Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 496 (2004) 55–61

www.elsevier.com/locate/ejphar

In vitro comparative assessment of the scavenging activity against three reactive oxygen species of non-steroidal anti-inflammatory drugs from the oxicam and sulfoanilide families

Pierre Van Antwerpen, Jean Nève*

Laboratory of Pharmaceutical Chemistry, Institute of Pharmacy, Université Libre de Bruxelles, Campus Plaine 205-5, B-1050 Brussels, Belgium

Received 24 May 2004

Abstract

The study of the interaction of non-steroidal anti-inflammatory drugs (NSAIDs) with several reactive oxygen species is of great interest in inflammatory conditions where an uncontrolled release of these potentially damaging intermediates has been documented. This study focused on the scavenging of three species (hydroxyl radical, hydrogen peroxide and hypochlorous acid) with several members of the oxicam family and with the sulfoanilide nimesulide. Reaction with hydroxyl radical was assessed by the modified deoxyribose assay, and rate constants were calculated showing values between 0.8 and 1.1×10^{10} M^{-1} s^{-1} for oxicams and of about 0.9×10^{10} M^{-1} s^{-1} for nimesulide and ibuprofen. These were consistent with those of the literature but in the same range as those for other NSAIDs and for several thiol-containing molecules. The study of hydrogen peroxide scavenging by the horseradish peroxidase (HRP) assay lacked specificity but no interaction could be evidenced by the glutathione peroxidase assay. The scavenging of hypochlorous acid was finally investigated by the recently developed *para*-aminobenzoic acid assay which demonstrated better performances for meloxicam (1.7×10^4 M^{-1} s^{-1}) as compared to the other oxicams (tenoxicam: 4.0×10^4 M^{-1} s^{-1}, piroxicam: 3.6×10^4 M^{-1} s^{-1}, lornoxicam: 4.3×10^4 M^{-1} s^{-1}) and nimesulide (2.3×10^3 M^{-1} s^{-1}). These rate constants were, however, lower than those for thiol-containing molecules and ascorbate. These results suggest that the antioxidant properties of NSAIDs could be influenced by a proper pharmacomodulation as far as the scavenging of hypochlorous acid is concerned while the interest is quite limited for the scavenging of hydroxyl radical.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Antioxidant activity; Non-steroidal anti-inflammatory drug; Hydroxyl radical; Hydrogen peroxide; Hypochlorous acid

1. Introduction

The human body is equipped with a complete arsenal of defences against external and internal aggressions. Those against the so-called reactive oxygen species such as superoxide anion ($O_2^{-\cdot}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) are crucial in inflammatory and antibacterial responses where they participate in physiological processes such as arachidonic acid cascade and phagocytosis (Halliwell and Gutteridge, 1999). The concentrations of these metabolic intermediates are actually kept under strict control by the activity of a complex defence system including enzymes (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic species such as glutathione, ascorbate, tocopherol or retinol. However, an uncontrolled production of reactive oxygen species is liable to occur in several conditions leading to a situation known as "oxidative stress" where the highly reactive species can attack many essential biomolecules (protein, DNA, RNA, lipids) and even cell structures, causing oxidative damages. As a matter of fact, many pathological processes are initiated or aggravated by such processes (Greene and Paller, 1991; Reiter, 1995; Galleron et al., 1999; Kampf and Roomans, 2001).

For several years, pharmacological investigations of exogenous compounds or therapeutical agents have focused on a possible interaction with reactive oxygen species (Aruoma et al., 1991; Kauder and Watts, 1996; Nève et al., 2001) in order to assess their capacity to prevent or minimize free radical damages to biological targets. As the inflammatory process is typically a situation of increased reactive oxygen species production that may aggravate the

* Corresponding author. Tel.: +32-2-650-51-77; fax: +32-2-650-52-49.
E-mail address: jneve@ulb.ac.be (J. Nève).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.06.017

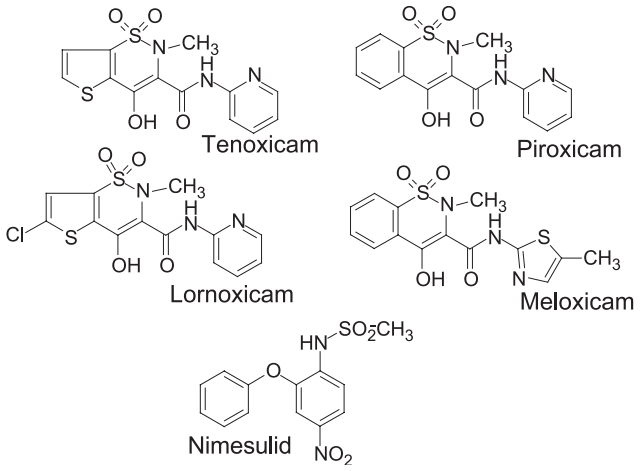

Fig. 1. Structures of the examined molecules.

