 min at 1250 g (20°C). The pellet was treated with a hypotonic solution ($NH_4Cl$ 0.15 mM; EDTA 0.1 mM; $NaHCO_3$ 10 mM) to eliminate the remaining erythrocytes. Neutrophils were then suspended in HBSS, counted and kept at room temperature. NIM and 4-HONIM were dissolved in alkaline solution (0.1 M NaOH), further diluted with phosphate buffer at pH 7.4, and tested at concentrations ranging from 10 to 200 μM. Drugs were preincubated with neutrophils for 10 min at 37°C before cell activation. The ROS and free radical production by activated neutrophils was monitored by CL and ESR techniques, respectively.

### Chemiluminescence assay

CL measurements were carried out with a Bio-Orbit 1251 luminometer (Turku-Finland) at 25°C. The reactions were performed in circular polystyrene tubes with neutrophils or a defined amount of NaOCl ($10^{-5}$ M), in the presence of $10^{-3}$ M luminol. The neutrophils ($10^6$ cells/ml) were activated by $10^{-6}$ M PMA and the light emission was monitored for 20 minutes. The CL induced by NaOCl was recorded during 60 seconds. The assays were performed in phosphate buffer (pH 7.4), in the absence or in the presence of the drugs, which were used at concentrations ranging from 10 to 100 μM.

### Peroxynitrite synthesis

$ONOO^-$ in alkaline solution was prepared from $NaNO_2$ and $H_2O_2$ as previously described[27]. To eliminate the excess of $H_2O_2$, $ONOO^-$ was treated with $MnO_2$, and $ONOO^-$ concentration was determined spectrophotometrically at 302 nm ($\varepsilon = 1700\ M^{-1}\ cm^{-1}$)[28]. Stock solutions were stored at –80°C.

### Peroxynitrite decomposition analysis

Peroxynitrite is stable in alkaline solution (pH ≥ 12) but decomposes when the pH decreases. In our experiments, the rate of $ONOO^-$ decomposition was monitored by recording the peak absorbance of $ONOO^-$ at 302 nm. In these conditions, the concentration of $ONOO^-$ decreased

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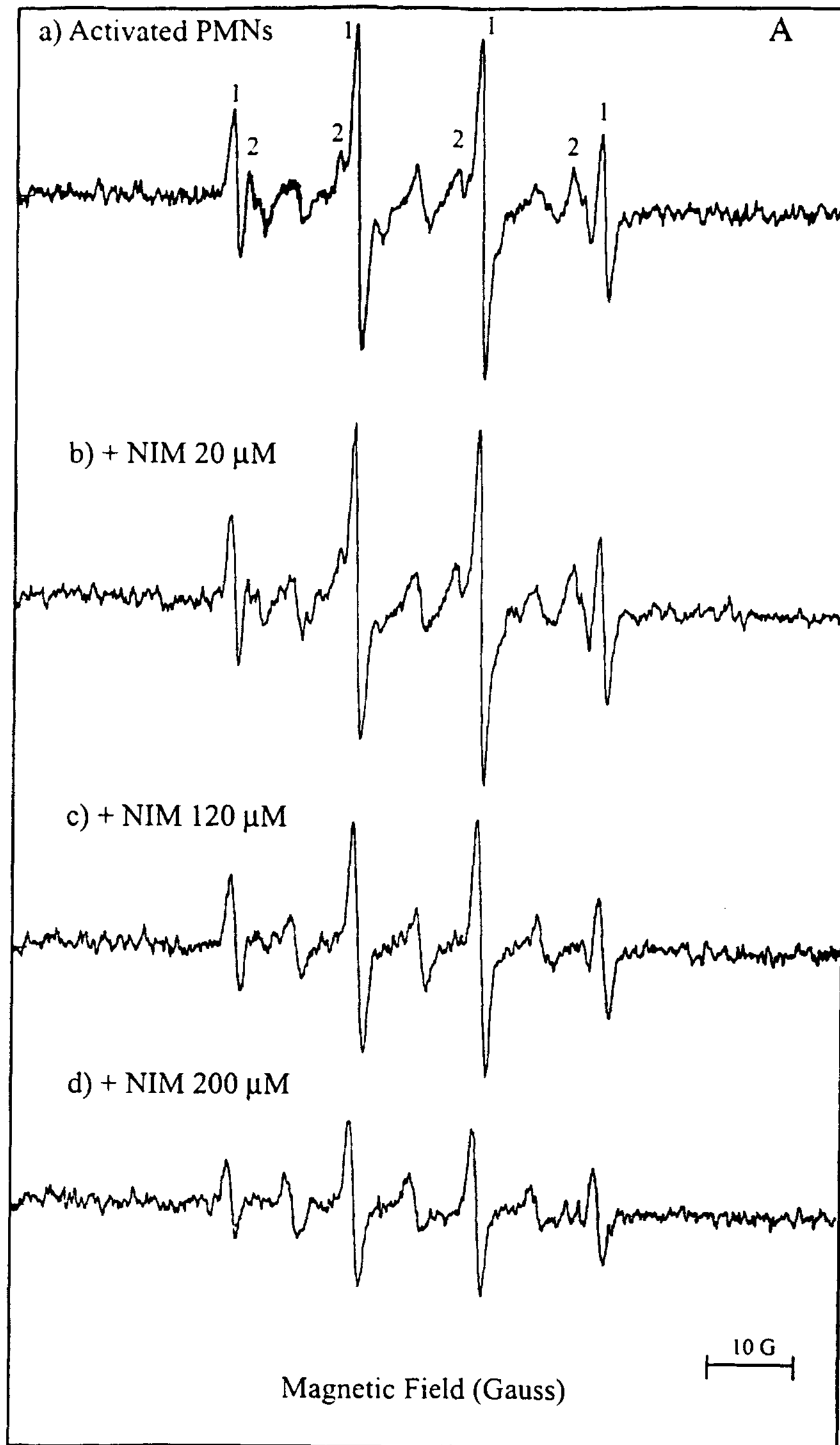


