

Antioxidant Activity and Inhibition of Human Neutrophil Oxidative Burst Mediated by Arylpropionic Acid Non-steroidal Anti-inflammatory Drugs

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It has been suggested that the anti-inflammatory activity of some non-steroidal anti-inflammatory drugs (NSAIDs) may be partly due to their ability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as to inhibit the respiratory burst of neutrophils triggered by various activating agents. Therefore, the aim of the present work was to evaluate and compare the potential scavenging activity for an array of ROS ($O_2^{\cdot-}$, H_2O_2 , HO^{\cdot} , ROO^{\cdot} and $HOCl$) and RNS ($\cdot NO$ and $ONOO^-$) using *in vitro* non-cellular screening systems as well as a cellular screening system (human neutrophil oxidative burst), mediated by the arylpropionic acid derivatives (APAs) NSAIDs ibuprofen, flurbiprofen, fenoprofen, fenbufen, ketoprofen, naproxen and indoprofen. The results obtained in the present work demonstrate that under the present experimental conditions, many of the studied APA NSAIDs showed $O_2^{\cdot-}$ scavenging activity (fenbufen \approx flurbiprofen \approx indoprofen \approx ketoprofen), H_2O_2 (ketoprofen \approx indoprofen \approx fenbufen $>$ flurbiprofen $>$ naproxen), HO^{\cdot} (fenoprofen \approx ibuprofen $>$ fenbufen \approx flurbiprofen $>$ ketoprofen $>$ indoprofen \approx naproxen), $\cdot NO$ (indoprofen $>$ naproxen), $ONOO^-$ (indoprofen $>$ naproxen $>$ fenoprofen \approx flurbiprofen \approx ibuprofen), as well as inhibit myeloperoxidase (MPO) activity (indoprofen) and scavenge human neutrophil derived ROS (ketoprofen $>$ indoprofen $>$ fenbufen $>$ flurbiprofen). The observed effects, if confirmed *in vivo*, may strongly contribute to the anti-inflammatory therapeutical activity observed with these NSAIDs.

Key words non-steroidal antiinflammatory drug; reactive species; human neutrophils; oxidative burst; myeloperoxidase

Non-steroidal anti-inflammatory drugs (NSAIDs) are amongst the most widely prescribed drugs for the treatment of pain, fever, and inflammation. The arylpropionic acid derivatives (APAs) (e.g. fenbufen, fenoprofen, flurbiprofen, ibuprofen, indoprofen, ketoprofen and naproxen, Fig. 1) constitute an important group of NSAIDs widely used for the treatment of pain and inflammation and represent the drugs of choice commonly used in the management of musculoskeletal traumatism, rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, acute gouty arthritis and dysmenorrhoea.^{1–3)} Chemically, these derivatives are weak acids and by virtue of a chiral carbon atom on the propionic acid side chain, exist as enantiomer pairs with (*S*)-isomers being primarily responsible for inhibition of prostaglandin production and inflammatory events, although the majority of these acids continue to be employed as racemate.⁴⁾

Whereas low doses of NSAIDs markedly inhibit the *in vitro* and *in vivo* synthesis of prostaglandin, higher doses are required for an anti-inflammatory effect *in vivo*, suggesting that at higher concentrations other biological activities contribute to the final anti-inflammatory effects mediated by these compounds.⁵⁾ Accordingly, it is well known that other factors beyond prostaglandin synthesis, contribute strongly to the pathophysiology of inflammatory processes, as is the case of overproduction of reactive oxygen species (ROS), namely superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^{\cdot}), peroxy radical (ROO^{\cdot}) and hypochlorous acid ($HOCl$)^{6–9)} as well as nitrogen reactive species (RNS), namely nitric oxide ($\cdot NO$) and peroxynitrite anion ($ONOO^-$).^{10–12)} ROS and RNS are generated by different cell types, with especial emphasis to neutrophils during the respiratory burst.^{13–16)} Thus, it has been suggested that the anti-inflammatory activity of some NSAIDs may also be partly due to their ability to scavenge ROS and RNS, as well as inhibit the respiratory burst of neutrophils triggered by

various activating agents. In fact, it has been demonstrated that at high (antirheumatic) concentrations, several NSAIDs are potent scavengers of ROS and RNS,^{10,17–21)} inhibit the oxidative burst induced by a range of stimuli in human neutrophils^{5,21,22)} and bovine neutrophils,²³⁾ and prevent LDL oxidation.²⁴⁾

The scavenging activities of NSAIDs from the APA group against ROS and RNS, as well as their putative inhibition of neutrophil burst have only been sparsely and incompletely evaluated, which makes it very difficult to compare the relative antioxidant potency among them. Although the effectiveness has already been reported for some NSAIDs (as previously reported²⁵⁾), it is possible to present original and rele-

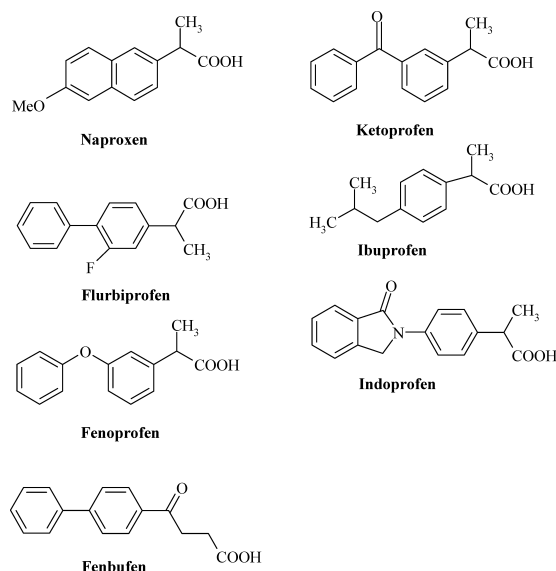


Fig. 1. Chemical Structures of Naproxen, Ketoprofen, Flurbiprofen, Ibuprofen, Fenoprofen, Indoprofen and Fenbufen

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vant data by comparing a large panel of drugs using optimised systems and establishing complete dose-effect curves, with determination of median effective concentrations (IC_{50} s). Therefore, the aim of the present work was focused on evaluating and comparing the potential scavenging activity for an array of ROS ($O_2^{\cdot-}$, H_2O_2 , HO^{\cdot} , ROO^{\cdot} and $HOCl$) and RNS (NO and $ONOO^-$) using *in vitro* non-cellular screening systems as well as a cellular screening system (human neutrophil oxidative burst), mediated by the APAs ibuprofen, flurbiprofen, fenoprofen, fenbufen, ketoprofen, naproxen and indoprofen.

MATERIALS AND METHODS

Materials The following reagents were obtained from Sigma Chemical Co. (St. Louis, U.S.A.): Flurbiprofen, fenoprofen, fenbufen, ketoprofen, naproxen, indoprofen, ibuprofen, nitroblue tetrazolium chloride (NBT), β -nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), 30% hydrogen peroxide, ferric chloride anhydrous, ethylenediaminetetraacetic acid (EDTA) disodium salt, ascorbic acid, trichloroacetic acid, thiobarbituric acid (TBA), deoxyribose, sodium hypochlorite solution with 4% available chlorine ($NaOCl$), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), mannitol, cysteine, lipoic acid, carboxy-PTIO, sodium borohydride, sodium bicarbonate, fluorescein sodium salt, dihydrorhodamine 123 (DHR), 4,5-diaminofluorescein (DAF-2), sodium nitroprusside dihydrate (NPS), Histopaque 1077, Histopaque 1119, Dulbecco's Phosphate Buffer saline, without calcium chloride and magnesium chloride (PBS), Trypan blue solution 0.4%, luminol, phorbol myristate acetate (PMA), tiron, diphenyleiiodonium chloride (DPI) and myeloperoxidase (MPO) from human leukocytes (EC 1.11.1.7). 4-Aminobenzoic acid hydrazide (ABAH) was obtained from Calbiochem® (Postfach, Germany). Sodium chloride, sodium acetate, ethanol, sulphuric acid, hydrochloric acid, sodium hydroxide, sodium carbonate and sodium nitrite were obtained from Merck (Darmstadt, Germany). Lucigenin, α,α' -azodiisobutyramidine dihydrochloride (AAPH), dimethylsulfoxide (DMSO), TRIS-hydroxymethylaminomethane (TRIS), potassium chloride and Trolox were supplied by Fluka Chemie GmbH (Steinheim, Germany).