pathological manifestations of the diseases (Halliwell and Gutteridge, 1999), several commonly used or potential non-steroidal anti-inflammatory drugs (NSAIDs) have been tested as potential free radical scavengers. Their capacity to react with some of these species and therefore to prevent oxidative damage in vivo has also been comparatively determined (Parij et al., 1995; Parij and Nève, 1996; Nève et al., 2001). More recent drugs such as several of the oxicam family (tenoxicam, lornoxicam, piroxicam and meloxicam) and the sulfoanilide nimesulide (Fig. 1) are nowadays rather frequently prescribed because they specifically inhibit the 2-isoform of cyclo-oxygenase, which could imply a lower incidence of undesirable side-effects such as gastric intolerance. It is therefore of interest to more precisely assess their capacity to interact with the main reactive oxygen species. This is precisely the scope of the present study that investigates their interactions with $^{\cdot}OH$, H_2O_2 and HOCl, taking ibuprofen as a reference molecule since its activity has already been largely documented in previous studies (Aruoma and Halliwell, 1988; Parij et al., 1995; Parij and Nève, 1996; Nève et al., 2001). Furthermore, some structure–activity relationships will be examined and the scavenging activity against reactive oxygen species will be discussed in comparison with literature results for non-selective cyclo-oxygenase inhibitors.

2. Materials and methods

2.1. Chemicals and apparatus

Tenoxicam (Roche, Basle, Switzerland), lornoxicam (Nycomed, Linz, Austria), piroxicam (Pfizer, Brussels, Belgium), meloxicam (Boehringer Ingelheim, Biberach, Germany), nimesulide (Helsinn, Biasca, Switzerland) and ibuprofen (Boots, Nottingham, England) were the tested molecules. They were dissolved in a Na_2CO_3 aqueous solution at the lowest possible concentration of the alkaline agent; thereafter, the pH of the solution was rapidly adjusted to 7.4. Deoxyribose, *para*-aminobenzoic acid, NaOCl, 5,5′-dithio-bis-2-nitro-benzoic acid (DTNB), guaiacol, Trizma base, horseradish peroxidase (HRP) type VI-A (230–330 U/mg protein) and glutathione peroxidase from bovine erythrocytes were purchased from Sigma (Bornem, Belgium). The following reagents were obtained from VWR International (Leuven, Belgium): KI, ammonium acetate, acetic acid, NaCl, KH_2PO_4 and KOH. Finally, LC analytical-grade acetonitrile was purchased from VWR International (Fontenay sous Bois, France). All reagents were of analytical grade.

The measurement of ultra-violet and visible absorbance was performed on a Shimadzu UV-160 (Antwerp, Belgium). The 1100 series LC system coupled with a fluorimeter from Agilent (Palo Alto, CA, USA) was used in the *para*-aminobenzoic acid assay. The chromatographic system consisted in a flow rate of 1 ml/min, an Alltima C18 column 15 cm × 4.6 mm, 5 μm with a guard column, 5 cm × 4.6 mm, 5 μm (Alltech, Deerfield, IL), and the mobile phase was a mixture of acetate buffer (acetic acid 5% v/v and acetate ammonium 2 g/l) and acetonitrile, the proportion depending on the drug (80:20 to 60:40).

2.2. Deoxyribose assay

The reactivity of selected NSAIDs with $^{\cdot}OH$ was assessed by the deoxyribose assay (Halliwell et al., 1981, 1987; Parij et al., 1995). The reaction mixture in a final volume of 1.2 ml contained the following reagents at the stated concentrations: deoxyribose (15 mmol/l), a pH 7.4 KH_2PO_4/KOH buffer (20 mmol/l), $FeCl_3$ (20 μmol/l), EDTA (100 μmol/l), H_2O_2 (2.8 mmol/l), the drug (0 to 0.8 mmol/l) and ascorbic acid (200 μmol/l). All solutions were mixed up in deoxygenated water. $FeCl_3$ and EDTA were premixed prior to their addition and ascorbic acid was finally added to start the reaction. After incubation during 1 h at 37 °C, 1 ml of thiobarbituric acid (TBA, 1% w/v) in NaOH 0.05 mM and 1 ml of trichloracetic acid (TCA, 2.8% w/v) in water were added, and the mixture was heated to 100 °C for 20 min. After cooling, 5 ml of water was added, and the absorbance of the pink chromogen formed was measured at 532 nm. The graph of $1/A_{532}$ vs. the drug concentrations gives a linear plot, allowing to calculate the second-order rate constant for the reaction of the drug with $^{\cdot}OH$.

