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Interactions of sulindac and its metabolites with phospholipid membranes: An explanation for the peroxidation protective effect of the bioactive metabolite

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Abstract
Non-steroidal anti-inflammatory drugs (NSAIDs) treat inflammatory processes by inhibition of cyclooxygenase (COX). However, their action against lipid peroxidation can be an alternative pathway to COX inhibition. Since inflammation and lipid peroxidation are cell-surface phenomena, the effects of NSAIDs on membrane models were investigated. Peroxidation was induced by peroxyl radical (ROO\(^{\cdot}\)) derived from AAPH and assessed in aqueous or lipid media using fluorescence probes with distinct lipophilic properties: fluorescein; HDAF and DPH-PA. The antioxidant effect of Sulindac and its metabolites was tested and related with their membrane interactions. Drug-membrane interactions included the study of: drug location by fluorescence quenching; drug interaction with membrane surface by zeta-potential measurements; and membrane fluidity changes by steady-state anisotropy. Results revealed that the active NSAID (sulindac sulphide) penetrates into the lipid bilayer and protects the membrane against oxy-radicals. The inactive forms (sulindac and sulindac sulphone) present weaker interactions with the membrane and are better radical scavengers in aqueous media.

Keywords: Lipid peroxidation, liposome, non-steroidal anti-inflammatory drugs (NSAIDs), fluorescence, peroxyl radical, AAPH

Introduction
Reactive oxygen species (ROS) play an important role in several pathophysiological processes causing oxidation of biomolecules such as proteins, lipids and deoxyribose nucleic acids (DNA) and leading to cell injury, cancer and death [1]. Via lipid peroxidation, ROS cause the release of arachidonic acid from membrane phospholipids and may increase the formation of prostaglandins and leucotrienes. Therefore, ROS are considered as mediators of inflammation in vivo [2,3]. Evidence also suggests that oxidative damage contributes to neurodegenerative disorders like the Alzheimer’s disease [4,5], since the brain is particularly vulnerable to oxidation due to its high content of easily peroxidable unsaturated fatty acids and to the fact that is not as highly enriched with antioxidant defence mechanisms compared to other organs [6].

Non-steroidal anti-inflammatory drugs (NSAIDs) are routinely prescribed to reduce swelling and pain in patients suffering from inflammatory conditions such as arthritis. These compounds are thought to exert their effects by interfering with the cyclooxygenase (COX) pathway, thus inhibiting the synthesis...
of pro-inflammatory prostaglandins. In addition to their prescribed role, NSAIDs have been shown to reduce the risk of developing colorectal cancer and adenoma [7]. Furthermore, the chronic use of NSAIDs has been shown to decrease the risk of developing Alzheimer’s disease by unknown mechanisms [8].

Sulindac is an inactive prodrug of the therapeutic class of NSAIDs that is rapidly metabolized following oral administration (Figure 1). Hence, sulindac can be irreversibly oxidized in vivo to an inactive sulphone (Figure 1C) metabolite which is not a NSAID because it does not inhibit prostaglandin synthesis [9]. Additionally, sulindac can be reversibly reduced in vivo to the pharmacologically active metabolite sulindac sulphide (Figure 1A) [9].

Like many anti-inflammatory agents, sulindac sulphide inhibits the biosynthesis of prostaglandins by the membrane-associated enzyme COX [9]. Originally, the effects of sulindac sulphide were only ascribed to its inhibitory influence on COX, but since many inflammatory processes are cell-surface phenomena [10] the possible effects of this NSAID on model membrane systems should also be investigated. Indeed, further understanding of factors influencing the behaviour of a drug molecule in a membrane environment is obviously needed to facilitate the future design of more effective drugs. Moreover, although the inhibition of prostaglandin synthesis constitutes the primary mechanism of action of NSAIDs, it is suggested that their antioxidant activity against reactive oxygen species (ROS) and reactive nitrogen species (RNS) is also implicated in the effects exerted in inflammation and cancer [11]. For the presented reasons, the focus of the current study was to define the location and fluidity effects of sulindac and its metabolites within membranes and to evaluate the antioxidant capacity of these drugs once this information is not always considered and may be useful in the development of new therapeutic strategies.