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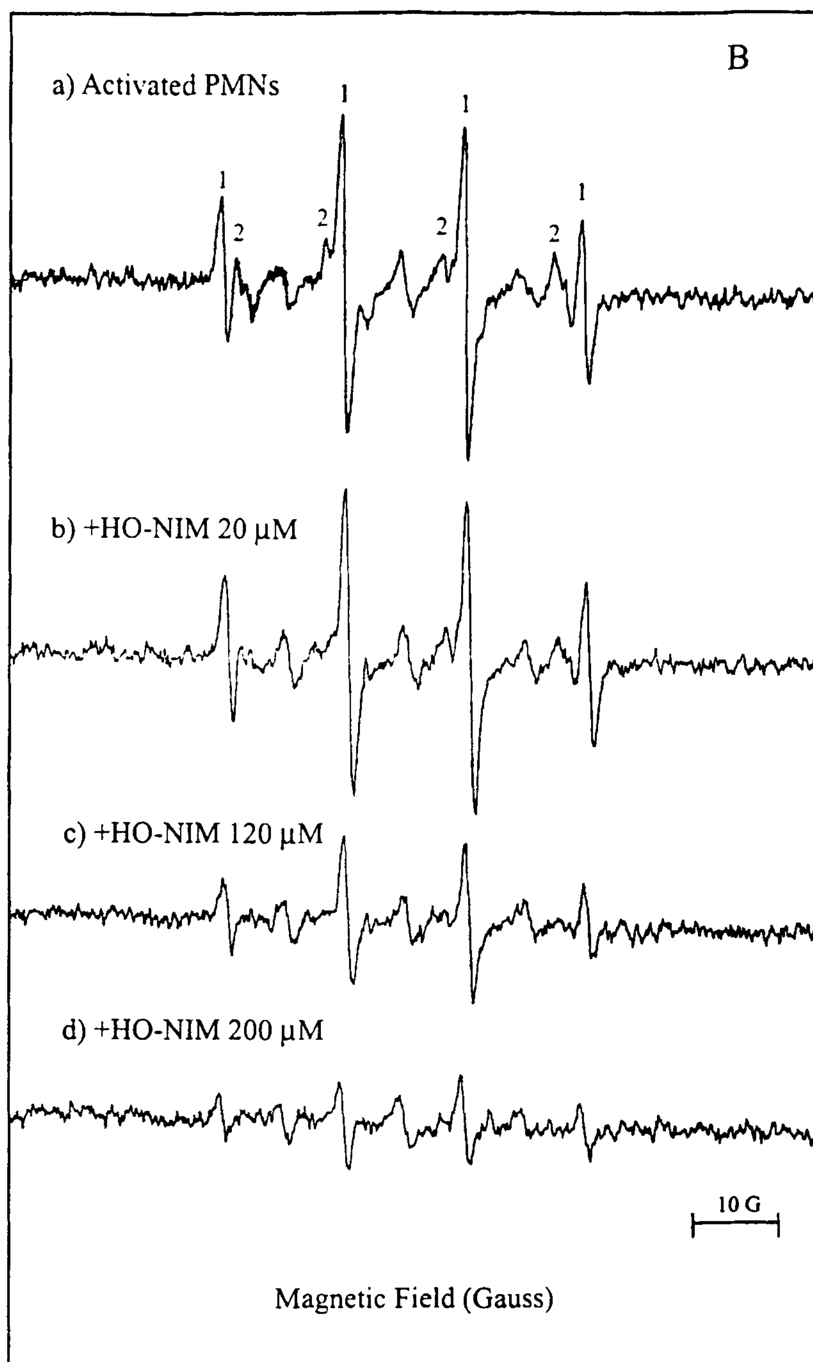


FIGURE 1 ESR spectra resulting from PMA-activated human neutrophils (PMNs) incubated in the presence of DMPO (100 mM), with or without increasing amounts of NIM (A) or 4-HONIM (B). The experimental settings were: receiver gain $2.10^4$, time constant 81.92 ms, conversion time 164 ms. The number of scans was 4. 1. DMPO-OH adduct ($a_N = a_H = 14.89$ G); 2. DMPO-OOH adduct ($a_N = 14.3$ and $a_H = 1.3$ G)

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exponentially with time. The kinetics of the reactions of $ONOO^-$ were recorded at 25°C using a Kontron UV-visible spectrophotometer (UVIKON 941) equipped with a thermostated (circulating water) cuvette holder (1 cm) (Hellma, Aartselaar-Belgium). Absorbance was monitored at the rate of 50 measures/min. The reaction started by the addition of the buffered NSAID solution to the alkaline solution of 0.28 mM $ONOO^-$. The final pH of the reaction mixture (1 ml) was 8.0. For these experiments, concentrated ($10^{-2}$ M) drug solutions were prepared in methanol, and diluted with phosphate buffer to the final concentration of $10^{-5}$ M. Reported results were the average of at least 4 separate assays.

### ESR experiments

ESR experiments were performed using two different models: isolated human neutrophils and linoleic acid peroxidation. The neutrophils ($4.10^6$ cells/ml) were activated by $5.10^{-7}$ M PMA, with or without addition of the drug. The production of free radicals was monitored in the presence of the spin trap agent DMPO (100 mM). The linoleic acid emulsion was prepared by mixing 200 mg of CHAPS and 0.2 ml of linoleic acid in 100 ml of chelexed phosphate buffer at pH 7.4 (final concentration of linoleic acid: 6.4 mM). The lipoperoxidation started by adding $10^{-5}$ M $Fe^{2+}$ to the emulsion in the presence of $10^{-3}$ M ascorbate and 50 mM spin trap 4-POBN (final volume 1 ml). The reaction mixture was immediately transfered into the flat cell in the $TM_{110}$cavity of the spectrometer. ESR spectra were monitored with a Bruker spectrometer, operating at non-saturating microwave power (20 mW). The experimental settings were as follows: 9.75 GHz microwave frequency, 3480 G centre of field, 100 G sweep width. The other parameters are listed in the legends of the figures.

### Statistical analysis

The results were expressed as the mean values with the standard deviation. Statistical analysis was performed by the unpaired Student's *t* test with a limit of significance at $p < 0.05$. Dose-reponse curves were fitted by regression models (Pearson's test) using drug concentrations in logarithmic scale.

## RESULTS

### Effects of NSAIDs on ROS and free radical production by PMA-activated neutrophils

Figure 1 shows the high intensity ESR spectrum resulting from the activation of neutrophils by PMA. This spectrum was characteristic for DMPO-OOH radical adducts ($a_N = 14.3$ G, $a_H = 1.3$ G) and/or their decomposition adducts DMPO-OH ($a_H = a_N = 14.89$ G) with a major part of the spectrum (75%) formed by the DMPO-OH adducts. The intensity of the ESR spectrum was strongly reduced in the presence of 30 U/ml SOD (90 % inhibition) and to a lesser degree by·OH scavengers (30 % inhibition with $10^{-4}$ M mannitol). The addition of NIM (Figure 1A) or its metabolite 4-HONIM (Figure 1B), at the concentrations ranging from 10 to 200 µM, resulted in a dose-dependent inhibition of the ESR signal intensity. At 200µM, NIM and 4-HONIM decreased the signal height by 52 and 73% respectively. By comparison, ACLO exhibited a weak effect (7% inhibition) and 4-HOACLO reduced by 35% the signal intensity (Table I).