2.3. Horseradish peroxidase assay and interaction of the drug with horseradish peroxidase

The scavenging activity against H_2O_2 was firstly assessed by the HRP assay (Aruoma et al., 1989), as modified by Parij and Nève (1996). The reaction mixture in a final volume of 3 ml contained the following reagents at the stated final concentrations: a pH 7.4 KH_2PO_4/KOH buffer (20 mmol/l), H_2O_2 (0.5 mmol/l), drug (0 to 4 mmol/l) and guaiacol (0.01% v/v). This mixture was incubated for 30 min at 37 °C. After the addition of 150 μl of HRP buffered

solution (0.05% w/v), the absorbance of the resulting brown chromogen was monitored at 436 nm during 20 min for each concentration of the drug.

The interaction of the drug with horseradish peroxidase was studied at 25 °C by a method derived from Yamada et al. (1991). The spectrum of the native enzyme (HRP^{3+}, λ_{max}: 500 and 645 nm) was first recorded with a reaction mixture containing 6.0 mg of native enzyme ($\approx 4.65 \times 10^{-5}$ M) in 2.8 ml of 20 mM KH_2PO_4/KOH buffer at pH 7.0 containing 150 mM NaCl. One hundred microliters of H_2O_2 solution (100 μM) was then added and the spectrum of the compound formed was recorded after 1 min and every 20 min. At the same time, 100 μl of H_2O_2 solution (100 μM) was also added at the reaction mixture described above and the spectrum was recorded before the addition of 100 μl of the drug solution (2 mM) and every 2 min.

2.4. Glutathione peroxidase assay

The glutathione peroxidase (GSHPx) assay was performed according to Miles and Grisham (1994) to further study the antioxidant capacity against H_2O_2. The reaction mixture in a final volume of 1 ml contained the following reagents at the stated final concentrations: a pH 7.4 KH_2PO_4/KOH buffer (10 mmol/l), H_2O_2 (0.2 mmol/l) and the drug (0 to 4 mmol/l). After an incubation of 1 h at 37 °C, 100 μl of both glutathione (1 mM) and GSHPx buffered solution in pH 8.5 Tris–HCl (1 U/ml) was added and re-incubated 5 min at 37 °C. The mixture was then diluted 10 times with pH 8.5 Tris–HCl buffer (0.2 M) and 1 ml of DTNB in the same Tris buffer (1 mM) was added. After 5 min at 37 °C, the absorbance at 412 nm was measured.

2.5. para-Aminobenzoic acid assay

The reactivity of the drugs with HOCl was assessed by the *para*-aminobenzoic acid method such as the one recently developed by us (Van Antwerpen et al., 2004). The reaction mixture contained in 2 ml the following reagents at the stated final concentrations: a pH 7.4 KH_2PO_4/KOH buffer (10 mmol/l), *para*-aminobenzoic acid (30 μmol/l), the drug (50 to 400 μmol/l) and HOCl (29 μmol/l). The mixture was incubated at 37 °C before the addition of HOCl that initiated the reaction. After 5 more minutes at 37 °C, 40 μl of the mixture was injected in the LC system with a mobile phase depending on the studied drug and a fluorimetric signal (*p*-aminobenzoic acid) was measured at an excitation and an emission wavelength of 280 and 340 nm, respectively.

3. Results

3.1. Deoxyribose assay

The deoxyribose assay is based on a Fenton reaction that produces $^{\cdot}OH$ in the presence of H_2O_2 and Fe(II). Starting from Fe(III), ascorbic acid initiates the reaction by reduction of Fe(III) (Eq. (1)) and the production of $^{\cdot}OH$ by the Fenton reaction (Eq. (2)) oxidizes deoxyribose and gives rise to malondialdehyde (MDA, Eq. (3)). This derivative can be determined by the formation of a chromogen after reaction with thiobarbituric acid (TBA, Eq. (4)):

$$Fe^{3+} + \text{ascorbic acid} \rightarrow Fe^{2+} + \text{ascorbyl radical} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \quad (2)$$

$$^{\cdot}OH + \text{Deoxyribose} \rightarrow \text{MDA} \quad (3)$$

$$\text{MDA} + 2\ \text{TBA} \rightarrow \text{Chromogen}\ (A_{\lambda 532nm}) \quad (4)$$