The location of drugs and the charge type of target membrane affect substantially their behaviour as antioxidants in heterogeneous systems due to the type of interactions between the drug and the lipid bilayers surface [12–14]. According to this, the location of sulindac and its metabolites within EPC liposomes used as membrane models was determined by fluorescence quenching of diphenylhexatriene propionic acid probe (DPH-PA) inserted across the lipid bilayer. In addition, zeta-potential measurements were used to evaluate changes in membrane surface and thus obtain more information about the drugs’ binding behaviour, already gathered by the fluorescence technique. Steady-state anisotropy measurements were also made to determine the drugs induced perturbation in membrane structure. Finally, to gain a deeper insight into the anti-radical properties of the assayed drugs their antioxidant activity was evaluated in liposomes where the peroxidative degradation of a probe was initiated by peroxy radicals generated by the compound 2, 2′-azobis (2-amidinopropanane) dihydrochloride (AAPH). Three different probes, specifically diphenylhexatriene propionic acid (DPH-PA), hexadecanoylaminofluorescein (HDAF) and fluorescein, with different lipophilic properties and thus different locations (aqueous or lipidic) were used. Peroxidation was followed by a decrease in the probe’s fluorescence [15].

The results indicate that the active NSAID studied (sulindac sulphide—Figure 1A), which possesses a hydrophobic sulphide substituent with a smaller dipole moment [10,16] than the sulphoxide (Figure 1B) or sulphone (Figure 1C), can penetrate into the lipid bilayer and leave the ionized carboxyl group near the polar surface of the membrane and this effect reflects in a better antioxidant protection towards the most lipophilic probes. In contrast the inactive forms studied (sulindac and sulindac sulphone) do not present significant membrane effects and interact weakly only with the boundary region of the bilayer [10,16]. Therefore, these inactive drugs have shown better antioxidant protection towards the most hydrophilic probe and this is a possible reason for these drugs being less effective as antioxidants against lipid peroxidation in vivo since the antioxidant efficiency is linked to their proximity with the oxy-radical.

Figure 1. Chemical structures of sulindac sulphide (A), sulindac (B) and sulindac sulphone (C).
Materials and methods

Materials

The drugs sulindac ([Z]-5-fluoro-2-methyl-1-[(p-(methylsulphonyl)-benzylidene]indene-3-acetic acid), sulindac sulphone ([Z]-5-fluoro-2-methyl-1-[(p-(methylsulphonyl)benzylidene]indene-3-acetic acid), sulindac sulphide ([Z]-5-fluoro-2-methyl-1-[(p-(methylthio)benzylidene]indene-3-acetic acid); EPC (Egg L-α-phosphatidylcholine) and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO). Trolox (2-Carboxy-2,5,7,8-tetramethyl-6-chromanol) and AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) were purchased from Fluka (St. Louis, MO). Fluorescein sodium salt and HDAF (5-hexadecanoyl aminofluorescein) were obtained from Aldrich (St. Louis, MO). DPH-PA (diphenylhexatriene propionic acid) was obtained from Molecular Probes (Invitrogen Corporation, Carlsbad, CA). All other chemicals were from Merck (Darmstadt, Germany); all were used without additional purification. Solutions were prepared with double-deionized water (conductivity < 0.1 μS/cm) and for all solutions used, the ionic strength was adjusted to 0.1 M with NaCl.

Preparation and fluorescence labelling of liposomes

Large unilamellar vesicles (LUV) containing the desired mole ratio of EPC in buffer solution were prepared by extrusion of freeze–thawed lipid dispersions through 100-nm pore diameter polycarbonate membranes, as previously described [15,17].