The chemiluminescence results are presented on figure 2 : the control value (without drug addition) is the mean of 3 assays performed with 3 different cell batches, with the individual absolute values obtained for each cell batch ranging from 2100 to 1950 mV. This mean value was taken as 100 % and the effects of drugs were expressed in % of this control value. NIM and 4-HONIM exhibited a significant dose-dependent inhibiting effect on the CL. The inhibiting effect was more important compared to ESR studies. At 10 µM, corresponding to the plas-

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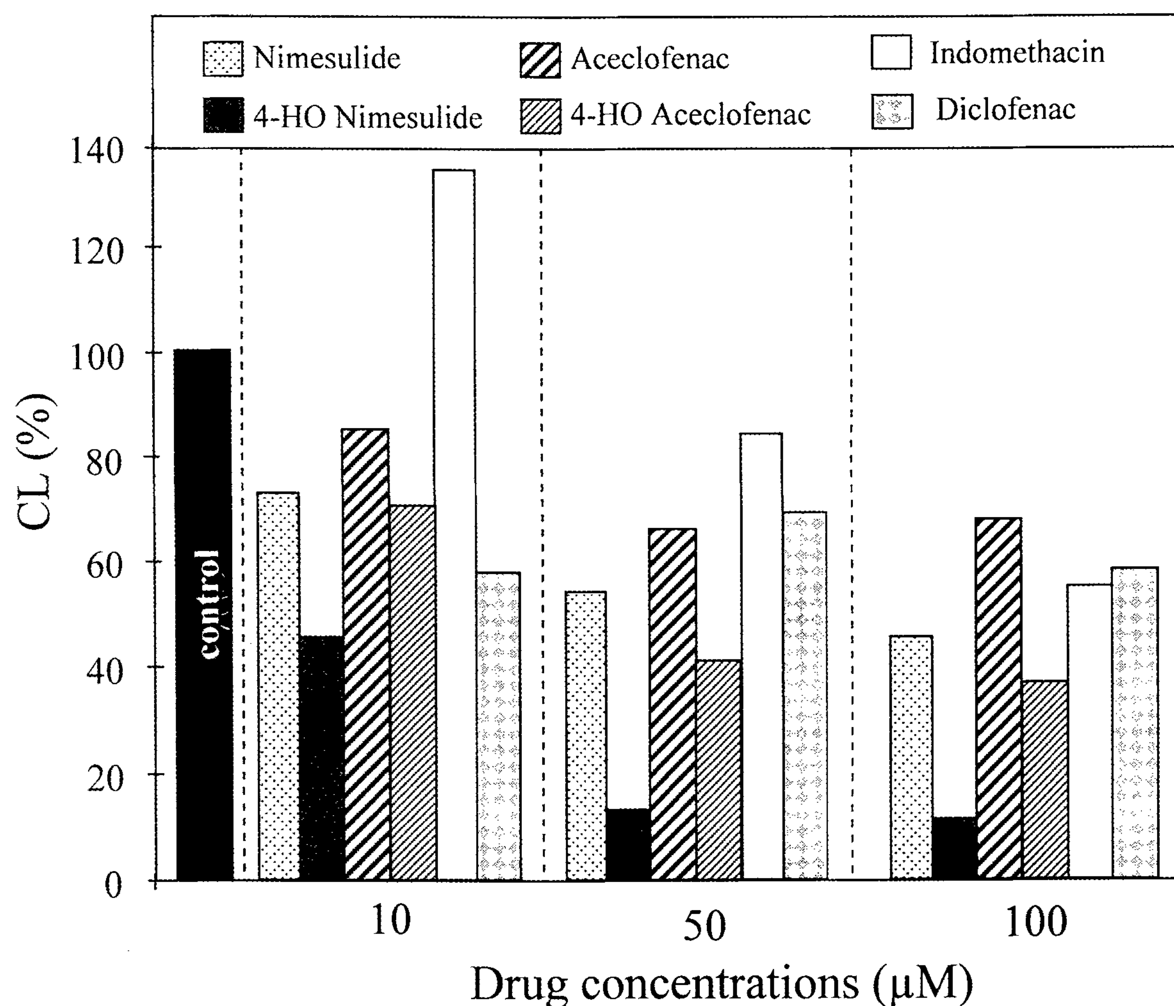


FIGURE 2 Effects of increasing concentrations of NSAIDs on luminol-enhanced chemiluminescence generated by PMA-activated neutrophils ($10^6$ cells/ml). Results are expressed as the percentage of the control value (100%). The values are the means of at least 3 independent experiments conducted with neutrophils coming from 3 different healthy donors

matic NIM peak value after therapeutic dose administration (29), NIM inhibited by 25 ± 5 % and 4-HONIM by 55 ± 9 % the CL intensity. By comparison, ACLO or 4-HOACLO (10 µM) reduced the CL emission by 16 ± 4 % and 30 ± 7 %, respectively and DICLO by 40 ± 9 %. Surprisingly, INDO showed a biphasic effect on this parameter. At low concentration (10 µM), INDO increased by +30% the CL resulting from activated PMN but induced a progressive inhibition at high concentration (100 µM).

Furthermore, this experiment clearly demonstrated that the hydroxylated metabolites were more efficient antioxidants than the parent drugs. This finding suggests that the phenolic structure resulting from the drug metabolization played a key role in the antioxidant properties of the NSAIDs.

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TABLE I Comparative effects of 200 μM of NSAIDs on the ROS production by PMA-activated neutrophils ($4.10^6$ cells/mL). The production of free radicals was monitored by ESR experiment using DMPO as spin trap agent. The results are expressed as the % of inhibition of the ESR signal height. The experimental settings were: receiver gain $2.10^4$, time constant 81.92 ms, conversion time 164 ms. The number of scans was 6

| *Compound* | *Inhibition of the ESR signal (%)* |
|---|---|
| None | 0 |
| Nimesulide | 52 |
| 4-HO-nimesulide | 73 |
| Aceclofenac | 7 |
| 4-HO-aceclofenac | 35 |

TABLE II Comparative effects of 10 μM NSAIDs on the inhibition of ESR signal resulting from linoleic acid peroxidation induced by $Fe^{2+}$/ascorbate system. In this model, 4-POBN was used as spin trap agent. The experimental settings were: receiver gain $2.10^4$, time constant 40.96 ms, conversion time 40.96 ms. The number of scans was 4

| *Compound* | *Inhibition of the ESR signal (%)* |
|---|---|
| None | 0 |
| Nimesulide | 50 |
| 4-HO-nimesulide | 50 |
| Aceclofenac | 0 |
| 4-HO-aceclofenac | 45 |
| Indomethacin | 34 |
| Diclofenac | 38 |

### Effects of NSAIDs on lipid peroxidation induced by iron (II) and ascorbate

In the absence of $Fe^{2+}$, no signal of lipoperoxidation was observed (Figure 3, spectrum A). The addition of 10 μM $Fe^{2+}$ induced linoleic acid peroxidation resulting in the appearance of a six-line ESR spectrum. This signal is characteristic of the 4-POBN radical adducts with the following coupling constants: $a_N = 16.0$ G and $a_H = 2.6$ G (Figure 3, spectrum B). Moreover, a two-line ESR spectrum centered at 3480 G ($a_H = 1.7$ G), characteristic for the ascorbyl free radical (AFR), was also observed. The addition of NIM or 4-HONIM, at the final concentration of 10 μM, decreased by 50 % the ESR spectrum intensity (Figure 3, spectra C and D). INDO and DICLO also exhibited a good inhibiting effect on the ESR signal intensity, but they were less active than NIM or 4-HONIM (table II). 4-HOACLO was also a potent scavenger of the lipid peroxides (45% inhibition of the signal height) whereas ACLO was without effect.

