Evaluation of NSAIDs antioxidant activity on lipid peroxidation in splenocyte membranes

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Abstract

Lipid peroxidation disrupts the structural and protective functions of biomembranes, leading to several oxidative stress mediated diseases. In this work, kinetic methods were used to assess the antioxidant activities of non-steroidal anti-inflammatory drugs (NSAIDs) - clonixin, tenoxicam, piroxicam, indoprofen and etodolac - during free radical peroxidation of splenocyte membranes. These studies allow getting insights into drugs effect at the cellular membrane level and a more realistic evaluation of their antioxidant activity, since these assays are commonly executed in aqueous buffer media or with membrane mimetic systems. Lipid peroxidation was initiated using peroxyl radicals (ROO$^{\bullet}$) derived from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and the protective effect of the drugs were assessed using the fluorescent probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) by fluorescence intensity and steady-state anisotropy measurements. The results obtained show that the anti-inflammatory drugs etodolac, piroxicam and tenoxicam (indoprofen and clonixin did not present an evident antioxidant activity) were able to inhibit lipid peroxidation in a concentration dependent manner. Additionally, it is possible to conclude that the protective effect of drugs on the lipid peroxidation is related with their chemical structure, but also with their ability to interact and/or to modify the physical properties of the membrane.

Keywords: NSAIDs, splenocytes, lipid peroxidation, peroxyl radicals, antioxidant activity.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to treat inflammatory diseases, which include a huge amount of disorders and conditions, such as asthma, autoimmune diseases, neurodegenerative diseases and transplant rejection [**1, 2**]. During inflammation, the uncontrolled release of reactive oxygen species (ROS; such as superoxide that reacting with nitric oxide leads to the production of reactive nitrogen species) leads to tissue damage [**2, 3**]. One of the cell components particularly susceptible to ROS are the biomembranes [**4**]. The attack of these species affects membrane functions by inducing continuous lipid peroxidation and this uncontrolled reaction causes or amplifies pathological phenomena [**5, 6**]. Indeed, increased lipid peroxidation markers have been detected, for example, in low-density lipoprotein (LDL) samples or biopsies of patients suffering from rheumatic arthritis, atherosclerosis or inflammatory bowel disease [**7, 8**]. To counteract reactive species and also to limit their damaging effects, living organisms developed complex antioxidant systems [**9, 10**]. However, these endogenous antioxidant defense systems may be decreased in free radicals mediated diseases, increasing the need of exogenous antioxidants. Therefore, increased attention has been focused in the investigation of antioxidant effect of NSAIDs, besides their anti-inflammatory, analgesic and antipyretic activities.

In the current work the protective effect of the NSAIDs etodolac, piroxicam, tenoxicam, clonixin and indoprofen (**Fig. 1**) on lipid peroxidation in splenocyte membranes is evaluated. To initiate the oxidative damage were used Peroxyl radicals (ROO$^{\bullet}$) generated by thermal decomposition of the water-soluble azo compound 2, 2'-azobis(2-amidinopropane) dihydrochloride (AAPH). The evaluation of the antioxidant effect of the NSAIDs against radicals generated in the extracellular environment constitutes an important study since that *in vivo* biomembranes are continuously attacked by free radicals that are produced in the aqueous space of cellular and/or subcellular compartments. Additionally, the ROS under study, ROO$^{\bullet}$, may act as an important initiator of lipid peroxidation *in vivo* [**11, 12**]. AAPH, although not biologically relevant, generates well-defined radicals at a constant rate [**13, 14**], allowing quantitative analyses of drugs antioxidant activity. Indeed, this free radical

initiator has been generally used to estimate drugs antioxidant activity, for instance in homogeneous solution or in the presence of model membranes or LDL [**7, 15-20**]. Moreover, the radicals derived from AAPH are similar to those existing in biological systems [**21**].