Measurement of ROS and RNS Scavenging Activity Using *in Vitro* Non-cellular Screening Systems. **$O_2^{\cdot-}$ Scavenging Assay** $O_2^{\cdot-}$ scavenging activity was measured spectrophotometrically in a microplate reader (Synergy HT, BIO-TEK) by monitoring the $O_2^{\cdot-}$ -induced reduction of nitroblue tetrazolium chloride (NBT) at 560 nm for 2 min, as previously reported.¹⁹⁾ $O_2^{\cdot-}$ radicals were generated by a β -nicotinamide adenine dinucleotide (NADH)/phenazine methosulfate (PMS) system. The reaction mixture in the sample wells contained the following reagents at the indicated final concentrations (in a 300 μ l final volume): NADH (166 μ M), NBT (43 μ M), tested compound, dissolved in DMSO, at various concentrations and PMS (2.7 μ M). No direct effect was observed between DMSO and $O_2^{\cdot-}$ in the present assay conditions. All other components were dissolved in 19 mM phosphate buffer, pH 7.4. The assay was performed at room temperature. The selective effect of superoxide on the reduction of NBT to diformazan was confirmed by using superoxide dismutase, which completely in-

hibited this effect. Tiron was used as a positive control. Effects are expressed as the percentage inhibition of the $O_2^{\cdot-}$ -induced NBT reduction to diformazan. Each study corresponds to four experiments, performed in triplicate.

H_2O_2 Scavenging Assay H_2O_2 scavenging activity was measured by chemiluminescence in a microplate reader (Synergy HT, BIO-TEK) by monitoring the oxidation of lucigenin by H_2O_2 , as previously reported.²⁰⁾ The reaction mixtures in the sample wells contained the following reagents at the indicated final concentrations (in a final volume of 250 μ l): 50 mM Tris buffer, pH 7.4, lucigenin (3 mM), tested compound at various concentrations, dissolved in buffer and H_2O_2 (2%). The resulting chemiluminescence was followed for 5 min. Trolox was used as a positive control. Effects are expressed as the percentage inhibition of the H_2O_2 -elicited lucigenin oxidation. Each study corresponds to four experiments, performed in triplicate.

HO^{\cdot} Scavenging Assay HO^{\cdot} scavenging activity was measured spectrophotometrically using a spectrophotometer (Lambda 45, Perkin-Elmer) as previously reported.¹⁹⁾ HO^{\cdot} radicals were generated by a Fenton system (ascorbic acid- $FeCl_3$ -EDTA). When exposed to HO^{\cdot} , the sugar deoxyribose is degraded to malonaldehyde, which generates a pink chromogen on heating with thiobarbituric acid (TBA), at low pH. Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 1 ml): 10 mM phosphate buffer, pH 7.4, deoxyribose (2.8 mM), H_2O_2 (1.42 mM), tested compounds in various concentrations, $FeCl_3$ -EDTA (20 μ M, 100 μ M) and ascorbic acid (50 μ M). The iron salt was premixed with the chelator dissolved in water before addition to the reaction mixture. All other components were dissolved in 10 mM phosphate buffer, pH 7.4. After incubation at 37 °C for 1 h, 1 ml of trichloroacetic acid (2.8%, w/v) and 1 ml of TBA (1%, w/v) were added and the mixture heated in a water bath at 100 °C for 15 min. Absorbance of the resulting solution was measured at 532 nm. This assay was also performed without ascorbic acid or EDTA to evaluate a possible pro-oxidant and/or iron chelation activity. Mannitol was used as a positive control. Effects are expressed as the percentage inhibition of the deoxyribose degradation to malonaldehyde with the second-order rate constants (K_s) being calculated according to Halliwell *et al.*⁶⁾ Each study corresponds to four experiments, performed in duplicate.

ROO^{\cdot} Scavenging Assay ROO^{\cdot} scavenging activity was measured by monitoring fluorescence decay in a microplate reader (Synergy HT, BIO-TEK), due to the oxidation of fluorescein by ROO^{\cdot} generated by α,α' -azodiisobutyramidine dihydrochloride (AAPH) thermo-decomposition, according to a procedure termed the Oxygen Radical Absorbance Capacity (ORAC) assay, as previously reported.¹⁹⁾ The reaction mixtures in the sample wells contained the following reagents dissolved in 75 mM phosphate buffer, pH 7.4, at the indicated final concentrations (in a final volume of 200 μ l): fluorescein (61 nM), tested compound at various concentrations and AAPH (19 mM). Fluorescence measurements were carried out at a constant temperature of 37 °C with excitation and emission wavelengths of 485 nm and 528 nm respectively. Effects are expressed as the relative Trolox equivalent ORAC value which is calculated by the following equation, where AUC represents the area under the curve¹⁹⁾:

$$\text{relative ORAC value} = \frac{[(AUC_{\text{sample}} - AUC_{\text{blank}}) / (AUC_{\text{Trolox}} - AUC_{\text{blank}})]}{\times (\text{molarity of Trolox} / \text{molarity of compound})}$$

Each study corresponds to four experiments, performed in triplicate.

HOCl Scavenging Assay. Production of HOCl Solution HOCl was prepared immediately before use by adjusting the pH of a 1% (v/v) solution of NaOCl to 6.2 with diluted sulphuric acid. The concentration of HOCl was further determined spectrophotometrically using a spectrophotometer (Lambda 45, Perkin-Elmer) at 235 nm and the molar absorption coefficient of $100 \text{ M}^{-1} \text{ cm}^{-1}$.²⁶ For each assay, HOCl was appropriately diluted with 50 mM phosphate buffer, pH 7.4.

Synthesis of 5-Thio-2-nitrobenzoic Acid (TNB) TNB was prepared according to a described procedure⁷ with modifications. Briefly, 50 mM sodium borohydride was added to a 1 mM solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 50 mM phosphate buffer, pH 6.6, containing 5 mM EDTA. The solution was incubated at 37 °C for 30 min. TNB concentration was determined by measuring the absorbance at 412 nm in a spectrophotometer (Lambda 45, Perkin-Elmer) using the molar absorption coefficient of $13600 \text{ M}^{-1} \text{ cm}^{-1}$. For each assay, TNB was appropriately diluted with 50 mM phosphate buffer, pH 7.4.

Measurement of HOCl Scavenging Activity HOCl scavenging activity was measured using a spectrophotometer (Lambda 45, Perkin-Elmer) monitoring the oxidation of TNB to DTNB, as previously reported.¹⁹ Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 1 ml): TNB (70 μM), tested compound at various concentrations, dissolved in ethanol or buffer, and HOCl (25 μM). The assay was performed at room temperature. No direct effect was observed between ethanol and HOCl in the present assay conditions. Absorbance was measured at 412 nm, 5 min after the addition of HOCl. Lipoic acid was used as a positive control. Effects are expressed as the percentage inhibition of TNB oxidation to DTNB. Each study corresponds to four experiments, performed in duplicate.

$\cdot\text{NO}$ Scavenging Assay $\cdot\text{NO}$ scavenging activity was measured by monitoring the $\cdot\text{NO}$ -induced oxidation of 4,5-diaminofluorescein (DAF-2) to triazolo fluorescein by fluorimetry as previously reported.¹⁹ In this assay, DAF-2 does not react directly with $\cdot\text{NO}$ but rather with the oxidized form of $\cdot\text{NO}$, involving N_2O_3 .²⁷ One milligram of DAF-2 in 0.55 ml DMSO was diluted with 50 mM phosphate buffer (pH 7.4) to 1/400-fold. Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 1.5 ml): DAF-2 (3.14 μM), sodium nitroprusside (NPS) (20 μM) and tested compounds in various concentrations, dissolved in DMSO. No direct effect was observed between DMSO and $\cdot\text{NO}$ in the present assay conditions. All other components were dissolved in 50 mM phosphate buffer, pH 7.4. The reaction mixture was incubated for 10 min at 37 °C, under tungsten light. Light is of prime importance for the sustained release of $\cdot\text{NO}$ by NPS.²⁸ The fluorescence signal caused by DAF-2 reacting with $\cdot\text{NO}$ was measured using a spectrofluorimeter (LS-50B, Perkin-Elmer) with excitation and emission wavelengths of 495 nm and 521 nm respectively and excitation and emission slit widths of 8 nm, at

37 °C. Effects are expressed as the percentage inhibition of DAF-2 oxidation. Carboxy-PTIO was used as a positive control. Each study corresponds to four experiments, performed in triplicate.