To prepare DPH-PA or HDAF labelled liposomes a chloroform/methanol (3:2) solution of the probes was previously added to the EPC in the same solvent mixture. The resultant mixture was used to prepare a dried lipid film in the same way as previously described [15,17] and dispersed into Hepes buffer (10 mM, I = 0.1 M, pH 7.4) when the fluorescence probe was DPH-PA and into potassium phosphate buffer (75 mM, I = 0.1 M, pH 7.4) when the fluorescent probe was HDAF. In the assays using fluorescein, the resultant dried lipid film was dispersed into potassium phosphate buffer (75 mM, I = 0.1 M, pH 7.4) and prepared in the same way, but without adding the probe to the organic solution. Fluorescein solution was only added to the LUV suspension immediately before the measurements were taken. LUV suspensions were left at room temperature for 30 min in the dark under magnetic stirring to allow a complete incorporation of the probe into the membranes [18]. The ratio of lipid-to-probe was always greater than 300:1 to prevent changes in the structure of the liposomes [17,19]. Although no bleaching phenomena were observed during the time course of the measurements, the determinations were made immediately after incubation.

Drug location and changes in membrane fluidity

The interactions of sulindac and its metabolites with membranes were studied by fluorescence quenching and steady-state anisotropy measurements using liposomes as membrane model systems. These studies were carried out with suspensions of labelled liposomes with DPH-PA probe. EPC concentration was set to 500 μM. Buffered solutions of the drugs studied were added to the membrane model systems in a total drug concentration of 50 μM. Fluorescence quenching and steady-state fluorescence anisotropy studies were performed in a Perkin Elmer LS-50 spectrofluorimeter equipped with a constant-temperature cell holder. All data were recorded at a controlled temperature (37.0°C) in a 1 cm path length cuvette. Excitation and emission wavelength was set to 384 nm and 435 nm, respectively. For each measurement or data point, fluorescence emission was automatically acquired during 50 s. Fluorescence values were corrected for light scattering contributions by subtraction of intensities from unlabelled samples at the same conditions. These contributions were always negligible (less than 0.5%).

Lipoperoxidation by fluorescence measurements

The antioxidant activity of sulindac and metabolites was evaluated by different methodologies using hydro and lipophilic fluorescent probes (fluorescein, HDAF and DPH-PA). In all cases, peroxidation was initiated by addition of the water-soluble azocompound AAPH used to generate peroxyl radicals by thermal decomposition. Initiator solution was freshly prepared before experiments by dissolution of AAPH in Hepes buffer (10 mM, I = 0.1 M, pH = 7.4).

Fluorescein was used as hydrophilic probe and the studies were carried out in buffer solution and in the liposome media. An analogous probe of fluorescein, HDAF, was also used in the liposome media, where the probe is located with its oxidizable portion at the membrane surface [12,20,21]. In both cases, the measurement of scavenging activity of the assayed drugs was achieved by monitoring the decay in fluorescence due to oxidation of the probe according to a described procedure called the oxygen radical absorbance capacity (ORAC) assay [22].

Studies in aqueous media were performed in a reaction mixture (200 μL) containing the following final concentrations of reagents dissolved in potassium phosphate buffer (75 mM, I = 0.1 M, pH = 7.4): 48 nM of fluorescein; 15 mM of AAPH and different concentrations of sulindac and its metabolites (0–20 μM). Trolox was used as a reference antioxidant. Controls were prepared in the same way, but without the drugs studied.