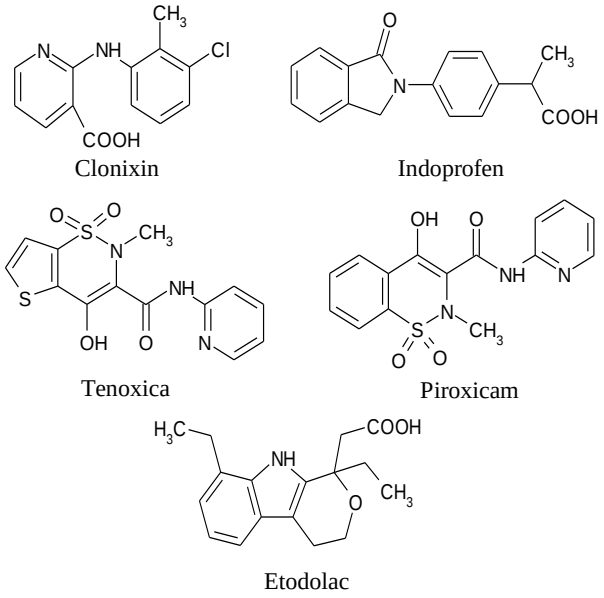

Fig. 1. Chemical structures of clonixin, piroxicam, tenoxicam, etodolac and indoprofen.

To evaluate the NSAIDs effectiveness in avoiding radical chain reactions, the fluorescent probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) was used. Peroxidation was monitored by means of fluorescence intensity decay of the DPH-PA probe, due to its oxidative degradation, and additionally by the study of the membrane fluidity, by steady-state fluorescence anisotropy measurements. Indeed, lipid peroxidation and membrane fluidity are closely related [**22-27**]. Therefore, drugs antioxidant activity can be related with their chemical properties, but also with their ability to interact with and partition into the lipid bilayers. In fact, the importance of cell membrane properties changing by drugs in counteracting peroxidative injury is clearly exhibited in several studies using not free radical scavengers or chain-breaking antioxidants [**28**]. This physical mechanism has been postulated for cholesterol and their derivatives [**29, 30**]. Additionally, a disordered lipid bilayer can lead to a better interaction between antioxidants and lipid radicals [**26**]. Thus, the fluidifying or disordering effect of the NSAIDs on the bilayer [**31-33**] could be responsible for their antioxidant activity or for promoting their free radical scavenging characteristics. Hence, the objective of this work was to study the antilipoperoxidation activity of different NSAIDs, namely etodolac, piroxicam, tenoxicam, clonixin and infoprofen, on splenocyte membranes to obtain a better correlation of their effects at cellular level and to understand as much as possible their pharmacological action. Indeed, despite free radical peroxidation of unsaturated lipids in biomembranes might be the responsible factor of their structural and protective function disruption, leading consequently to important pathological events [**5, 34-36**], there are few works in the literature using cell membranes, and thus more realistic correlations between pharmacological action of drugs with their effects at cellular level are lacking.

Experimental

Reagents

Clonixin and etodolac were generously supplied by their manufacturers, Janssen-Cilag Pharmaceutica and Sofex Farmacêutica Lda, respectively. Diclofenac, tenoxicam, piroxicam, indoprofen, glutamine, penicillin and streptomycin were acquired from Sigma. The fetal calf serum (FCS) was purchased from Gibco. The fluorescent probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenilpropionic acid (DPH-PA) was obtained from Molecular Probes. 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), trolox and dimethylsulfoxide (DMSO) were obtained from Fluka. The other reagents were purchased from Merck (pro analysi); all were used as received. Double-deionised water (conductivity less than 0.1 μS cm^{-1}) was used to prepare Hepes buffer with an ionic strength of 0.1 M by adding NaCl.