### Effects of NSAIDs on the NaOCl-induced CL

In a range of concentrations from 10 to 100 μM, NIM and 4-HONIM dose-dependently inhibited the luminol-enhanced HOCl generated CL ($r=0.97$ for NIM and 0.88 for 4-HONIM; $p<0.001$) (Figure 4). At 10 μM, NIM inhibited by 15% the CL whereas 4-HONIM reduced it by 60%. At all concentrations, 4-HONIM was significantly more efficient than the parent molecule ($p<0.0001$). ACLO and DICLO, even at the highest concentration tested (100 μM), did not significantly reduced the HOCl-induced CL, while INDO was slightly but significantly active at the doses of 50 and 100 μM. 4-HOACLO was significantly active in a dose-dependent manner ($r=0.97$; $p<0.001$), and was more efficient at each dose than its parent drug.

### Effects of NSAIDs on the kinetic curve of peroxynitrite decomposition

All the NSAIDs tested were capable to accelerate the decomposition of $ONOO^-$ showing their capacity to scavenge peroxynitrite. The apparent first-order kinetic constant was $3.57\ 10^{-2}\ s^{-1}$ for NIM at the final concentration of $10^{-5}$ M. The other drugs had a weaker effect on the rate of $ONOO^-$ decomposition, and exhibited similar rate constants: $2.28\ 10^{-2}\ s^{-1}$ for 4-HONIM, $2.24\ 10^{-2} s^{-1}$ for ACLO, $2.21\ 10^{-2}\ s^{-1}$ for 4-HOACLO and $2.48\ 10^{-2}\ s^{-1}$ for INDO. Furthermore, metab-

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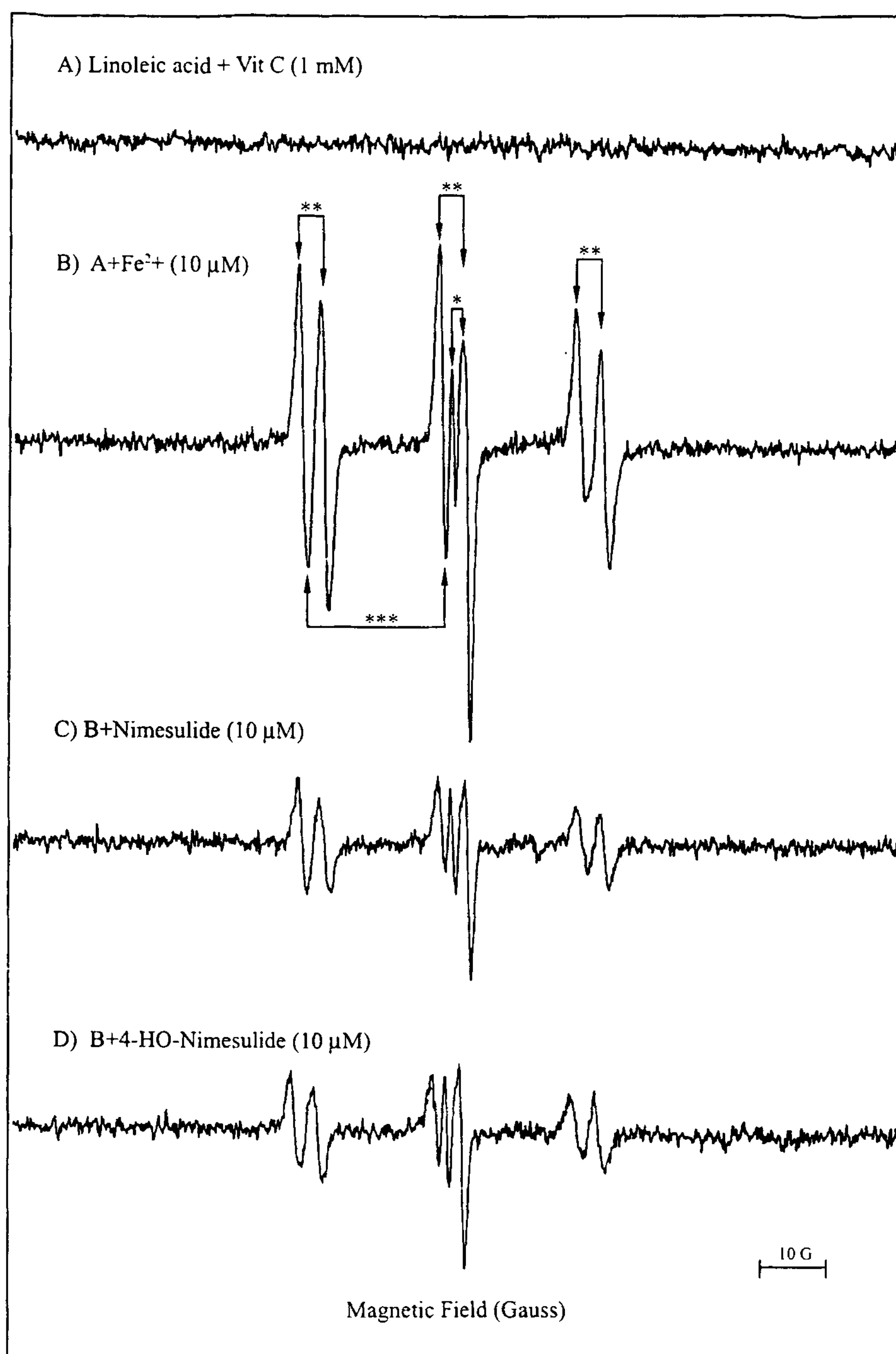


FIGURE 3 ESR spectra resulting from the peroxidation of linoleic acid by the $Fe^{2+}$ / ascorbate system. 4-POBN was used as spin trap. A) control: in the absence of $Fe^{2+}$; B) in the presence of $Fe^{2+}$ ($10^{-5}$M); C) same as B with $10^{-5}$ M NIM; D) same as B with $10^{-5}$ M 4-HONIM. * ESR spectrum of ascorbyl free radical (coupling constant aH = 1.7 G). ** and *** ESR spectrum of 4-POBN spin adduct resulting from the reaction of 4-POBN with ROO· radicals (** $a_N$ = 16.0 G and *** $a_H$ = 2.6 G). The experimental settings were: receiver gain $2.10^4$, time constant 40.96 ms, conversion time 40.96 ms. The number of scans was 4