Such as previously observed by some of us with several examined NSAIDs (Parij et al., 1995), drugs of the oxicam family and nimesulide behaved as pro-oxidants in this system as they were able to reduce iron(III). A modified procedure therefore had to be used to avoid the interferences, and the concentration of ascorbic acid had to be increased up to 200 μM. In these conditions, linear plots were obtained (Fig. 2). Moreover, such as already observed by Parij et al. (1995), the concentration of deoxyribose had to be increased up to 15 mM. The slope of the linear plot of $1/A_{532}$ vs. drug concentrations (S) permitted to calculate the second-order rate constant for the drugs, k_s (Halliwell et al., 1981):

$$1/A = 1/A^\circ + k_s\ [S]/k_{\text{deoxyribose}}\ [\text{Deoxyribose}]A^\circ \quad (5)$$

In this equation, k_s and $k_{\text{deoxyribose}}$ are the rate constants for the drug and for deoxyribose, respectively, A is the absorbance measured when the drug is present and A°, the

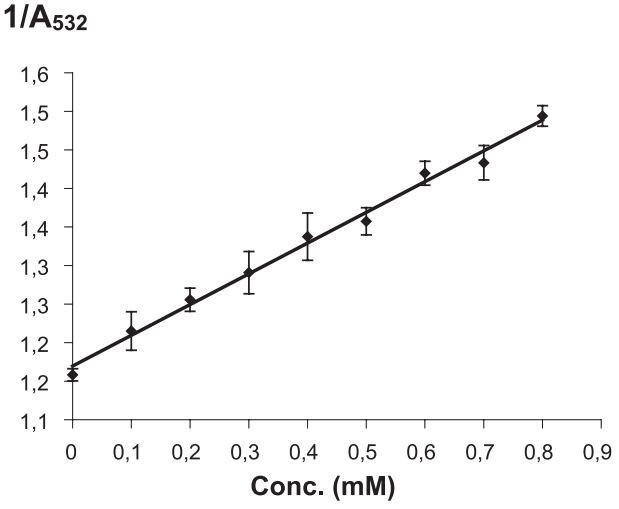

Fig. 2. Effect of meloxicam in the deoxyribose system with 15 mM deoxyribose and 200 μM ascorbic acid. Each point is the mean value of three independent measurements.

absorbance when no drug is present. The k_s can be calculated from the slope by replacing $k_{deoxyribose}$ by its experimentally determined value, $3.1 \times 10^9\ M^{-1}\ s^{-1}$ (Ebert et al., 1965). Table 1 gives the experimental values obtained with the different studied drugs as well as several literature values for comparison.

3.2. Horseradish peroxidase assay

The horseradish peroxidase system is based on the oxidation by H_2O_2 of the native enzyme (HRP^{3+}) into compounds I (HRP^{5+}) and II (HRP^{4+}). The first is very unstable and rapidly transformed into compound II. A substrate that reacts with the last compound restores the native enzyme. In the selected system, guaiacol acts as a substrate giving an oxidation product absorbing at 436 nm. Any compound added to the system and able to react with H_2O_2 will decrease the colour formation by reducing the quantity of compound II. The following reactions take place:

$$H_2O_2 + HRP^{3+} \rightarrow HRP^{5+} + H_2O \quad (6)$$

$$HRP^{5+}\ (\text{compound I}) + \text{guaiacol} \xrightarrow{\text{fast}} HRP^{4+}\ (\text{compound II}) + \text{Ox. Guaiacol} \quad (7)$$

$$HRP^{4+} + \text{guaiacol} \underset{\text{slow}}{\rightarrow} HRP^{3+} + \text{Ox. Guaiacol} \quad (8)$$

Before any measurement, it is important to check the possible occurrence of a direct interaction between the studied NSAIDs and the enzyme. Indeed, some drugs can directly interact with compound II, making the assay completely unsuitable (Yamada et al., 1991; Parij and Nève, 1996). This was precisely the case for the presently examined drugs. Comparison of the time needed for transformation of compound II into the native enzyme in the absence or presence of drugs showed that about 2 h was necessary in the absence of drugs while the same phenomenon only took a few minutes in the presence of drugs. The demonstration that compound II is able to oxidize the drug renders the method inadequate.