Isolation and fluorescence labeling of mouse splenocytes

The isolation and fluorescence labelling of splenocytes was performed as previously described [**31**]. Balb/c mice (Harlen Iberica, Barcelona, Spain), treated and handled in accordance with institutional ethical guidelines, were sacrificed by cervical dislocation. After removal and homogenization of their spleens, splenocytes were isolated and washed (centrifugation conditions: 260 g, 10 min, 4 ºC) in RPMI 1640 culture medium supplemented with glutamine (2 mM), penicillin and streptomycin (100 U/mL and 100 μg/mL, respectively), Hepes (2 mM) and FCS (10 %). To fluorescently label splenocytes membranes, the cells were centrifuged (using the same conditions previously referred), resuspended and adjusted to a concentration of $1x10^7$ cells/mL in Hepes buffer (10 mM, I = 0.1 M, pH 7.4), in order to abolish culture medium interferences, such as intrinsic fluorescence.

A 1:2 dilution of the DPH-PA solution ($4x10^{-5}$ M) in 1% (**v**/**v**) of dimethylsulfoxide in Hepes buffer (10 mM, I = 0.1 M, pH 7.4) was performed with the cellular suspension. To ensure a full integration of the probe into the biomembranes, the cell suspension was left in an ice bath and in the dark for 1 h.

Lipoperoxidation assay

The NSAIDs ability to inhibit lipoperoxidation was quantified according to Lucio *et al.* [**15**], namely through the measurement of fluorescence intensity decay, and fluorescence anisotropy values to infer about the fluidity of the splenocytes membrane.

Labeled cells ($5x10^6$ splenocytes/mL) were incubated with different concentrations of Hepes buffered solutions (10 mM, I = 0.1 M, pH 7.4) of NSAIDs or trolox, used as a reference antioxidant. The final drug concentrations varied between 0-106 μM for tenoxicam, 0-50 μM for piroxicam, 0-169 μM for etodolac, 0-170 μM for indoprofen, 0-222 μM for clonixin and 0-18 μM for trolox. After incubation in the dark and with continuous stirring for 10 min in a thermostatted holder (37.0 ± 0.1 ºC), AAPH in Hepes buffer (15 mM) was added to initiate lipid peroxidation. The solution of AAPH was freshly prepared before experiments. Controls were prepared as described, but without drugs.

The fluorescence intensity and steady-state anisotropy monitoring were performed for 120 min, at 37 ºC, in a Perkin-Elmer LS 50B steady-state fluorescence spectrometer at excitation and emission wavelengths of 384 nm and 435 nm, respectively. At least three independent experiments were performed.

Data analysis

The fluorescence intensity and anisotropy values obtained in the absence or in the presence of different concentrations of NSAIDs or trolox were converted to relative data. For that, the fluorescence intensity or anisotropy values obtained at a particular time were divided by the fluorescence intensity or anisotropy values acquired at 0 min. Then, was determined the area under the curve (AUC) in a plot of relative fluorescence or anisotropy *versus* time, for control (without NSAIDs or trolox) and each drug concentration tested. After that, it was possible to analyse the results obtained from fluorescence decay studies using the following equation: $(AUC_{NSAIDs/Trolox}-AUC_{Control})/AUC_{Control}$. For the anisotropy studies the numerator was inverted to avoid achieving negative values: $(AUC_{Control} - AUC_{NSAIDs/Trolox})/AUC_{Control}$. Indeed, the AUC value of the control is the highest possible, since peroxidation results in higher anisotropy data. In fact, in the control a complete oxidation of the system can occur, reaching anisotropy the maximum value.

The linear fitting of the fluorescence intensity and anisotropy data versus increasing concentrations of NSAIDs or trolox allowed the calculation of the compounds IC_{15} (concentration, in μM, to obtain a result of 15% in the previous equations) values.