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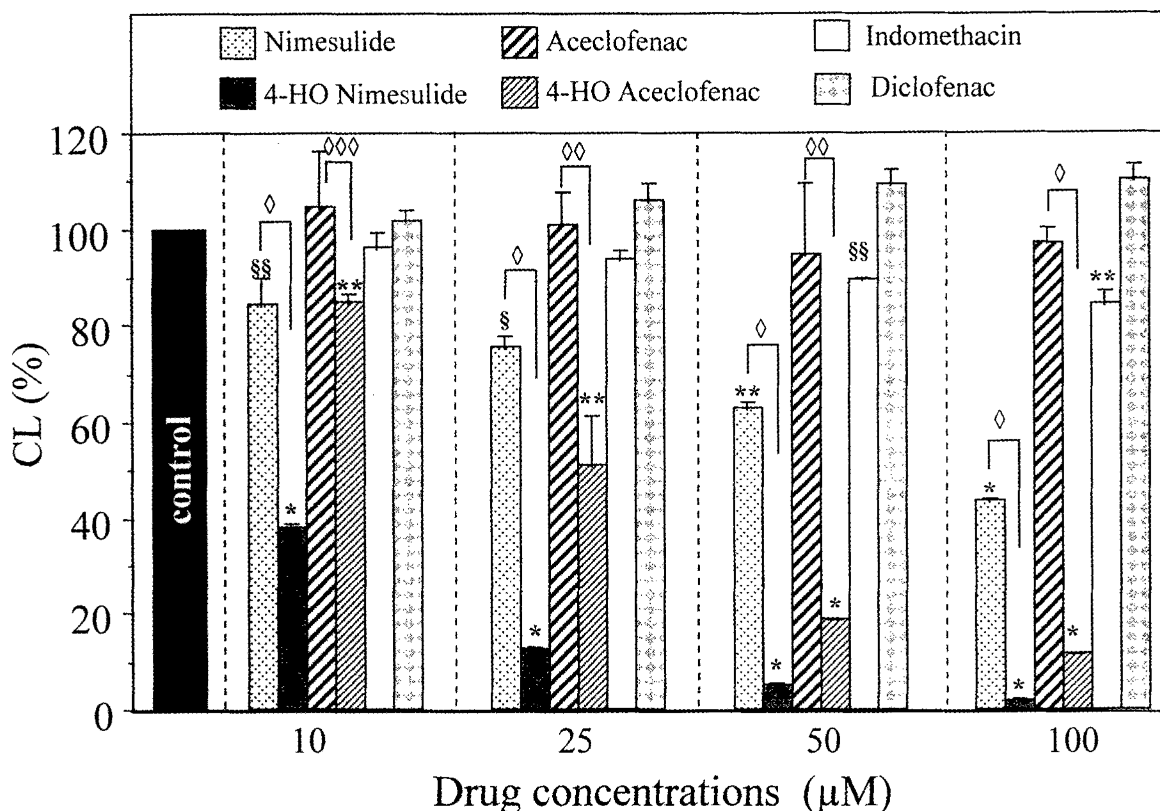


FIGURE 4 Effects of increasing amounts of NSAIDs on luminol-enhanced chemiluminescence (CL, expressed in % of control) produced by $10^{-4}$ M HOCl, in phosphate buffer at pH 7.4. Results are expressed as the percentage of the control values (n= 5). Drug-treated groups are significantly different from the control: * $p<0.0001$; ** $p<0.001$; § $p<0.01$; §§ $p<0.05$. The hydroxylated metabolite effects are significantly different from the parent drug effects: ◊ $p<0.0001$; ◊◊ $p<0.005$; ◊◊◊ $p<0.05$

olites and parent drugs showed similar activity to scavenge $ONOO^-$.

## DISCUSSION

Neutrophils, recruited to tissue sites of inflammation, release a variety of oxidants and enzymes, which are responsible for tissue damage. Among the oxidants released are potent chlorinated compounds (such as hypochlorous acid and chloramines), nitric oxide-derived inflammatory oxidants, including peroxynitrite, nitryl chloride and nitrogen dioxide but also hydroxyl radicals, superoxide anions and hydrogen peroxide which directly induce tissue cell damage but also inactivate $\alpha_1$-antiproteinase, the major inhibitor of elastase[30–34]. We demonstrated herein that many NSAIDs possess antioxidant activities and therefore, could limit the oxidative stress generated by activated neutrophils[35,36]. These findings are in agreement with previous papers showing that NSAIDs were capable to inhibit the respiratory burst of neutrophils triggered by various activator agents[8,10,11,23]. The finding that NSAIDs are able to blunt ROS production from activated neutrophils, leads to suggest that the inhibition of oxidative burst may represent an additional mechanism of the antiinflammatory activity of

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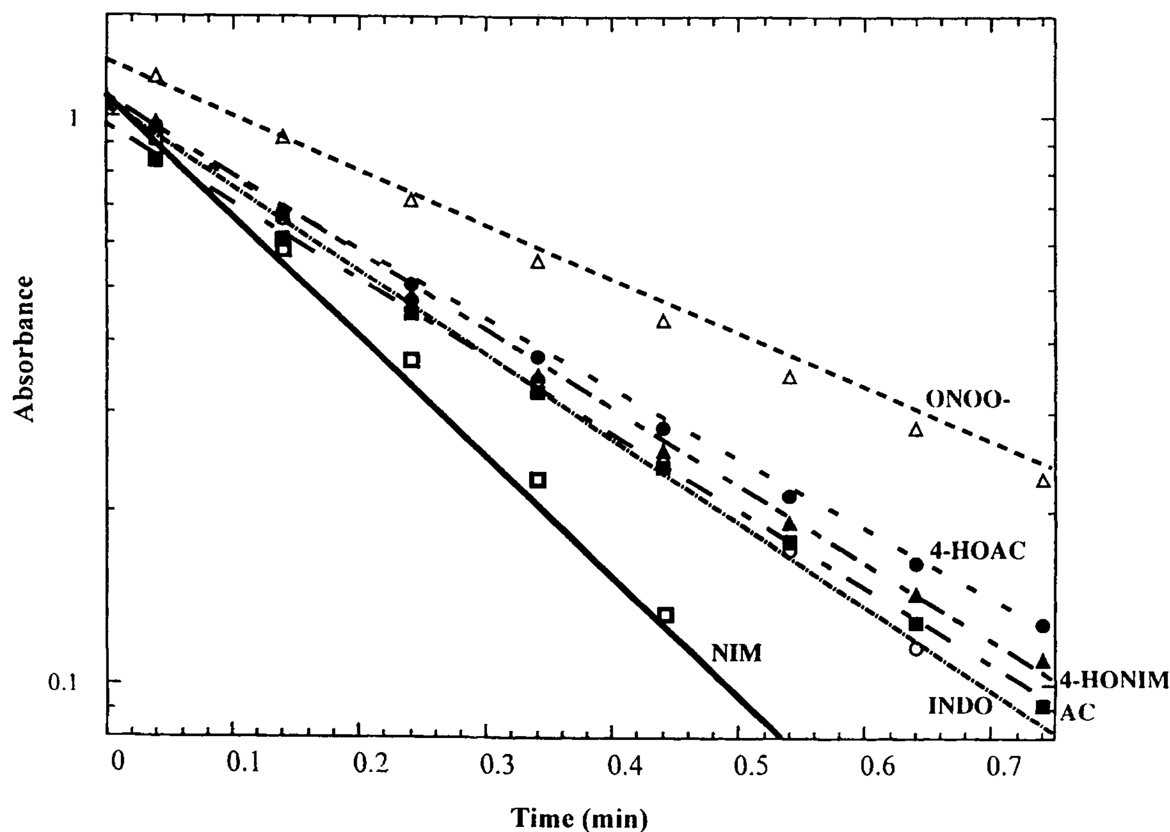