ONOO⁻ Scavenging Assay. Synthesis of ONOO⁻ Synthesis of ONOO⁻ was essentially performed as described before by Beckman *et al.*²⁹ Briefly, an acidic solution (HCl 0.7 M) of 0.6 M H_2O_2 was mixed with NaNO_2 (0.66 M) on ice for one second and the reaction quenched with ice-cold 3 M NaOH. Residual H_2O_2 was removed by mixing with granular MnO_2 pre-washed with NaOH 3 M. The ONOO⁻ stock solution was filtered and then frozen (-20 °C) with the top layer of the solution collected for the experiment. ONOO⁻ concentration was determined by measuring the absorbance at 302 nm ($\epsilon = 1670 \text{ M}^{-1} \text{ cm}^{-1}$). The typical yield of freshly prepared ONOO⁻ ranged from 60–80 mM. Higher concentrations (>200 mM) of ONOO⁻ can be obtained by freeze fractionation. However, in the present study only freshly prepared ONOO⁻ solution was used to minimise nitrite ion contamination. Prior to each study, the concentration of the ONOO⁻ stock was determined spectrophotometrically in 0.1 M NaOH.

Measurement of ONOO⁻ Scavenging Activity ONOO⁻ scavenging activity was measured by monitoring the ONOO⁻-induced oxidation of dihydrorhodamine 123 (DHR) to rhodamine 123 by fluorimetry, as previously reported.¹⁹ A stock solution of 2.89 mM DHR in dimethylformamide was purged with nitrogen and stored at -20 °C. Working solutions of DHR diluted from the stock solution were placed on ice in the dark immediately before the determinations. Buffer (90 mM sodium chloride, 50 mM sodium phosphate and 5 mM potassium chloride, pH 7.4) was purged with nitrogen and placed on ice before use. At the outset of the experiments, 100 μM diethylenetriaminepentaacetic acid (DTPA) was added to the buffer. The assay was performed at 37 °C. Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 1.5 ml): DHR (50 μM), tested compound, dissolved in DMSO, at various concentrations and ONOO⁻ (600 nM) in 0.1 M NaOH. In the present assay conditions, less than 10% of the effect was found for DMSO itself, which was subtracted in the results obtained for the compounds. Cysteine was used as a positive control. The fluorescence signal induced by DHR reacting with ONOO⁻ was measured using a spectrofluorimeter (LS-50B, Perkin-Elmer) with excitation and emission wavelengths of 500 nm and 536 nm respectively and excitation and emission slit widths of 2.5 nm and 3.0 nm respectively. Background and final fluorescent intensities were measured 5 min after treatment. The effects are expressed as the percentage inhibition of DHR oxidation. In a parallel set of experiments the assays were performed in the presence of 25 mM NaHCO_3 to simulate physiological conditions with high CO_2 concentrations *in vivo*. This evaluation is important because under physiological conditions, the reaction of ONOO⁻ with bicarbonate is predominant, with the reaction rate constant of CO_2 with ONOO⁻ being very rapid ($k_2 = 3\text{--}5.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$).³⁰ Thus, the reactivity of the putative scavengers for ONOO⁻ should be able to match or exceed that of bicarbonate. Each study corresponds to four experiments, performed in triplicate.

Measurement of PMA-Induced Oxidative Burst in

Human Neutrophils This study was performed using neutrophils isolated from healthy human volunteers. Essentially, the study was based on the stimulation of human neutrophils with phorbol 12-myristate 13-acetate (PMA) with subsequent measurement of the neutrophil-ROS-generating-capacity. For this purpose, chemiluminescence methods, using luminol and lucigenin as probes, were adapted to a microplate reader (Synergy HT, BIO-TEK).

Isolation of Neutrophils Venous blood was collected from healthy human volunteers by antecubital venipuncture, into EDTA vacuum tubes. Whole venous blood was separated into cell types using Histopaque solutions 1077 and 1119 in 12 ml polypropylene centrifuge tubes, as previously reported.²¹⁾ Briefly, 3 ml of Histopaque 1077 was carefully layered on top of 3 ml Histopaque 1119 in a 12 ml polypropylene tube. 6 ml of the collected blood was decanted on top of this discontinuous density gradient. The tube was centrifuged at 800 *g* for 30 min at 20 °C. On completion of centrifugation, the neutrophils were carefully removed using a Pasteur pipette. The neutrophil pellet was removed and doubled in volume using PBS (this reduces the viscosity of the Histopaque-neutrophil suspension so that the cells can be centrifuged without the need for high *g* forces) before the neutrophils were centrifuged at 700 *g* for 5 min at 4 °C. The supernatant was decanted and a mixture of 1.25 ml PBS+5.25 ml sterile distilled water added to the neutrophil pellet to lyse any remaining red blood cells. The tube was gently inverted for 1 min after which isotonicity was re-established by adding 2.2 ml of NaCl 3%. This suspension was then subjected to a further centrifugation at 700 *g* for 5 min at 4 °C, after which the supernatant was decanted and the neutrophil pellet re-suspended in PBS. The obtained cell suspensions contained more than 99% neutrophils and the control of their viability showed more than 95% of the cells excluding Trypan blue (0.4%). Isolated neutrophils were maintained in ice until use. Neutrophils were used from one volunteer per experiment.

Oxidation of Luminol by Neutrophils—Generated ROS The measurement of neutrophil burst was undertaken by chemiluminescence, by monitoring ROS-induced oxidation of luminol, according to a previously described procedure.²¹⁾ Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 200 μ l): neutrophils (final suspension = 1×10^6 cells/ml), tested compounds at various concentrations (0–2 mM), luminol (500 μ M) and PMA (1.6×10^{-7} M). Cells and tested compounds were pre-incubated for 5 min at 37 °C before the addition of luminol and PMA and the measurements were carried out at 37 °C. Kinetic readings were initiated immediately after cell stimulation. Measurements were taken at the peak of the curve. In all experiments, this peak was observed at around 5 min. Effects are expressed as the percentage inhibition of luminol oxidation. Lipoic acid (a HOCl scavenger) and 4-aminobenzoic acid hydrazide (ABAH) (a myeloperoxidase (MPO) inhibitor) were used as positive controls. Each study corresponds to four individual experiments, performed in triplicate in each experiment.

Oxidation of Lucigenin by Neutrophils—Generated $O_2^{\cdot-}$ The measurement of neutrophil burst was measured by chemiluminescence through monitoring the $O_2^{\cdot-}$ -induced oxidation of lucigenin, according to a previously described

procedure.²¹⁾ Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 200 μ l): neutrophils (final suspension = 5×10^6 cells/ml), tested compounds at various concentrations (0–2 mM), lucigenin (125 μ M) and PMA (1.6×10^{-7} M). Kinetic readings were initiated immediately after cell stimulation. All measurements were carried out at 37 °C and taken at the peak of the curve. In all experiments, this peak was observed at around 5 min. Effects are expressed as the percentage inhibition of lucigenin oxidation. Tiron (scavenger of $O_2^{\cdot-}$) and diphenyleioidonium chloride (DPI) (inhibitor of NADPH oxidase) were used as positive controls. Each study corresponds to four experiments, performed in triplicate.