Results and discussion

The induction of lipid peroxidation in splenocyte membranes by peroxyl radicals, at physiological pH, using the lipophilic DPH-PA probe, allowed evaluating etodolac, clonixin, piroxicam, tenoxicam and indoprofen antioxidant activity against this oxidative process. On the one hand, membrane peroxidation originates a fluorescence intensity decay of the probe, due to the presence in its chemical structure of a conjugated double bound susceptible to free radical

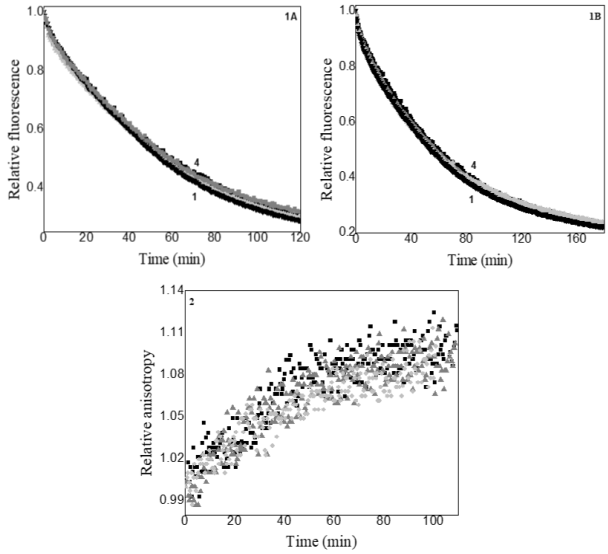

Fig. 2. [1] Relative fluorescence intensity of the DPH-PA probe observed in splenocyte membranes peroxidation induced by AAPH (15 mM), at 37 ºC, in the presence of different concentrations of clonixin (A: (1) 0; (2) 55; (3) 111; (4) 222 μM) and indoprofen (B: (1) 0; (2) 24; (3) 85; (4) 170 μM). [2] Relative anisotropy obtained for control (■) and for clonixin (▲; 222 μM) and indoprofen (♦; 170 μM), in the same experimental conditions described for 1.

species. On the other hand, membrane peroxidation leads to an increase in membrane rigidity, due to the oxidation of the double bounds [**26, 37-39**]. Consequently, the higher the ability of a compound to circumvent the probe fluorescence intensity decay or the increment of the membrane anisotropy the higher is its antioxidant activity. Therefore, the ability of NSAIDs and trolox in counteracting lipid peroxidation initiated by $ROO^{\bullet}$ at 37 ºC can be easily observed from the comparison of the decay of fluorescence intensity or from an increase in the anisotropy values (rigidity of the membrane) in the presence of increasing concentrations of NSAIDs or trolox with the results obtained in the control assay. For example, from **Fig. 2** it is possible to conclude that clonixin and indoprofen did not present an evident antioxidant activity under the experimental conditions of this study once that for all tested concentrations, their fluorescence intensity/increase in anisotropy profile was always similar to the control. Contrariwise, etodolac, tenoxicam, piroxicam and trolox presented a measurable antioxidant activity against lipid peroxidation in splenocyte membranes, in a concentration dependent manner. **Fig. 3** shows an example of concentration dependent peroxidation inhibition obtained in the presence of etodolac and trolox.

A better comparison of the antioxidant efficiency of each NSAID and trolox can be obtained through their IC_{15} values analyses. These data were calculated from the linear fit equations of % $(AUC_{AINE/Trolox}-AUC_{Control})/AUC_{Control}$ or % $(AUC_{Control}-AUC_{AINE/Trolox})/AUC_{Control}$ *versus* compound concentration (μM) for fluorescence and anisotropy measurements, respectively (**Fig. 4**). Comparing the