Table 1
Second-order rate constant $10^{10}\ M^{-1}\ s^{-1}$ of the reaction of NSAIDs with $^{\cdot}OH$ determined by the deoxyribose assay using two different ascorbic acid (100 or 200 μM) and deoxyribose (2.8 and 15 mM) concentrations

Drug	This study	Literature		
	15 mM, 200 μM	15 mM, 200 μM	15 mM, 100 μM	2.8 mM, 100 μM
Ibuprofen	0.86 ± 0.06		1.3 ± 0.1[a]	1.5–1.8[b]
Tenoxicam	0.84 ± 0.02	0.8 ± 0.1[a]		
Lornoxicam	0.99 ± 0.07[z]			
Piroxicam	1.1 ± 0.1[y]	1.17 ± 0.04[a]		0.5–0.7[b]
Meloxicam	1.1 ± 0.2			
Nimesulide	0.87 ± 0.08			
Flurbiprofen			0.78 ± 0.09[a]	0.8±0.1[a]
Naproxen			1.119 ± 0.006[a]	1.2–2.2[b]
Flufenamic acid			2.94 ± 0.07[a]	1.3–1.9[b]
Indometacin			1.8 ± 0.1[a]	1.0–1.2[b]
Niflumic acid			1.7 ± 0.2[a]	
Captopril				3.6±0.2[c]
N-Acetylcystein				1.4–2.3[d,e]
Nacystelyn				1.9±0.2[d]

Mean ± S.D. for at least three daily measurements; [y] is significantly different than ibuprofen, tenoxicam and nimesulide ($P<0.05$); [z] is significantly different than tenoxicam ($P<0.05$). Literature values are from (a) Parij et al. (1995), (b) Aruoma and Halliwell (1988), (c) Aruoma et al. (1990), (d) Vanberbist et al. (1996) and (e) Aruoma et al. (1991).

3.3. Glutathione peroxidase assay

Glutathione peroxidase (GSHPx) has an absolute specificity for glutathione (GSH), which is an electron-donating substrate in the reaction with H_2O_2. Other compounds would theoretically not interact with this enzyme, which ensures the specificity of the method and avoids problems described in the horseradish peroxidase assay.

$$H_2O_2 + 2\ GSH \underset{GSHPx}{\rightarrow} H_2O + GSSG \quad (9)$$

$$GSH + DTNB \rightarrow TNB + GSTNB \quad (10)$$

According to Miles and Grisham (1994), the quantity of GSH consumed by the reaction can be indirectly measured by DTNB which gives rise to 5-thio-2-nitrobenzoic acid absorbing at 412 nm (Eqs. (9) and (10)). The possible interaction of NSAIDs with H_2O_2 will therefore attenuate the oxidation of GSH and increase the absorbance. The application of this assay to the studied NSAIDs showed no interaction with H_2O_2 (200 μM) for concentrations around 4 mM. The activity of the drugs against H_2O_2 can therefore be considered as undetectable in these conditions.

3.4. para-Aminobenzoic assay

para-Aminobenzoic acid interacts with HOCl to produce 3-chloro-4-aminobenzoic acid (She et al., 1997). This reaction can be monitored by fluorimetry. It has been developed to assess the antioxidant activity of some NSAIDs that interfered in existing procedures for assessing HOCl scavenging such as the taurine chlorination system (Van Antwerpen et al., 2004). A competition is installed between *para*-aminobenzoic acid and the drug for HOCl, which can be expressed by:

$$1/(\text{Int}^{\circ} - \text{Int}) = 1/(\text{Int}^{\circ} - \text{Int}') + k_s\ [S]/k_{PABA} \times\ [PABA]\ (1/(\text{Int}^{\circ} - \text{Int}')) \quad (11)$$

In this equation, k_s and k_{PABA} are the rate constant of the reaction of NSAID ([S]) and *para*-aminobenzoic acid with

Table 2
Second-order rate constants (M^{-1} s^{-1}) for the reaction of NSAIDs with HOCl

Drugs	*p*-Aminobenzoic acid method[a]	Literature[b,c]	
	k_s, pH 7.4	pH	k_s
Ibuprofen	ND		
Tenoxicam	$4.0 \pm 0.7 \times 10^3$		
Lornoxicam	$4.3 \pm 0.7 \times 10^3$		
Piroxicam	$3.6 \pm 0.7 \times 10^3$		
Meloxicam	$1.7 \pm 0.3 \times 10^4$ [z]		
Nimesulide	$2.3 \pm 0.6 \times 10^2$ [z]		
Taurine		7.0	$4.8 \pm 0.1 \times 10^5$ [b]
Glutathione		7.4	$>10^7$ [b,c]
Thiols		7.4	$>10^7$ [b,c]
Ascorbate		7.4	$\sim 6 \times 10^6$ [b]
Methionine		12.0	$\sim 2 \times 10^4$ [c]
Cysteine		12.0	$\sim 2 \times 10^5$ [c]

ND = No interaction detected.
Literature values are from (a) Van Antwerpen et al. (2004), (b) Folkes et al. (1995) and (c) Peskin and Winterbourn (2001).
[z] Is significantly different ($P < 0.05$).