With the assays performed in liposome media, using the probe fluorescein the reaction mixtures...
(300 μL) contained the following volumes of reagents, prepared in potassium phosphate buffer (75 mM, I = 0.1 M, pH = 7.4): 80 μL of 3 mM LUV suspension; 30 μL of 480 mM fluorescein; 40 μL of 187.5 μM AAPH and 150 μL of tested drug achieving the following final concentrations: 800 μM of LUV suspension; 48 nM of fluorescein; 25 mM of AAPH and increasing concentrations of sulindac and its metabolites (0–15 μM). When the assays were performed in liposome media, using the probe HDAF the reaction mixtures (300 μL) contained the following volumes of reagents, prepared in potassium phosphate buffer (75 mM, I = 0.1 M, pH = 7.4): 80 μL of 3 mM LUV suspension labelled with 0.5 μM of HDAF; 110 μL of 545.5 μM AAPH and 110 μL of tested drug achieving the final concentrations: 800 μM of LUV suspension; 0.13 μM of HDAF; 200 mM of AAPH and increasing concentrations of sulindac and its metabolites (0–30 μM). Trolox was used as a reference antioxidant. In all assays, the LUV/drugs mixtures were shaken for 10 min at 37°C before the addition of the radical initiator. Controls were prepared in the same way, but without the drugs studied.

The decay in fluorescence intensity of the probes was monitored as a function of time, in a multiplate reader (H.T. Synergy, BIO-TEK). All data was recorded at 37°C. Excitation wavelength was set at 485 ± 20 nm and emission wavelength, 528 ± 20 nm and 521 ± 20 nm when using fluorescein or HDAF, respectively.

Results obtained by these methods correspond to the mean of three independent experiments, performed in quadruplicate.

DPH-PA was used as a lipophilic fluorescent probe and the antioxidant activity of sulindac and its metabolites against lipoperoxidation was evaluated by fluorescence decay due to DPH-PA oxidation [23] and by monitoring the membrane fluidity changes measured by fluorescence anisotropy. Fluorescence studies were carried out in a Perkin-Elmer LS 50B steady-state fluorescence spectrometer, equipped with a constant-temperature cell holder. All data was recorded at 37°C in 1-cm cuvettes. Excitation wavelength was set at 399 nm and emission wavelength at 435 nm.

An aliquot of 1.0 mL LUV suspension containing the probe DPH-PA (final concentrations of 800 μM and 2.7 μM, respectively) was incubated with different concentrations of sulindac and its metabolites (total concentrations of 0–150 μM) in a thermostatted holder (37°C) for 10 min with continuous stirring and away from light. Trolox was used as a reference. Peroxidation was initiated by addition of the water soluble azoinitiator AAPH (final concentration of 15 mM). Controls were prepared in the same way, but without the drugs studied.

The decay in DPH-PA fluorescence intensity and increase in steady-state anisotropy (both due to lipoperoxidation by peroxy radicals) were monitored at 37°C for 60 min. Results obtained by this method correspond to the mean of three independent experiments.

Data obtained for the fluorescence intensity or anisotropy was converted in all the assays (drug location studies; membrane fluidity studies and studies of lipoperoxidation by fluorescence measurements) to relative fluorescence or anisotropy values. Relative values were obtained by dividing the fluorescence intensity or anisotropy at a given time by the fluorescence intensity or anisotropy at 0 min to have always comparable anisotropy/fluorescence profiles, independent of the experimental conditions.

Zeta-potential and size determinations

Zeta-potential (ζ-potential) values and size distribution of EPC liposomes, with and without drug incorporated, were determined in Hepes buffer (10 mM, I = 0.1 M, pH = 7.4) and at 25.0 ± 0.1°C. Lipid concentration was kept constant at ~400 μM. All suspensions were then vortexed and incubated in the dark for 30 min. After equilibration the ζ-potential and size measurements were recorded by quasi-elastic light scattering analysis using a ZET 5104 cell in a Malvern Zetasizer 5000, with a 90° scattering angle. The values for viscosity and refractivity index were taken as 0.890 cP and 1.330, respectively [24]. The mean particle size of the vesicles was found to be constant with increasing concentrations of the drugs: 115 ± 5 nm (average and standard deviation of the measurements of six independently prepared suspensions).