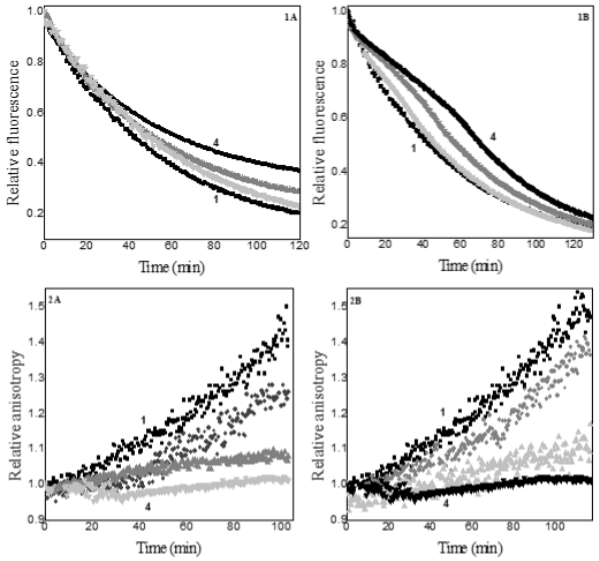

Fig. 3. Relative fluorescence intensity of the DPH-PA probe (1) and relative anisotropy (2) obtained in the presence of different concentrations of etodolac (A; (1) 0; (2) 21; (3) 84; (4) 169 μM) or trolox (B; (1) 0; (2) 5; (3) 9; (4) 18 μM) and of the oxidative system AAPH (15 mM) and splenocytes ($5x10^6$ cells/mL), at 37 ºC.

NSAIDs' IC_{15} values (**Table 1**) it is possible to conclude that etodolac has the highest antioxidant activity against lipoperoxidation induced by ROO•, since it has a smaller IC_{15} value. Additionally, by the analysis of **Table 1** and **Fig. 4** it is evident that the order of NSAIDs and trolox efficiency on peroxidation inhibition for both fluorescence and fluorescence anisotropy studies is the following: trolox > etodolac > tenoxicam > piroxicam.

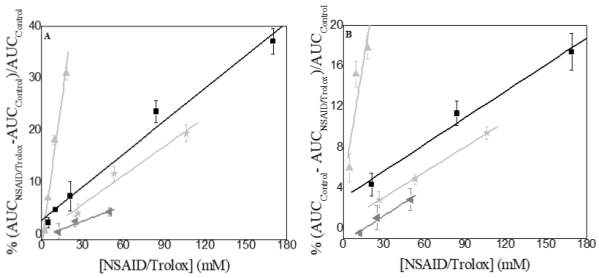

Fig. 4. Dependence of the $(AUC_{NSAID/Trolox}-AUC_{Control})/AUC_{Control}$ and $(AUC_{Control}-AUC_{AINE/Trolox})/AUC_{Control}$ ratio (%) with the increasing concentrations (μM) of etodolac (■), piroxicam (►), tenoxicam (●) and trolox (▲), respectively, in the fluorescence (A) and anisotropy (B) measurements, at pH 7.4. Mean ± standard error of three independent experiments.

The differences of NSAIDs reactivity towards the AAPH derived radical species can explain the obtained results in the lipoperoxidation assays. Moreover, the location into the membrane and the hydrophobicity or partition coefficient of the drugs as well as their ability to disturb the membrane fluidity are important factors contributing to their antioxidant efficiency. The scavenging ability of the compounds depends on their ability to transfer an H atom to peroxyl radicals [**21, 40**]. Therefore, the presence of H-donating groups (-OH, -NH, -SH) will increase the antioxidant ability of a compound. Indoprofen is the only NSAID studied that does not have any H-donating group, providing this fact an explanation for the absence of antioxidant activity. The higher antioxidant activity of etodolac can also be explained by the presence of the pyran ring in its structure. Indeed, pyran derivatives have been described as strong ROS scavengers [**7**]. Contrariwise, the presence of electron withdrawing substituents (-COOH, -Cl) directly bind to the aromatic ring, can justify the antioxidant inefficiency of clonixin, since these substituents can decrease the chemical stability of the clonixin's radical, which is a parameter very important for the antioxidant activity of any compound [**28**].

Table 1. IC_{15} (± standard deviation) values obtained for etodolac, piroxicam, tenoxicam and trolox in counteracting lipid peroxidation induced by ROO•, by fluorescence and anisotropy measurements of DPH-PA-labelled splenocyte membranes.