Luminol Oxidation by Purified-Myeloperoxidase—Generated HOCl The oxidation of luminol by purified MPO-generated HOCl was performed according to a previously described procedure.²¹⁾ Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 250 μ l): NaCl (100 mM), luminol (250 μ M), tested compounds at various concentrations (0–2 mM), MPO (0.5 U/ml) and H_2O_2 (100 μ M). Kinetic readings were initiated immediately after addition of H_2O_2 . All measurements were carried out at a constant temperature of 37 °C and were taken at the peak of the curve. In all experiments, this peak was observed at around 7 to 9 min. The inhibitory effects of the tested compounds are expressed as the percentage inhibition of luminol oxidation. ABAH was used as a positive control. Each study corresponds to four experiments, performed in triplicate.

RESULTS

ROS and RNS Scavenging Activity Using *in Vitro* Non-cellular Systems. $O_2^{\cdot-}$ Scavenging Activity Figure 2 shows the results obtained in the $O_2^{\cdot-}$ scavenging assay. It was observed that flurbiprofen, fenbufen, ketoprofen and indoprofen prevented the $O_2^{\cdot-}$ -induced reduction of NBT in a concentration dependent manner, indoprofen being the most potent, with an IC_{50} of 3387 ± 191 μ M, followed by fenbufen, ketoprofen and flurbiprofen with equivalent potencies (IC_{50} s of 3481 ± 268 , 3631 ± 633 and 3753 ± 282 μ M, respectively) (mean \pm S.E.M.) (Table 1). No activity was observed for fenoprofen up to 1250 μ M and for naproxen and ibuprofen up to 5000 μ M. The positive control (Tiron) provided an IC_{50} of 167 ± 7 μ M (mean \pm S.E.M.) (Table 1).

H_2O_2 Scavenging Activity Figure 3 shows the results obtained in the H_2O_2 scavenging assay. It was observed that fenbufen, flurbiprofen, ketoprofen, naproxen and indoprofen prevented the H_2O_2 -induced oxidation of lucigenin in a concentration dependent manner, ketoprofen being the most potent with an IC_{50} of 2847 ± 142 μ M, followed by indoprofen, fenbufen, flurbiprofen and naproxen (IC_{50} s: $3050 \pm 87 \approx 3220 \pm 175 \approx 3698 \pm 355 > 5343 \pm 199$ μ M, respectively) (mean \pm S.E.M.) (Table 1). No activity was observed for fenoprofen and ibuprofen up to 5 mM. The positive control (Trolox) provided an IC_{50} of 2535 ± 94 μ M (mean \pm S.E.M.) (Table 1).

HO^{\cdot} Scavenging Activity Figure 4 shows the results obtained in the HO^{\cdot} scavenging assay. All assayed compounds prevented the HO^{\cdot} -induced degradation of deoxyribose into malonaldehyde in a concentration dependent manner. The

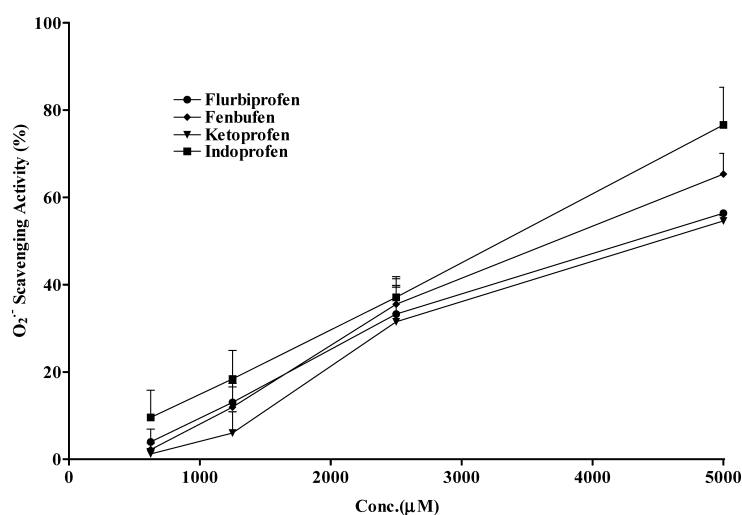


Fig. 2. O₂⁻ Scavenging Activity of Flurbiprofen, Fenbufen, Ketoprofen and Indoprofen

Each point represents the values obtained from four experiments, performed in triplicate (mean ± S.E.M.).

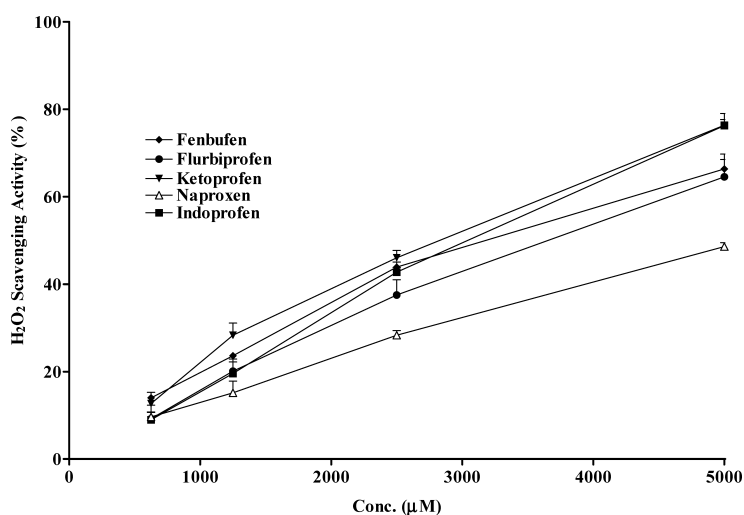


Fig. 3. H₂O₂ Scavenging Activity of Fenbufen, Flurbiprofen, Ketoprofen, Naproxen and Indoprofen

Each point represents the values obtained from four experiments, performed in triplicate (mean ± S.E.M.).

Table 1. ROS Scavenging Effects (IC₅₀, ks and ORAC Activity; Mean ± S.E.M.) of Fenbufen, Fenoprofen, Flurbiprofen, Ibuprofen, Indoprofen, Ketoprofen, Naproxen, Mannitol, Trolox, Tiron GSH and Ascorbic Acid

Tested compound	O ₂ ⁻	H ₂ O ₂	HO [•]		ROO [•]
	IC ₅₀ (μM)	IC ₅₀ (μM)	IC ₅₀ (μM)	ks (M ⁻¹ ·s ⁻¹)	ORAC activity ^{a)}
Fenbufen	3481 ± 268	3220 ± 175	93 ± 16	(4.9 ± 0.5) × 10 ¹⁰	0.16 ± 0.10
Fenoprofen	NB	NA	52 ± 2	(2.8 ± 0.2) × 10 ¹⁰	0.09 ± 0.06
Flurbiprofen	3753 ± 282	3698 ± 355	100 ± 1	(2.7 ± 0.3) × 10 ¹⁰	0.14 ± 0.05
Ibuprofen	NA	NA	61 ± 11	(8.3 ± 1.6) × 10 ¹⁰	0.02 ± 0.01
Indoprofen	3387 ± 191	3050 ± 87	320 ± 27	(2.6 ± 0.6) × 10 ¹⁰	0.04 ± 0.02
Ketoprofen	3631 ± 633	2847 ± 142	258 ± 12	(4.4 ± 0.6) × 10 ¹⁰	0.02 ± 0.01
Naproxen	NA	5343 ± 199	309 ± 64	(1.2 ± 0.2) × 10 ¹⁰	0.36 ± 0.03
Mannitol	—	—	3272 ± 215	(0.2 ± 0.03) × 10 ¹⁰	—
Trolox	—	2535 ± 94	—	—	1
Tiron	167 ± 7	—	—	—	—
GSH	—	—	—	—	0.34 ± 0.10
Ascorbic acid	—	—	—	—	0.25 ± 0.07

NA, no activity was found up to 5000 μM. NB, no activity was found up to 1250 μM. a) μM Trolox equivalent/μM compound.