HOCl, respectively, Int° is the relative intensity of *para*-aminobenzoic acid without HOCl and NSAID, Int′ is the relative intensity of *para*-aminobenzoic acid when it is oxidised by HOCl in absence of drug and Int is the relative intensity of *para*-aminobenzoic acid when it is oxidised in the presence of the NSAID.

The graph of $1/(\text{Int}° - \text{Int})$ vs. [S] gives a linear plot where the slope depends on the second-order rate constant of the NSAIDs. The rate constant k_s can be calculated from this slope since the provided k_{PABA} is known (4.3×10^3 M^{-1} s^{-1}) (Van Antwerpen et al., 2004). However, as numerous oxidation products interfered on the fluorimetric signal, a LC procedure was developed to separate the different products and the method was validated. Table 2 assembles the values of the second-order rate constant for reaction of NSAID with HOCl in the conditions discussed above. Meloxicam appears as a significantly ($P < 0.05$) better antioxidant than the other oxicams that have quite comparable antioxidant capacities and nimesulide, which significantly ($P < 0.05$) is the weakest antioxidant of the studied series of drugs. Ibuprofen has no detectable activity against HOCl.

4. Discussion

The rate constants for reaction of tenoxicam and piroxicam with ·OH are comparable to those obtained by Parij et al. (1995) who used a similar procedure. The lower value obtained for ibuprofen could be explained by the different amount of ascorbic acid presently used (200 μM) instead of 100 μM in the previous study (Table 1). Indeed, the pro-oxidant effect of the studied drugs obliged us to increase the concentration of ascorbic acid up to 200 μM, which decreased the rate constant for ibuprofen. Table 1 shows that oxicams have experimentally determined rate constants in the range 0.84 to 1.1×10^{10} M^{-1} s^{-1}, while nimesulide has a rate constant of 0.87×10^{10} M^{-1} s^{-1}. Piroxicam has a significantly ($P < 0.05$) higher antioxidant activity than tenoxicam, ibuprofen and nimesulide. Moreover, lornoxicam appears as a significantly ($P < 0.05$) better antioxidant than tenoxicam. However, the range of the determined rate constants is not very widespread and the values are quite similar to the ones previously determined for other NSAIDs and for molecules containing a free thiol group, i.e. from 0.8 to 3.6×10^{10} M^{-1} s^{-1} (Table 1) (Aruoma and Halliwell, 1988; Aruoma et al., 1989, 1991; Parij et al., 1995; Vanberbist et al., 1996). Such a statement is rather astonishing considering the different structures. Indeed, the molecules containing a thiol group would have been expected to have remarkable antioxidant capabilities linked to their transformation into sulfoxide or sulfone by the ·OH (Barnes et al., 1996). A lower reactivity would be linked to the attack of the aromatic ring of NSAIDs of the arylacetic, arylpropionic or indolic acids as well as of sulfoanilide such as proposed by Marusawa et al. (2002). Concerning oxicams, a radical attack on the thieno-thiazine (tenoxicam) or benzothiazine (piroxicam) rings is proposed together with a breakage of the enol function, as expected with an intermediary reactivity (Gaudiano et al., 2003; Facino et al., 1996). An attempt to classify the different drugs according to their reactivity with ·OH would give the following classification: thiol-containing molecules>oxicams>other examined NSAIDs. However, this reactive oxygen species actually seems to be one of the most reactive and this quite independently of the chemical structure of the scavenger.

The scavenging of H_2O_2 was measured by the horseradish peroxidase and the glutathione peroxidase assays. The first one is based on the oxidation capacity of this hemoprotein to oxidize many electron-donating substrates (e.g. guaiacol) chosen as detectors in the presence of H_2O_2. When dealing with this assay, an interaction of the drugs with H_2O_2 could actually be observed but, such as previously mentioned for other drugs (Yamada et al., 1991; Parij and Nève, 1996), all derivatives directly reacted with compound II, therefore making the assay completely unsuitable to our purpose. The GSHPx assay was then used as a highly specific method for the determination of interaction with H_2O_2. The assay is based on the specific oxidation of GSH in GSSG and the enzyme has an absolute specificity for its substrate. In these conditions, no interaction of the drugs with H_2O_2 could be detected suggesting that they do not react with this reactive oxygen species in physiological conditions. This is not the case for thiol-containing molecules that interacted with a first-order rate constant of 0.009 to 0.03 min^{-1} (Vanberbist et al., 1996). The present results further illustrate the weak oxidation capacity of H_2O_2 since none of the examined NSAIDs (Parij and Nève, 1996), and even those possessing an enol function (oxicams), is able to react with this species.