Compounds	IC_{15}	
	Fluorescence	Anisotropy
Etodolac	58.0 ± 3.9	137.3 ± 10.3
Piroxicam	n.r.*	n.r.*
Tenoxicam	79.8 ± 5.0	n.r.*
Trolox	9.0 ± 1.4	13.2 ± 3.5

* Not reached

As previously referred, the antioxidant efficacy of a compound is closely related with its chemical structure, but also with its concentration in the neighborhood of the oxidizable lipids and with its efficiency to increase membrane fluidity, opposing the resultant rigidity in peroxidized membranes. Nevertheless, in spite of oxicams were the most effective as membrane fluidizing agents and presented the highest partition coefficient [**31, 33**], they were not the more potent NSAIDs antioxidants. In fact, in the same studies performed for etodolac, the NSAID demonstrated partition into membranes in a considerably minor extent and was not able induce measurable membrane fluidity changes [**15**]. However, etodolac was the NSAID with less IC_{15}. This result highlights that the prevention of the initial attack of aqueous radicals on the membrane phospholipids is fundamental, since the lipid peroxidation once initiated is an auto-catalytic reaction. Therefore, just one peroxyl radical can lead to the transformation of hundreds of polyunsatured fatty acids in lipid hydroperoxides. Indeed, after formation of a alkyl radical by ROS, a succession of reactions occur originating new reactive species that are able to initiate others peroxidation reactions [**41**]. Hence, it is crucial to avoid the initiation of the lipid peroxidation. In this sense, despite its minor hydrophobicity, etodolac was the NSAID which presented a higher capacity to protect

membrane against lipid peroxidation. In fact, trolox, known as an efficient water-soluble antioxidant (trolox is mainly present in the aqueous environment) [**7, 42, 43**], also led to an effective inhibition of splenocyte membranes peroxidation.

Regarding oxicams, tenoxicam was more efficient than piroxicam at inhibiting AAPH-induced peroxidation, notwithstanding their structural similarities. This observation can be related with the higher capacity of tenoxicam to increase membrane fluidity [**31**], which corroborate the relation suggested in the literature between drugs ability to induce changes on membrane fluidity and their antioxidant activity [**28, 37, 44**]. Therefore, fluidizing or disordering the membrane, drugs can interact more efficiently with lipid radicals enhancing their ability to inhibit the oxidative damage of cellular membranes [**26, 37**].

Notwithstanding that the NSAIDs concentration used in these studies are higher than those observed in plasma and synovial fluid of patients (for example the average peaks of tenoxicam concentration were 4.3 and 1.4 μg/mL, respectively, in plasma and synovial fluid of patients with rheumatoid arthritis and osteoarthritis) [**45**], much lower NSAIDs concentrations can be active, since that the antioxidant efficacy of a drug is deeply related with the amount of reactive species being produced, giving rise consequently to different IC [**46**]. Consequently, it is possible that lower concentrations of the anti-inflammatory drugs have antioxidant activity, because the amount of the ROS produced in the inflamed tissues must be considerably less than that generated in the assays performed in the current work. Additionally, others factors as the administration of NSAIDs for longer periods of time can reduce the quantity of ROS [**47**] and, consequently, it will be required minor concentrations of the drugs than those used in this study. Indeed, despite the high concentrations of indomethacin needed *in vitro*, it has a strong antioxidant activity *in vivo* [**7, 48**].

Conclusion

This work contributes to discern the mechanism of action of NSAIDs since that the antioxidant properties of these drugs can act synergistically with their ability to inhibit cyclooxygenase and lipoxygenase in counteracting acute and chronic inflammatory reactions. Indeed, as peroxidation increases inflammatory diseases severity, the NSAIDs antioxidant activity may be of extreme significance in decreasing the accumulation of free radicals and consequently in reducing their deleterious effects in the surrounding tissues. Additionally, this work demonstrated that some drugs, such as oxicams, can protect membranes against oxidative damage only by a physical process, namely by increasing membrane fluidity.

In summary, the present study provides more realistic results about NSAIDs antioxidant activity against lipid peroxidation, since the majority of the assays present in the literature have been performed in aqueous solutions or with membrane models, which have a lesser correlation with the biological environment.

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