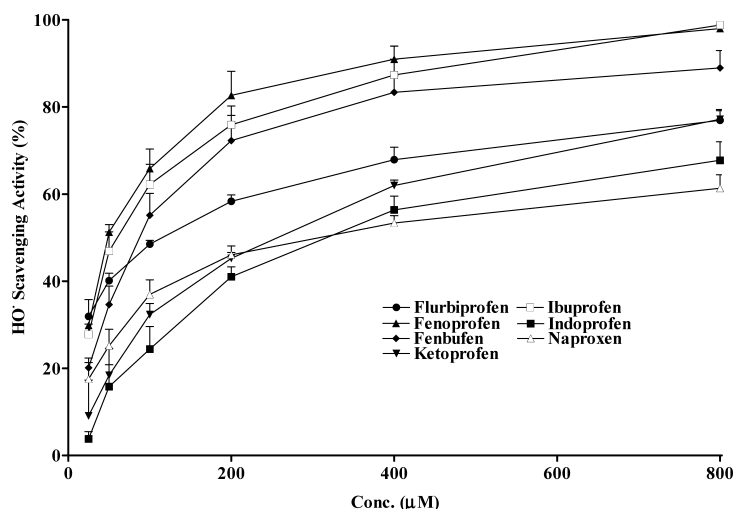


Fig. 4. HO· Scavenging Activity of Flurbiprofen, Fenoprofen, Fenbufen, Ketoprofen, Ibuprofen, Indoprofen and Naproxen

Each point represents the values obtained from four experiments, performed in duplicate (mean \pm S.E.M.).

order of potencies observed was fenoprofen \approx ibuprofen $>$ fenbufen \approx flurbiprofen $>$ ketoprofen $>$ naproxen \approx indoprofen (IC_{50} s of $52 \pm 2 \approx 61 \pm 11 > 93 \pm 16 \approx 100 \pm 1 > 258 \pm 12 > 309 \pm 64 \approx 320 \pm 27 \mu M$, respectively) (mean \pm S.E.M.) (Table 1).

The second-order rate constants (k_s) calculated were $(4.9 \pm 0.5) \times 10^{10}$, $(2.8 \pm 0.2) \times 10^{10}$, $(2.0 \pm 0.2) \times 10^{10}$, $(8.3 \pm 1.6) \times 10^{10}$, $(2.6 \pm 0.6) \times 10^{10}$, $(3.3 \pm 0.3) \times 10^{10}$ and $(1.2 \pm 0.2) \times 10^{10} M^{-1} \cdot s^{-1}$, for fenbufen, fenoprofen, flurbiprofen, ibuprofen, indoprofen, ketoprofen and naproxen respectively (Table 1). The positive control (mannitol) provided an IC_{50} of $3272 \pm 215 \mu M$ and a k_s of $(0.20 \pm 0.03) \times 10^{10} M^{-1} \cdot s^{-1}$ (mean \pm S.E.M.) (Table 1).

The assay performed without ascorbic acid or EDTA did not indicate any pro-oxidant and/or iron chelation activity, respectively, for the NSAIDs studied, since no increased malonaldehyde formation was observed in their presence.

ROO· Scavenging Activity The results obtained from the ROO· scavenging assay are shown in Table 1. It can be observed that ROO· is effectively scavenged by naproxen, while the other compounds were much less active against this ROS. Nevertheless, the activity observed for all assayed compounds was concentration-dependent. The order of potencies was found to be naproxen $>$ fenbufen $>$ flurbiprofen $>$ fenoprofen $>$ indoprofen $>$ ketoprofen \approx ibuprofen (ORAC values of 0.36 ± 0.03 , 0.16 ± 0.10 , 0.14 ± 0.05 , 0.09 ± 0.06 , 0.04 ± 0.02 , 0.02 ± 0.01 and 0.02 ± 0.01 , respectively) (mean \pm S.E.M.) (Table 1). The positive controls GSH and ascorbic acid yielded ORAC values of 0.34 ± 0.10 and 0.25 ± 0.07 (mean \pm S.E.M.), respectively (Table 1).

HOCl Scavenging Activity No scavenging activity against HOCl was observed for any of the NSAIDs studied. The positive control (lipoic acid) provided an IC_{50} of $59.5 \pm 4.7 \mu M$ (mean \pm S.E.M.).

·NO Scavenging Activity The results obtained in the ·NO scavenging assay are presented in Fig. 5 and Table 2. It was observed that indoprofen and naproxen are efficient ·NO scavengers (Fig. 5), while the other NSAIDs have lower but effective activities, except fenoprofen for which no activity was found up to 2.5 mM. The order of potencies found was indoprofen $>$ naproxen (IC_{50} s: 968 ± 104 and $3192 \pm 852 \mu M$ respectively) (mean \pm S.E.M.) (Table 2). The other NSAIDs

Table 2. RNS Scavenging Effects (IC_{50} , Mean \pm S.E.M.) of Fenbufen, Fenoprofen, Flurbiprofen, Ibuprofen, Indoprofen, Ketoprofen, Naproxen, Carboxy-PTIO and Cysteine

Tested compound	IC_{50} (μM)		
	·NO	ONOO ⁻	
		Absence NaHCO ₃	Presence of 25 mM NaHCO ₃
Fenbufen	<5000	NB	NB
Fenoprofen	NA	<5000	NA
Flurbiprofen	<5000	<5000	<5000
Ibuprofen	<5000	<5000	<5000
Indoprofen	968 ± 104	1497 ± 130	1174 ± 214
Ketoprofen	<5000	NB	NB
Naproxen	3192 ± 352	3944 ± 367	1997 ± 365
Carboxy-PTIO	1.8 ± 0.4	—	—
Cysteine	—	5.2 ± 0.1	4.1 ± 0.3

NA, no activity was found up to 2500 μM . NB, no activity was found up to 5000 μM .

did not attain 50% inhibition up to 5 mM (Table 1). The positive control (carboxy-PTIO) yielded an IC_{50} of $1.8 \pm 0.4 \mu M$ (mean \pm S.E.M.) (Table 2).

ONOO⁻ Scavenging Activity The results obtained in the ONOO⁻ scavenging assay in both the absence and presence of 25 mM NaHCO₃ are presented in Figs. 6, 7 and Table 2. In the absence of NaHCO₃, it was observed that the oxidation of DHR by ONOO⁻ was prevented by indoprofen, naproxen, flurbiprofen, ibuprofen and fenoprofen. On the other hand, fenbufen and ketoprofen exerted no effect up to 5 mM. The most effective compounds were shown to be: indoprofen $>$ naproxen (IC_{50} s: 1497 ± 130 and $3944 \pm 367 \mu M$, respectively) (mean \pm S.E.M.) \gg flurbiprofen \approx ibuprofen \approx fenoprofen (37, 25 and 23% of inhibitory effect at 5000 μM , respectively). In the presence of NaHCO₃, the inhibitor activities of indoprofen and naproxen were significantly increased (the resulting IC_{50} s were 1174 ± 214 and $1997 \pm 365 \mu M$, respectively) (mean \pm S.E.M.). The percentage inhibition for the other effective compounds at the maximum concentration of 5 mM was 40, 29 and 27% inhibition for flurbiprofen, ibuprofen and fenoprofen, respectively). The positive control

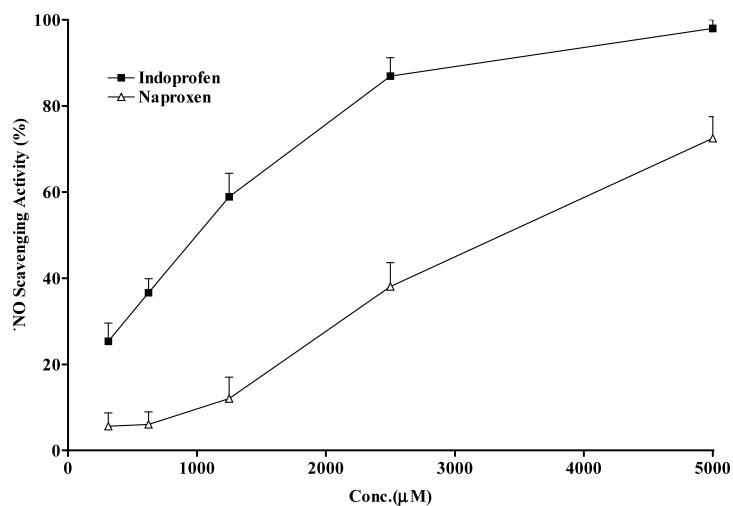


Fig. 5. \cdot NO Scavenging Activity of Indoprofen and Naproxen

Each point represents the values obtained from four experiments, performed in triplicate (mean \pm S.E.M.).