FIGURE 5 Rate of decomposition of 0.28 mM peroxynitrite in 50 mM phosphate buffer (pH 8.0), in the absence ($ONOO^-$ alone) or in the presence of the drug at 10 µM (n = 4). Absorbance decrease was followed at 302 nm

these drugs. This mechanism of action is particularly well documented for NIM, which has been shown to decrease FMLP and PMA-stimulated $O_2^{\bullet -}$ production in polymorphonuclear leukocytes[8,10]. The mechanism whereby NIM decreases $O_2^{\bullet -}$ generation is not yet fully elucidated although it seems related to the inhibition of the phosphodiesterase type IV and the subsequent increase in protein kinase A activity. The activation of protein kinase may prompt the phosphorylation of a number of substrates, thus inhibiting the assembly of NADPH-oxidase[10]. This effect could be mediated by the direct interaction of NIM with the adenosine receptor system on the cell membrane[8]. Furthermore, NSAIDs may exert their effects through a direct scavenging action reducing the extracellular avaibility of ROS largely involved in the stimulation, propagation and maintenance of both acute and chronic inflammatory diseases such as rheumatoid arthritis. This property was previously demonstrated for NIM, which reacts with HOCl but also with $^{\bullet}OH$ produced in vitro by the Fenton reaction[12,13,37]. To date, no specific mechanisms were proposed to explain the antioxidant activity of other NSAIDs such as ACLO, DICLO or INDO[38,39]. Furthermore, no informations are yet available concerning the reactivity of these NSAIDs with peroxynitrite, a newly identified and highly reactive ROS. In this work, we show

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that NIM, ACLO, INDO, 4-HONIM and 4-HOACLO, decreased chemiluminescence resulting from the activation of PMN, and that NIM and 4-HONIM were already active at 10 μM, a clinically relevant concentration (29). Neutrophil activation leads to the production of many ROS.[40–42], which may contribute to the enhancement of CL observed when neutrophils are activated. By ESR, we observed that the free radical species produced by the activated neutrophils was mainly the superoxide anion since SOD reduced for more than 90 % the ESR spectrum intensity (data not shown). The DMPO-OH adducts that we observed were thus likely to derive from the decomposition of the DMPO-OOH adducts, but we cannot exclude that a minor part of these adducts derived from a direct scavenging of ·OH (produced by a "Fenton-like" reaction or by the decomposition of peroxynitrite). The inhibiting effects of the drugs, especially NIM and 4-OH NIM, on the activation of the neutrophils were obtained at lower concentrations in the CL assays compared to ESR. But in the ESR studies, the free radical species produced by the activated neutrophils is mainly superoxide anion. Higher concentrations of NIM and its metabolite seem to be needed to inhibit this particular radical. The CL of activated neutrophils results from the total ROS produced by the cells and among these ROS, there are non radical species such as HOCl. As the CL method does not allow the identification of the ROS species targetted by NSAIDs, and to identify which of these ROS were scavenged by the drugs, we used three different in vitro methods: chemiluminescence generated by HOCl, the kinetics of peroxynitrite decomposition and the lipoperoxidation of linoleic acid induced by ascorbate/$Fe^{2+}$, a system which produces ferryl radicals responsible for triggering lipid oxidation.

In the model of CL induced by HOCl, NIM, 4-HONIM and 4-HOACLO showed a dose-dependent inhibiting effect whereas INDO, DICLO and ACLO were without significant effect. This higher reactivity of NIM and 4-OHNIM may be explained by the presence of a sulfonanilide function (figure 6, structure 1) which is more susceptible to react with HOCl[43]. All NSAIDs, excepted ACLO, were active in the lipid peroxidation model indicating that these drugs interact with the production of the ferryl radical. Two hypotheses can be advanced to explain this inhibitory effect. Firstly, NSAIDs act by chelating $Fe^{2+}$. This chelating property has been previously described for 4-HONIM[13]. Indeed, it is highly probable that NIM chelates $Fe^{2+}$, hindering it to participate in the redox cycle of ascorbate which is essential for inducing lipid peroxidation. The second explanation is a direct scavenging effect of NSAIDs towards the lipid radicals (ROO· or RO·) as previously reported for 4-HONIM[13]. In these two models of HOCl-generated CL and linoleic acid peroxidation, 4-OHNIM and 4-HOACLO were stronger scavengers of ROS than the parent molecules. These data suggest that the presence of a phenolic structure in the 4-OH-metabolites highly enhances the antioxidant properties of the drug (Figure 6). Finally, we observed that all the tested drugs accelerated the decomposion of $ONOO^-$, but that NIM was the most active. In this model, the 2 metabolites, 4-HONIM and 4-HOACLO, were less active than the parent compounds. The mechanisms of the decomposition of $ONOO^-$ remain discussed, even if it is now accepted that one of these mechanisms involves the formation of a radical pair, $NO^·_2$ and ·OH[44]. Therefore, an explanation for the increase of $ONOO^-$ decomposition observed in the presence of the NSAIDs could be an easy uptake and reaction with the peroxynitrite-derived radicals leading to nitration or hydroxylation of the cyclic part of the NSAID molecules. In this perspective, the higher efficacy of NIM on the decomposition of $ONOO^-$ could be explained by the presence of a sulfur atom that increases the drugs reactivity toward $ONOO^-$. Curiously, the 4-OHNIM had a weaker effect than NIM. To explain this difference, we