The scavenging of HOCl was assessed by the *para*-aminobenzoic acid method based on the oxidation of this compound into a chlorinated product, 3-chloro-4-aminobenzoic acid (She et al., 1997). This property was used to measure the interaction of drugs with HOCl by a simple chemical competition, and the second-order rate constant for the reaction with HOCl could be calculated and compared. Tenoxicam, piroxicam and lornoxicam have no statistically ($P>0.05$) different second-order rate constants. This has to be related to the oxidation of the C3-carbon of the molecules (Ichihara et al., 1985; Van Antwerpen et al., 2004). Meloxicam has a significantly ($P<0.05$) greater rate constant than the other molecules of the same family even if the mechanism of the reaction is similar. Ibuprofen shows no detectable interaction with HOCl while nimesulide has a significantly ($P<0.05$) weaker antioxidant ability. This is linked to an oxidation on C6-carbon of the molecule, which gives rise to a chlorinated product (Van Antwerpen et al., 2004). As compared to thiol-containing molecules, which have second-order rate constant values higher than 10^7 M^{-1} s^{-1} (Folkes et al., 1995; Peskin and Winterbourn, 2001), the presently determined constants (k_s between 2×10^2 and 2×10^4) are very significantly lower, demonstrating that examined oxicams and nimesulide are poor HOCl scavengers. This difference is clearly linked to the presence of the free thiol function (Folkes et al., 1995) when other NSAIDs from the arylacetic, arylpropionic and indolic acids showed no interaction with HOCl (Nève et al., 2001), suggesting they are not physiological scavengers of this species.

In conclusion and as far as the presently examined chemical structures are concerned, it seems obvious that oxicams are more reactive against reactive oxygen species than nimesulide and ibuprofen. This statement is not so pronounced for $^{\cdot}OH$ scavenging and the absence of interaction with H_2O_2 brings no further information concerning this reactive oxygen species. More interesting is the scavenging against HOCl since a substantial difference was observed between the oxicams and nimesulide. A further investigation of their oxidation products clearly demonstrates the importance of the enol function (Ichihara et al., 1985; Van Antwerpen et al., 2004). The oxidation by HOCl was carried out on the C-3 carbon of oxicam with a faster rate than the oxidation of nimesulide, which is a radical attack on the C-6 carbon giving rise to a chlorinated product. Nevertheless, the values of rate constants demonstrate that even if the investigated NSAIDs exert a significant effect at physiological concentrations, i.e. those inhibiting cyclo-oxygenase isoform-II (Warner et al., 1999), they remain poorer scavengers as compared to thiol-containing molecules. Therefore, there is a clear interest to investigate the pharmacomodulation of anti-inflammatory drugs in order to improve their interactions with reactive oxygen species and this mainly in relation to the scavenging activity against HOCl.

References

Aruoma, O.I., Halliwell, B., 1988. The iron-binding and hydroxyl radical scavenging action of anti-inflammatory drugs. Xenobiotica 18, 459–470.

Aruoma, O.I., Halliwell, B., Hoey, B.M., Butler, J., 1989. The antioxidant action of *N*-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide and hypochlorous acid. Free Radic. Biol. Med. 6, 593–597.

Aruoma, O.I., Akanmu, D., Cecchini, R., Halliwell, B., 1991. Evaluation of the ability of the angiotensin-converting enzyme inhibitor captopril to scavenge reactive oxygen species. Chem.-Biol. Interact. 77, 303–314.

Barnes, I., Becker, K., Patroescu, I., 1996. FTIR product study of the OH initiated oxidation of dimethyl sulphide: observation of carbonyl sulphide and dimethyl sulphoxide. Atmos. Environ. 30, 1805–1814.

Ebert, M., Keene, J.P., Swallow, A.J., Baxendale, J.H., 1965. Pulse Radiolysis. Academic Press, London.

Facino, R.M., Carini, M., Saibene, L., 1996. Scavenging of free radicals by tenoxicam: a participating mechanism in the antirheumatic/anti-inflammatory efficacy of the drug. Arch. Pharm. Pharm. Med. Chem. 329, 457–463.