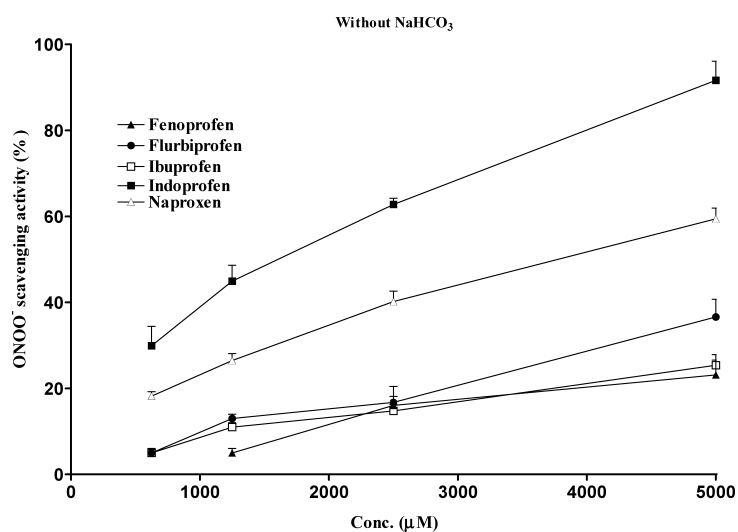


Fig. 6. ONOO^- Scavenging Activity of Fenoprofen, Flurbiprofen, Ibuprofen, Indoprofen and Naproxen

Each point represents the values obtained from four experiments, performed in triplicate (mean \pm S.E.M.).

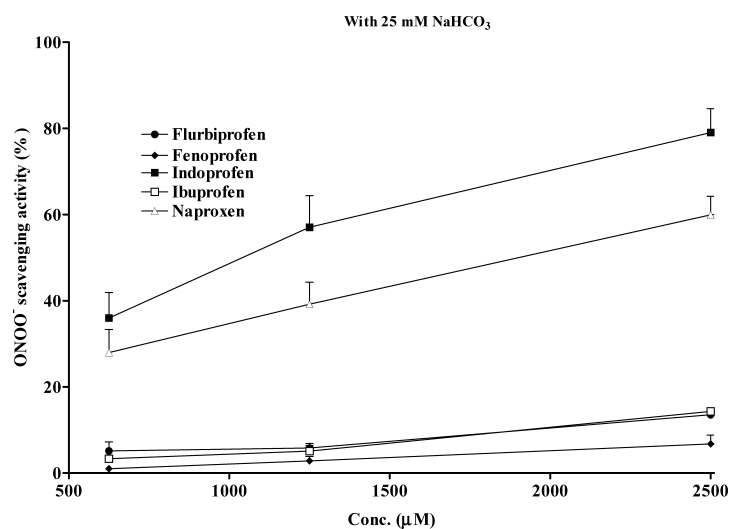


Fig. 7. ONOO^- Scavenging Activity of Flurbiprofen, Fenoprofen, Indoprofen, Ibuprofen and Naproxen in the Presence of 25 mM of Bicarbonate

Each point represents the values obtained from four experiments, performed in triplicate (mean \pm S.E.M.).

cysteine, provided an IC_{50} of $5.2 \pm 0.1 \mu M$ and $4.1 \pm 0.3 \mu M$, in the absence and presence of $NaHCO_3$, respectively (Table 2).

Inhibition of Neutrophil Burst The measurement of neutrophil burst was performed in two complementary assays. In the first assay the neutrophil burst was measured by chemiluminescence, through monitoring the ROS-induced oxidation of luminol (Fig. 8, Table 3). It can be observed that from all the compounds tested, only fenbufen was shown to be effective in preventing the neutrophil burst, but even here only a weak inhibitory activity was observed (46% inhibitory effect at the maximum concentration of $500 \mu M$).

The positive controls ABAH and lipoic acid provided IC_{50} s of 18 ± 4 and $943 \pm 271 \mu M$ (mean \pm S.E.M.), respectively.

In a second assay, the neutrophil burst was also measured by chemiluminescence, through monitoring the $O_2^{\cdot-}$ -induced oxidation of lucigenin (Fig. 9, Table 3). In this assay, indoprofen, ketoprofen, fenbufen and flurbiprofen displayed inhibitory activity in a concentration dependent manner, while fenoprofen, ibuprofen and naproxen showed no effect. Ketoprofen and indoprofen were the most potent (IC_{50} s of

Table 3. Inhibition of PMA-Induced Neutrophil Burst by Fenbufen, Fenoprofen, Flurbiprofen, Ibuprofen, Indoprofen, Ketoprofen, Naproxen, Diphenyleiiodonium Chloride (DPI), Tiron, 4-Aminobenzoic Acid (ABAH) and Lipoic Acid (IC_{50} : Mean \pm S.E.M.).

Tested compound	IC_{50} (μM)		
	Neutrophils luminol	Neutrophils lucigenin	MPO luminol
Fenbufen	46% inhib. at $500 \mu M$	36% inhib. at $500 \mu M$	NA
Fenoprofen	NB	NB	NB
Flurbiprofen	NB	30% inhib. at $500 \mu M$	NB
Ibuprofen	NB	NB	NB
Indoprofen	NB	539 ± 32	4070 ± 505
Ketoprofen	NB	392 ± 66	NB
Naproxen	NB	NB	NB
ABAH	18 ± 4	—	0.34 ± 0.01
DPI	—	1.1 ± 0.1	—
Lipoic acid	943 ± 271	—	119 ± 10
Tiron	—	88 ± 10	—

NA, no activity was found up to $500 \mu M$. NB, no activity was found up to $1000 \mu M$.

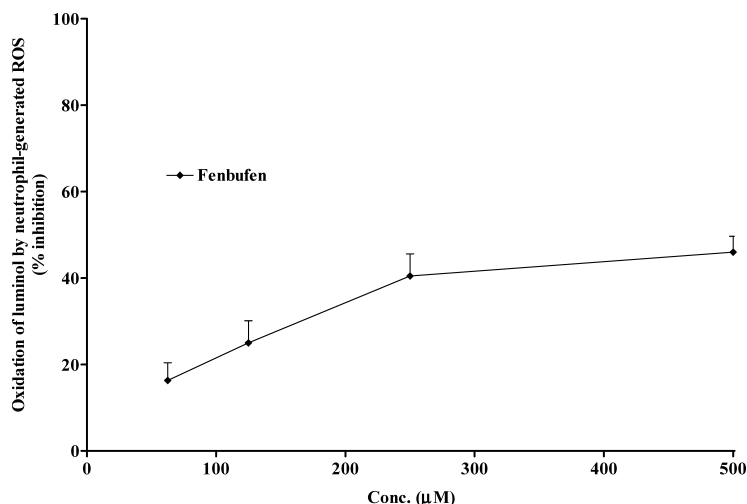


Fig. 8. Inhibitory Effect (%) of Fenbufen on Luminol Oxidation Mediated by Neutrophil-Generated ROS

Each point represents the values obtained from four experiments, performed in triplicate (mean \pm S.E.M.).

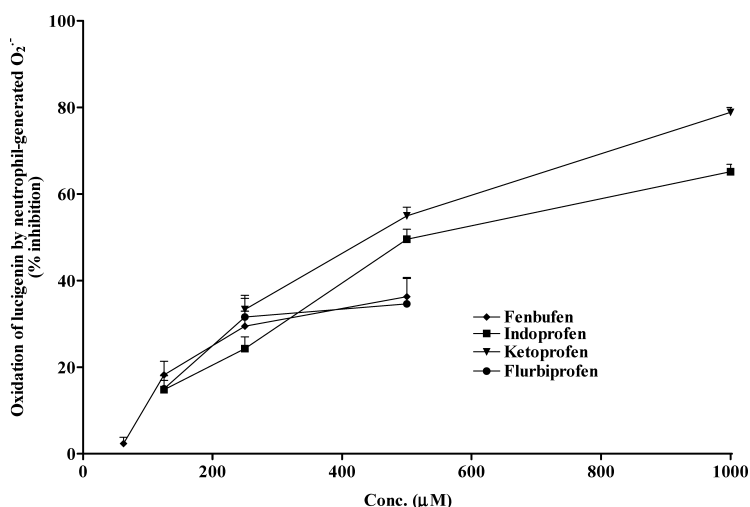


Fig. 9. Inhibitory Effect (%) of Fenbufen, Indoprofen, Ketoprofen and Flurbiprofen on Lucigenin Oxidation Mediated by Neutrophil-Generated $O_2^{\cdot-}$

Each point represents the values obtained from four experiments, performed in duplicate (mean \pm S.E.M.).