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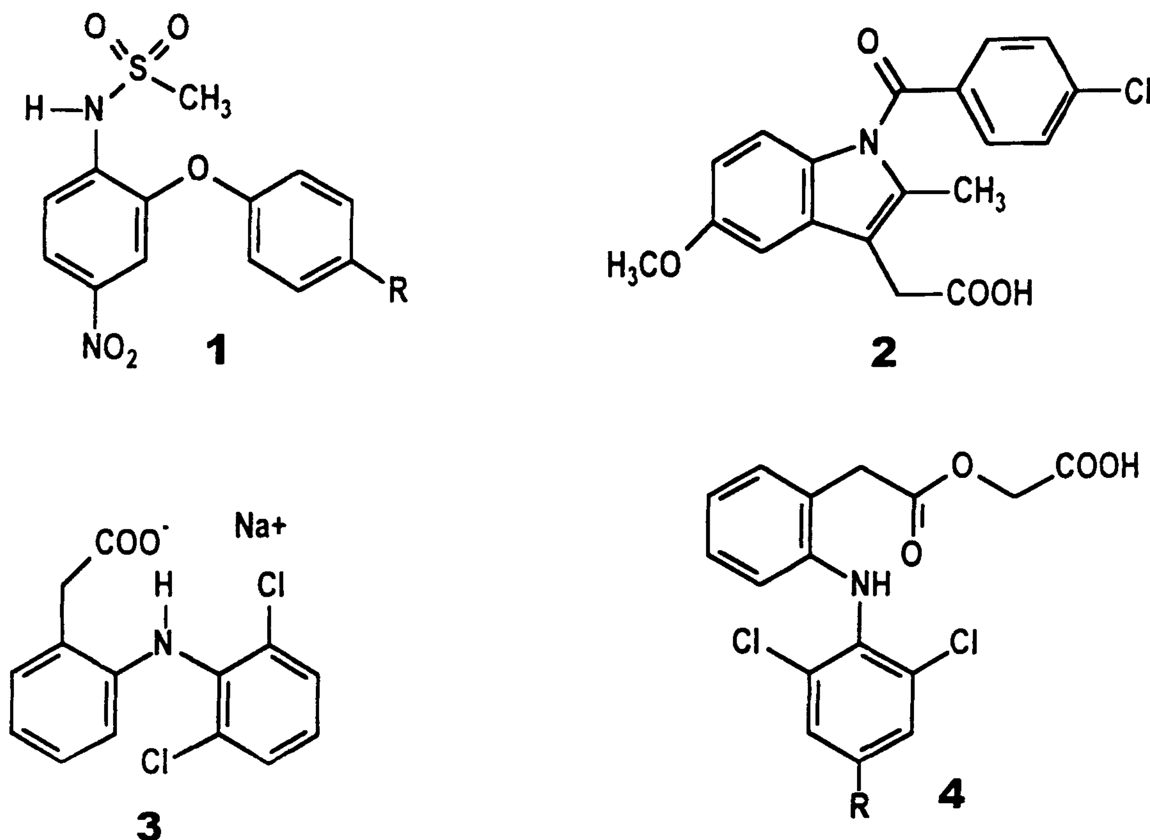


FIGURE 6 Chemical structures of the tested drugs (parent drugs and hydroxymetabolites). 1. with R = H: nimesulide [N-(4-nitro-2-phenoxyphenyl) methanesulfonamide]; with R = OH : 4-hydroxynimesulide. 2. indomethacin [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid] 3. diclofenac [2-[(2,6-dichlorophenyl)amino] benzene acetic acid monosodium salt] 4. with R = H: aceclofenac [2-(2,6-dichlorophenyl)amino acid carboxymethyl ester]; with R = OH: 4'-hydroxy-aceclofenac

suggest the existence of an interaction of the 4-hydroxyl group with $ONOO^-$, forming a hydrogen bond that would temporarily stabilize $ONOO^-$ and slower its decomposition. However, to support this hypothesis, more investigations are needed, particularly to identify the products of the reaction of NSAIDs with $ONOO^-$. But, taking into account the involvement of $ONOO^-$ in diverse pathophysiological disorders and joint inflammation diseases[3,20,45], our observations demonstrate the "anti-peroxynitrite" properties of NSAIDs.

In conclusion, this study demonstrated that many NSAIDs possess antioxidant properties but that their mechanisms of action can be different. For example, NIM is a potent scavenger of $ONOO^-$ and lipid radicals but is ineffective on $O_2^{\bullet -}$. Inversely, ACLO is a potent scavenger of $ONOO^-$ but does not act on lipid radicals and HOCl. We also demonstrated that NSAIDs may scavenge peroxynitrite. Finally, we conclude that hydroxylated metabolites are more potent scavengers of ROS than the parent molecules suggesting that they could be the active molecule *in*

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*situ*. Therefore, modulation of the local metabolization of NSAIDs by joint cells could be an interesting therapeutic approach.

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### *References*

[1] Y. E. Henrotin, A. H. Labasse, P. E. Simonis, S. X. Zheng, G. P. Deby, J-P. Famaey, J-M. R. Crielaard and J-Y. L. Reginster (1999) Effects of nimesulide and sodium diclofenac on interleukin-6, interleukin-8, proteoglycans and prostaglandin E2 production by human articular chondrocytes in vitro. *Clinical and Experimental Rheumatology*, 17, 151–160.

[2] J-P. Pelletier, F. Mineau, J. Fernandes, K. Kiansa, P. Ranger and J. Martel-Pelletier (1997) Two NSAIDs, nimesulide and naproxen, can reduce the synthesis of urokinase and IL-6 while increasing PAI-1, in human OA synovial fibroblasts. *Clinical and Experimental Rheumatology*, 15, 393–398.

[3] R. L. Dreiser and D. C. Benevelli (1993) Long term tolerability profile of nimesulide in the treatment of osteoarthritis. *Drugs*, 46, 270–274.

[4] J-P. Famaey (1996) In vitro and in vivo pharmacological evidence of selective cyclooxygenase-2 inhibition by nimesulide: An overview. *Inflammation Research*, 46, 437–446.

[5] J. A. Mitchell, P. Akarasereenont, C. Thiemermann and R. J. Flower (1994) Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 11693–11697.

[6] W. Wober (1999) Comparative efficacy and safety of nimesulide and diclofenac in patients with acute shoulder, and a meta-analysis of controlled studies with nimesulide. *Rheumatology*, 38, 33–38.

[7] F. R. Maffei, M. Caranini, G. Aldini, L. Saibene and A. Macciocchi (1993) Antioxidant profile of nimesulide, indomethacin and diclofenac in phosphatidylcholine liposomes (PLC) as membrane model. *International Journal of Tissue Reactions* 15, 225–234.

[8] P. L. Cappecchi, L. Ceccatelli, U. Beermann, P. L. Laghi and T. Di Perri (1993) Inhibition of neutrophil function in vitro by nimesulide. Preliminary evidence of an adenosine-mediated mechanism. *Arzneimittel-Forschung*, 43, 992–996.

[9] C.A. Ribeiro, A. Figeuiredo, P. Tavares, A. Poiares-Baptista and F. Teixeria (1992) Tiaprofenic acid-induced photohemolysis in vitro is inhibited by nimesulide. *Drug Metabolism and Drug Interactions*, 10, 293–305.

[10] M. Bevilacqua, T. Vago, G. Baldi, E. Renesto, F. Dallegri and G. Norblato (1994) Nimesulide decreases superoxide production by inhibiting phosphodiesterase type IV. *European Journal of Pharmacology*, 268, 415–423.

[11] F. Dallegri, F. Patrone, A. Ballestrero, L. Ottonello, F. Ferrando and C. Sacchetti (1990) Inactivation of neutrophil-derived hypochlorous acid by nimesulide: a potential mechanism for the tissue protection during inflammation. *International Journal of Tissue Reactions*, 12, 107–111.

[12] R. Maffei-Facino, M. Carini and G. Aldini (1993) Antioxidant activity of nimesulide and its main metabolites. *Drugs*, 46 (Suppl 1), 15–21.

[13] F. C. Maffei, M. Carini, G. Aldini, L. Saibene and M. Morelli (1995) Differential inhibition of superoxide, hydroxyl and peroxyl radicals by nimesulide and its main metabolite 4-hydroxynimesulide. *Arzneimittel-Forschung*, 45, 1102–1109.