Folkes, L.K., Candeias, P., Wardman, P., 1995. Kinetics and mechanisms of hypochlorous acid reactions. Arch. Biochem. Biophys. 323, 120–126.

Galleron, S., Borderie, D., Ponteziere, Ch., Lemarechal, H., Jambou, M., Roch-Arveiller, M., Ekindjian, O.G., Cals, M.J., 1999. Reactive oxygen species induce apoptosis of synoviocytes in vitro. α-Tocopherol provides no protection. Cell Biol. Int. 23, 637–642.

Gaudiano, M.C., Valvo, L., Bertocchi, P., Manna, L., 2003. RP-HPLC study of the degradation of diclofenac and piroxicam in the presence of hydroxyl radicals. J. Pharm. Biomed. Anal. 2, 151–158.

Greene, E.L., Paller, M.S., 1991. Oxygen free radicals in acute renal failure. Miner. Electrolyte Metab. 17, 124–132.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine, third ed. Clarendon Press, Oxford.

Halliwell, B., Gutteridge, J.M.C., Aruoma, O.I., 1981. The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. Anal. Biochem. 165, 215–219.

Halliwell, B., Grootveld, M., Gutteridge, J.M., 1987. Methods for the measurement of hydroxyl radicals in biochemical systems: deoxyribose degradation and aromatic hydroxylation. Methods Biochem. Anal. 33, 59–90.

Ichihara, S., Tomisawa, H., Fukasawa, H., Tateishi, M., 1985. Involvement of leukocyte peroxidases in the metabolism of tenoxicam. Biochem. Pharmacol. 34, 1337–1338.

Kampf, C., Roomans, G.M., 2001. Effect of hypochlorite on cultured respiratory epithelial cells. Free Radic. Res. 34, 499–511.

Kauder, W.F., Watts, J.A., 1996. Antioxidant properties of dihydropyridines in isolated rat hearts. Biochem. Pharmacol. 51, 811–819.

Marusawa, H., Ichikawa, K., Narita, N., Murakami, H., Ito, K., Tezuka, T., 2002. Hydroxyl radical as a strong electrophilic species. Bioorg. Med. Chem. 10, 2283–2290.

Miles, A.M., Grisham, M.B., 1994. Antioxidant properties of aminosalicylates. Methods Enzymol. 234, 555–572.

Nève, J., Parij, N., Moguilevsky, N., 2001. Inhibition of the myeloperoxidase chlorinating activity by non-steroidal anti-inflammatory drugs investigated with human recombinant enzyme. Eur. J. Pharm. 417, 37–43.

Parij, N., Nève, J., 1996. Nonsteroidal anti-inflammtory drugs interact with horseradish peroxidase in an in vitro assay system for hydrogen peroxide scavenging. Eur. J. Pharm. 311, 259–264.

Parij, N., Nagy, A.M., Nève, J., 1995. Linear and nonlinear competition plots in the deoxyribose assay for determination of rate constants for reaction of non steroidal anti-inflammatory drugs with hydroxyl radical. Free Radic. Res. 23, 571–579.

Peskin, A.V., Winterbourn, C.C., 2001. Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine and ascorbate. Free Radic. Biol. Med. 30, 572–579.

Reiter, R.J., 1995. Oxidative processes and antioxidative defense mechanisms in the aging brain. FASEB J. 9, 526–533.

She, Z.-W., Mays, D.C., Sagone, A.L., Davis, W.B., 1997. Aminobenzoic acid compounds as HOCl traps for activated neutrophils. Free Radic. Biol. Med. 22, 989–998.

Van Antwerpen, P., Gelbcke, M., Dubois, J., Nève, J., 2004. The reactions of oxicam and sulfoanilide non steroidal anti-inflammatory drugs with hypochlorous acid: determination of the rate constants with an assay based on the competition with *para*-aminobenzoic acid chlorination and identification of some oxidation products. Free Radic. Res. 38, 251–258.

Vanberbist, F., Maes, P., Nève, J., 1996. In vitro comparative assessment of the antioxidant activity of nacystelyn against three reactive oxygen species. Arzneim.-Forsch. Drug Res. 46, 783–788.

Warner, T.D., Giuliano, F., Vojnovic, I., Buksa, A., Mitchell, J.A., Vane, J.R., 1999. Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. Proc. Natl. Acad. Sci. U. S. A. 96, 7563–7568.

Yamada, T., Volkmer, C., Grisham, M.B., 1991. The effect of sulfasalazine metabolites on hemoglobin-catalysed lipid peroxidation. Free Radic. Biol. Med. 10, 41–49.