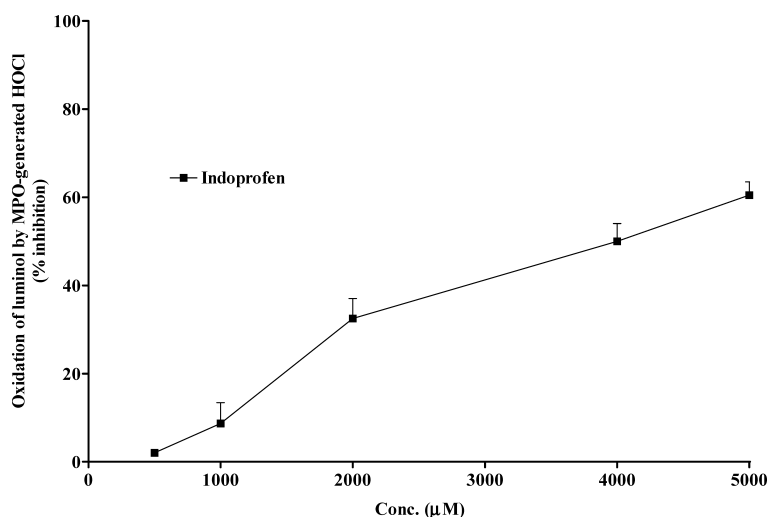


Fig. 10. Inhibitory Effect (%) of Indoprofen on Luminol Oxidation Mediated by Myeloperoxidase (from Human Leukocytes)-Generated HOCl
Each point represents the values obtained from four experiments, performed in triplicate (mean \pm S.E.M.).

392 \pm 66 and 539 \pm 32 μ M, respectively) (mean \pm S.E.M.). Fenbufen and flurbiprofen only displayed weak effects (36% and 30% inhibition, respectively, at the maximum concentration of 500 μ M). The IC₅₀ values found for the positive controls, DPI (inhibitor of NADPH oxidase), tiron (scavenger of O₂⁻) were 1.1 \pm 0.1 and 88 \pm 10 μ M (mean \pm S.E.M.), respectively (Table 3).

Luminol Oxidation by MPO-Generated HOCl Figure 10 and Table 3 show the results obtained in the oxidation of luminol by MPO-generated HOCl. Is this assay an effective activity was only observed for indoprofen, with an IC₅₀ value of 4070 \pm 505 μ M (mean \pm S.E.M.). The IC₅₀ values found for the positive control, ABAH and lipoic acid were 0.34 \pm 0.01 and 119 \pm 10 μ M (mean \pm S.E.M.), respectively (Table 3).

DISCUSSION

Although the inhibition of prostaglandin synthesis constitutes the primary mechanism behind the anti-inflammatory action of NSAIDs, it has been suggested that the activity of these compounds may be also partly due to their ability to scavenge ROS and RNS and to inhibit the respiratory burst of neutrophils triggered by various activator agents.^{10,18–21} As mentioned in the introduction, these activities have only been sparsely and inadequately evaluated for NSAIDs from the APA group, which prompted us to undertake a more systematic evaluation of their relative potencies. In considering the results obtained from the present assays using *in vitro* screening systems, the studied APA NSAIDs were shown to be scavengers of ROS and/or RNS and in some cases, inhibitors of the PMA-induced burst of human neutrophils, but only within the μ M to mM range. At first sight, it could be inferred that these effects, which were only obtained at high concentrations, would not be important for the therapeutic effect of these APA NSAIDs. However, by looking at the literature, it can be observed that the effective concentrations in *in vitro* studies do vary, depending on the methodology used. Furthermore, the effective concentrations are within those expected *in vivo* during anti-inflammatory therapy with APA NSAIDs,^{2,31} at least for HO[•]. HO[•] is one of the most reactive

radicals formed at the inflammation sites. Hiller and Wilson³² showed that various NSAIDs tested could scavenge HO[•] generated in a free solution by pulse radiolysis at an almost diffusion-controlled rate (k_2 values between 5×10^9 and $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$). Subsequently, it was shown that ketoprofen, ibuprofen, flurbiprofen and naproxen were effective HO[•] scavengers, using a methodology followed in the present study.^{6,33,34} Our results agree with those found elsewhere and further indicate that the other APA NSAIDs fenbufen, fenoprofen and indoprofen are also good HO[•] scavengers. Considering that the direct reactivity of the aryl ring with HO[•] is very important for this type of activity³⁵ and that the tested compounds contain two phenyl groups in their chemical structure (except ibuprofen, which only contains one phenyl group), this may explain the potent scavenging activity found. Notwithstanding, some compounds are capable of redox cycling the metal ion required for hydroxyl generation, thus increasing radical production, exhibiting a pro-oxidant activity.³⁶ With a view to evaluating the pro-oxidant potential of the APA NSAIDs, we modified the method by omitting ascorbic acid. Additionally, the assay performed in the absence of EDTA is also useful because it indicates the potential of the tested compounds to chelate iron ions. Fe(III) chelators will decrease the amount of thiobarbituric-reactive substances formed from deoxyribose as a result of Fe(III) removal from the sugar.³⁷ However, the assay performed without ascorbic acid or EDTA did not indicate any pro-oxidant and/or iron chelation activity respectively, for the studied NSAIDs, since no increased malonaldehyde formation was observed in their presence.

During inflammation, the activation of mast cells, macrophages, eosinophils, and neutrophils generate O₂⁻, with NADPH oxidase playing an important role.^{38,39} The importance of O₂⁻ scavenging activity in the therapy of inflammation has been well established.¹⁰ In the present study, it was observed that flurbiprofen, fenbufen, ketoprofen and indoprofen prevented the O₂⁻-induced reduction of NBT in a concentration dependent manner, indoprofen being the most potent, while no activity was observed for fenoprofen and naproxen.

Much of the damage caused by increased generation of $O_2^{\cdot-}$ and H_2O_2 in the inflammatory sites arises from their transition metal-dependent conversion into the highly reactive $HO\cdot$ ⁴⁰⁾ and into $HOCl$ by MPO catalysis.⁴¹⁾ In the present study, the scavenging effects observed for H_2O_2 were rather similar to those obtained for $O_2^{\cdot-}$ with the exception of naproxen, which exerted no effect against the later. On the other hand, no scavenging activity against $HOCl$ was observed for any of the studied NSAIDs. The lack of $HOCl$ scavenging activity agrees with a previous reported study, in which ibuprofen, naproxen, flurbiprofen and ketoprofen did not display scavenging activity for $HOCl$, using taurine chlorination as the detecting system.⁴²⁾

Alternatively, NSAIDs may also prevent the $HOCl$ damaging effects by inhibiting its production by MPO. To verify this possibility, the putative inhibitory effect of the tested APA NSAIDs on MPO activity was tested using both the purified enzyme and its activity in human neutrophils. Using the purified enzyme, an effective inhibitory activity was only observed for indoprofen. Notably, this is the only APA NSAID bearing an indolic structure. Other indolic NSAIDs like indomethacin and acemetacin have been shown to be strong MPO inhibitors.⁴²⁾ The absence of an inhibitory effect against purified MPO had already been observed for ibuprofen, ketoprofen and flurbiprofen.⁴²⁾ However, these authors observed an inhibitory effect for naproxen, which was not confirmed in the present study. The reason for this discrepancy is not clear as yet, though different methodologies were used to evaluate MPO activity.