[14] C. Guillen, I. B. McInnes, H. Kruger and J. H. Brock (1998) Iron, lactoferrin and iron regulatory protein activity in the synovium; relative importance of iron loading and the inflammatory response. *Annals of the Rheumatic Diseases*, 57, 309–314.

[15] C. Guillen, I. B. McInnes and J. H. Brock (1997) Iron in synovial fluid: removal by lactoferrin, and relationship to iron regulatory protein (IRP) activity. *Biochemical Society Transactions*, 25, 315S.

[16] A. J. Dabbagh, C. W. Trenam, C. J. Morris CJ and D. R. Blake (1993) Iron in joint inflammation. *Annals of the Rheumatic Diseases*, 52, 67–73.

[17] M. Rister, K. Bauermeister, U. Gravert and E. Gladtke (1978) Superoxide-dismutase deficiency in rheumatoid arthritis. *Lancet*, i, 1094.

[18] J. Aaseth, M. Haugen and O. Forre (1998) Rheumatoid arthritis and metal compounds – perspectives on the role of oxygen radical detoxification. *Analyst*, 123, 3–6.

[19] J. S. Beckman, T. W. Beckman, J. Chen, P. A. Marshall and B. A. Freeman (1990) Apparent hydroxyl radical production of peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences of the United States of America*, 87, 1620–1624.

[20] M. Oh, K. Fukuda, S. Asada, Y. Yasuda and S. Tanaka (1998) Concurrent generation of nitric oxide and superoxide inhibits proteoglycan synthesis in bovine articular chondrocytes: involvement of peroxynitrite. *Journal of Rheumatology*, 25, 2169–2174.

[21] D. Jang and G. A. C. Murrell (1998) Nitric oxide in arthritis. *Free Radical Biology and Medicine*, 24, 1511–1519.

[22] M. Whiteman, H. Kaur and B. Halliwell (1996) Protection against peroxynitrite dependent tyrosine nitration and alpha 1-antiproteinase inactivation by some antiinflammatory drugs and by the antibiotic tetracycline. *Annals of Rheumatologic Disesases*, 55: 383–387.

[23] L. Ottonello, P. Dapino, M. C. Scirocco, A. Balbi, M. Bevilacqua and P. Dallegri (1995) Sulphonamides as anti-inflammatory agents: old drugs for new therapeutic strategies in neutrophilic inflammation? *Clinical Science*, 88, 331–336.

[24] J. Schiller, J. Arnhold, J. Schwinn, H. Sprinz, O. Brede and K. Arnold (1998) Reactivity of cartilage and selected carbohydrates with hydroxyl radicals. An NMR study to detect degradation products. *Free Radical Research*, 28, 215–228.

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[25] M. J. Green and H. A. Hill (1984) Oxygen Radicals in Biological Systems. *Methods in Enzymology*, 105, 3–22.

[26] P. Borgeat and B. Samuelsson (1979) Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proceedings of the National Academy of Sciences od the United States of America*, 76, 2148–2152.

[27] R. M. Uppu, G. L. Squadrito, R. Cueto and W. A. Pryor (1996) Selecting the most appropriate synthesis of peroxynitrite. *Methods in Enzymology*, 269, 311–321.

[28] R. Kissner, T. Nauser, P. Bugnon, P.G. Lye and W. H. Koppenol (1997) Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. *Chemical Research in Toxicology*, 10, 1285–1292.

[29] R. Davis and R. N Brogden (1994) Nimesulide. An update of its pharmacodynamic and pharmacokinetic efficacy. *Drugs*, 48, 431–454.

[30] G. R. Areenwald and W. W. Moxx (1979) Inhibition of collagen gelation by action of superoxide radical. *Arthritis and Rheumatism*, 22, 251–259.

[31] J. A. Imlay and S. Linn (1988) DNA damage and oxygen radical toxicity. *Science*, 240, 1302–1304.

[32] H. Burkhardt, M. Schwingel, H. Menninger, H. W. Macartney and H. Tschesche 1986. Oxygen radicals as effectors of cartilage degradation. *Arthritis and Rheumatism*, 29, 379–387.

[33] H. Carp and A Janoff (1980) Potential mediator of inflammation: phagocyte derived oxidants suppress the elastase inhibitory capacity of α1-proteinase inhibitor in vitro. *Journal of Clinical Investigation*, 66, 987–995.

[34] J. P. Eiserich, M. Hristova, C. E. Cross, A. D. Jones, B. A. Freeman, B. Halliwell and A. van der Vliet (1998) Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature*, 391, 393–397.

[35] F. Dallegri, L. Ottenello, P. Dapino and C. Sacchetti (1992) Effect of nonsteroidal antiinflammatory drugs on the neutrophil promoted inactivation of alpha-1-proteinase inhibitor. *Journal of Rheumatology*, 19, 419–423.

[36] L. Ottonello, P. Dapino, G. Pastorino, G. Montagnani, F. Gatti, G. Guidi and F. Dallegri (1993) Nimesulide as a downregulator of the activity of the neutrophil myeloperoxidase pathway. *Drugs*, 46 (Suppl 1): 29–33.

[37] S. X. Zheng, A. Mouithys-Mickalad, G. Deby-Dupont, C. Deby, A. Maroulis, A. Labasse, M. Lamy, J-M. Crielaard, J-Y. Reginster and Y. Henrotin (2000). In vitro study of the antioxidant properties of nimesulide and 4-HO nimesulide: Effects on HRP-and luminol-dependent chemiluminescence produced by human chondrocytes. *Osteoarthritis and Cartilage* (in press).

[38] M. Bevilacqua and M. Eagni (1993) Recent contribution to knowledge of the mechanism of action of nimesulide. *Drugs*, 46 (Suppl 1), 40–47.

[39] B. Nandgaonkar, T. Rotschild, K. Yu and R. Higgins (1999) Indomethacin improves oxygen-induced retinopathy in the mouse. *Pediatric Research*, 46, 184–188.

[40] C. M. Carreras, G. A. Pargament, S. D. Catz, J. J. Poderoso and A. Boveris (1994) Kinetics of nitric oxide and hydrogen peroxide production and formation of peroxynitrite during the respiratory burst of human neutrophils. *FEBS Letters*, 341: 65–68.

[41] C. Gagnon, F. A. Leblond and J. G. Filep (1998) Peroxynitrite production by human neutrophils, monocytes and lymphocytes challenged with lipopolysaccharide. *FEBS Letters*, 431, 107–110.

[42] G. Deby-Dupont, C. Deby and M. Lamy (1999) Neutrophil myeloperoxidase revisited: it's role in health and disease. *Intensivmedizin*, 36, 500–513.

[43] C. C. Winterbourn and S. O. Brennan (1997) Characterization of the oxidation products of the reaction between reduced glutathione and hypochlorous acid. *Biochemical J ournal*, 326, 87–92.

[44] W. A. Pryor and G. L. Squadrito (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *American Journal of Physiology*, 268, L699–722.

[45] G.E. Arteel, K. Briviba and H. Sies (1999) Protection against peroxynitrite. *FEBS Letters*, 45, 226–230.

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