The majority of the neutrophil burst and the purified human MPO assays as well as those performed in the present study use the oxidation of luminol or lucigenin as the measured endpoints. While luminol can be oxidized by several ROS,¹⁶⁾ lucigenin is oxidized mainly by $O_2^{\cdot-}$ ^{16,43,44)} or H_2O_2 .²⁰⁾ When the neutrophil burst was measured by monitoring the $O_2^{\cdot-}$ -induced oxidation of lucigenin, the inhibitory activity of flurbiprofen, fenbufen, ketoprofen, and indoprofen, was displayed in a concentration dependent manner with ketoprofen and indoprofen being the most potent, while fenoprofen, ibuprofen and naproxen exerted no effect. Thus, these results may be explained, not as an inhibitory effect concerning neutrophil activation but rather as a reflection of a scavenging activity against neutrophil derived $O_2^{\cdot-}$ and/or H_2O_2 since the cellular and non-cellular assays are almost superimposed. Noteworthy, the observed IC_{50} s are much lower in the neutrophil assays (down to 10 times lower), which is a strong reminder that the concentrations tested *in vitro* may not be as important as the identification of the potential to scavenge reactive species. When the oxidation of luminol was used as the measured endpoint, it was observed that from all the tested compounds, only fenbufen was effective in preventing the neutrophil burst, but even here only a weak inhibitory activity was observed. These results agree with those previously reported for ibuprofen, flurbiprofen, ketoprofen and naproxen.²⁵⁾ It is generally agreed that luminol chemiluminescence in neutrophils results from intra and extra cellular events and depends mainly on the reactions of the MPO- H_2O_2 -Cl system (namely $HOCl$).^{16,25)} The absence of effect observed for indoprofen (shown to be inhibitor of MPO in the non-cellular assay) using neutrophils could be explained by a different sensitivity between the cellular and

non-cellular assays. Indeed, a higher concentration of ABAH (specific MPO inhibitor) is required for an effective inhibitory effect using neutrophils (IC_{50} of $18 \pm 4 \mu M$) compared to that for purified MPO (IC_{50} of $0.34 \pm 0.01 \mu M$).

The interaction of APA NSAIDs with human neutrophil functions has previously been tested *in vitro* and *in vivo*. The aggregation and degranulation of human neutrophils in response to the chemo attractant *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was shown to be inhibited at therapeutic concentrations by ibuprofen,^{45,46)} flurbiprofen and ketoprofen.⁴⁵⁾ On the other hand, ibuprofen had no effect on fMLP-induced $O_2^{\cdot-}$ generation at therapeutic concentrations⁴⁶⁾ and failed to show any effect against PMA stimulation of these cells.^{46,47)} Notably, neutrophils from patients taking therapeutic doses of ibuprofen showed profiles of inhibited responses to fMLP similar to those observed *in vitro*.⁴⁶⁾ Therefore, even if APA NSAIDs do not prevent the generation of ROS, their inhibition of aggregation and degranulation of human neutrophils may also contribute to their anti-inflammatory effects.

The $ROO\cdot$ scavenging activity may be of extreme value since, during the inflammatory processes, the walls of affected tissue cells involved become amenable to lipid peroxidation, explaining the increase of lipid peroxidation marker compounds in inflammatory diseases.¹⁹⁾ It was observed that $ROO\cdot$ is effectively scavenged by naproxen, while the other compounds were much less active against this ROS. Of all compounds tested, naproxen is the only one bearing a naphthalene moiety. Curiously, the β -blocker propranolol, which is another compound with a non-hydroxylated naphthalene moiety, has been shown to be a potent scavenger of $ROO\cdot$, with an ORAC value similar to that of naproxen.²⁷⁾ Although the chemical reactivity between $ROO\cdot$ and the naphthalene moiety is not clear as yet, it may be of great interest to further study it in the future.

RNS, namely $\cdot NO$ and $ONOO^-$ are also produced in the inflammatory sites and may contribute to the deleterious effects of inflammation. In fact, $\cdot NO$ is produced by nitric oxide synthases (NOS), which are typically induced during inflammation.⁴⁸⁾ Possible pro-inflammatory effects of $\cdot NO$ include augmentation of vascular permeability of inflamed tissues, the generation of other destructive free radicals (namely $ONOO^-$ by reaction with $O_2^{\cdot-}$), the induction of cyclooxygenase as well as angiogenic and inflammatory cytokines, activation of matrix metalloprotease and induction of chondrocyte apoptosis.⁴⁹⁾ $ONOO^-$ itself is a relatively long-lived cytotoxicant with strong oxidizing properties towards various cellular constituents, including sulphhydryls, lipids, amino acids, and nucleotides.⁵⁰⁾ In the present study, indoprofen was shown to be a potent scavenger of $\cdot NO$ and $ONOO^-$ as was naproxen, although to a lesser extent, while fenbufen, fenoprofen, flurbiprofen, ibuprofen and ketoprofen only exerted residual effects. Again, it appears that the indolic structure of indoprofen is a determining factor for its RNS scavenging activity, in line with that observed for other indolic NSAIDs.¹⁹⁾ In a previous study,⁵¹⁾ the scavenging effect of various NSAIDs for $\cdot NO$ was directly evaluated *in vitro*, by ESR spectrometry, using carboxy-PTIO as the $\cdot NO$ detecting reagent and NOC-7 as the $\cdot NO$ donor. In this study, both ibuprofen and ketoprofen displayed potent scavenging effects, well within therapeutic concentrations (IC_{50} s of 48

and 67 μM respectively), while naproxen only had low activity within the tested concentrations.⁵¹⁾ The differences in the effective concentrations observed, compared to the present study, strongly suggests that *in vitro* screening systems are much more important for determining relative potencies among putative scavengers than for extrapolating effective concentrations. In accordance with this, ketoprofen and ibuprofen were demonstrated to protect B65 neuronal cells (a rat neuroblastoma cell line) against 'NO induced cell death, again within therapeutic concentrations (333 nM—333 μM).⁵¹⁾ It is worth mentioning that the interference with 'NO by NSAIDs may also be due to the inhibition of inducible NO synthase (iNOS), which was already demonstrated for APA NSAIDs. Indeed, in an *in vitro* model involving RAW 264.7 macrophages, it was demonstrated that iNOS mRNA expression is suppressed by flurbiprofen and ibuprofen,^{52,53)} an effect related to the inhibition of transcription factor activation like NF- κB and AP-1, resulting in diminished formation of pro-inflammatory factors like iNOS and TNF- α .^{53,54)} However, the putative beneficial effects resulting from lower levels of 'NO should be interpreted cautiously since the possible depletion of physiological 'NO concentrations may also be harmful for the patient. 'NO, at physiological levels is mainly involved in homeostatic biochemical and physiological processes such as signal transduction, neurotransmission, smooth muscle relaxation, peristalsis, gastroprotective effects, inhibition of platelet aggregation, blood pressure modulation, immune system control as well as learning and memory.^{55,56)} Therefore, a possible variation of 'NO physiological levels by APA NSAIDs could potentially affect these physiological processes.

In conclusion, the results obtained in the present work demonstrate that under the present experimental conditions, many of the studied APA NSAIDs showed to be scavengers of O_2^- (fenbufen \approx flurbiprofen \approx indoprofen \approx ketoprofen), H_2O_2 (ketoprofen \approx indoprofen \approx fenbufen $>$ flurbiprofen $>$ naproxen), HO^\cdot (fenoprofen \approx ibuprofen $>$ fenbufen \approx flurbiprofen $>$ ketoprofen $>$ indoprofen \approx naproxen), 'NO (indoprofen $>$ naproxen), ONOO $^-$ (indoprofen $>$ naproxen $>$ fenoprofen \approx flurbiprofen \approx ibuprofen), to inhibit MPO activity (indoprofen) and to scavenge human neutrophil derived ROS (ketoprofen $>$ indoprofen $>$ fenbufen $>$ flurbiprofen). The observed effects, if confirmed *in vivo*, may strongly contribute to the anti-inflammatory therapeutical activity of these NSAIDs.

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