Results and discussion

Drug location and changes in membrane fluidity

The quenching of a membrane bound fluorophore provides a measure of its accessibility to the quenching molecule and can be related with the concentration of the quencher [Q] by the Stern-Volmer equation (1), where I_0 and I are, respectively, the fluorescence of the probe in the absence and presence of the drug and K_{SV} is the Stern-Volmer constant [25,26]:

\[ \frac{I_0}{I} = 1 = K_{SV}[Q] \]  

For this purpose, the Stern-Volmer constant was determined for liposomes labelled with DPH-PA using sulindac and its metabolites as quenchers. Figure 2 shows, as an example, the fluorescence spectra of the probe with increasing concentrations of sulindac sulphide and the correspondent Stern-Volmer linear plot where it is possible to observe that this drug was able to decrease the fluorescence of the
probe by fluorescence quenching. The Stern-Volmer constant value, obtained from the slope of the Stern-Volmer linear plots of sulindac sulphide was remarkably higher \((159 \pm 600)\) than the values of the Stern-Volmer constants obtained for the other two compounds which were similar \((6489 \pm 1100\) and \(5900 \pm 650\) for sulindac and sulindac sulphone, respectively).

If the location of the fluorophores used as probes is well characterized, the extent of quenching expressed by the values of the Stern-Volmer constants can reveal the accessibility of fluorophores to the quenchers. In the case of membrane-embedded DPH and its derivative DPH-PA, which has a polar anchoring group, their location and orientation in lipid bilayers has been experimentally studied rather widely \([27/34]\). The results of recent published investigation clarified the long-standing uncertainty about the DPH location referring the probe as being deeply buried in the hydrocarbon core aligned roughly parallel to the acyl chains, as expected from the fact that DPH is a long molecule with hydrophobic nature \([35]\). The polar group of DPH-PA is accommodated at the shallow end of the molecule close to the membrane surface without involving a large change in DPH location. For this reason, if the molecular location of probes (fluorophore) within membranes is known with certainty, quenching studies can be used to reveal the location of quenchers in membranes \([25]\).

Consequently, as the DPH-PA probe was quenched in a much smaller extension by sulindac and sulindac sulphone, it can be concluded that these drugs have a weaker penetration into the membrane bilayer. However, sulindac sulphide has shown higher quenching efficiency of the probe, which indicates that this drug is able to penetrate into the lipid bilayer, probably with its ionized carboxyl group near the polar surface of the membrane. These results are consistent with other reported observations \([10,16]\) which show that the active NSAID sulindac sulphide is able to partition deeply into the hydrocarbon region of the bilayer. In contrast, the prodrug sulindac and the inactive metabolite sulindac sulphone present less significant membrane effects \([10,16]\).

DPH-PA has also been established as an appropriate tool to detect the ‘fluidity’ of the lipid bilayer of liposomes, biological membranes and whole cells, by monitoring the anisotropy \((r)\) of its fluorescence \([36,37]\). In the present study the effect of sulindac and its metabolites in the fluidity of the membrane was measured by steady-state fluorescence anisotropy. Steady-state fluorescence anisotropy is a technique that assesses the range of rotational motion of the membrane-associated fluorescent probe, in this case DPH-PA, during the lifetime of its excited electronic state \([30,36]\). The probe was excited with vertically polarized light and resulting fluorescence intensities recorded with the analysing polarizer oriented parallel \((I_{VH})\) and perpendicular \((I_{VH})\) to the excitation polarizer allowed the determination of steady-state fluorescence anisotropy, \(r_s\), for each labelled sample by equation (2) \([25]\):

\[
r_s = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2 G I_{VH}}
\]

where \(G = I_{HV}/I_{HH}\) is the grating correction factor \([25]\) and the subscripts \(H\) and \(V\) stand, respectively, for the horizontal or vertical orientation of the polarizers. The greater the extent of probe rotation during its excited state lifetime, the smaller will be the observed fluorescence anisotropy \((r_s)\), to the extent that \(r_s = 0\) for complete DPH-PA reorientation. The probe rotational motions are tightly coupled to the phospholipid fatty acid chain fluctuations, which provide a measure of membrane ‘fluidity’ \([30]\). Results were analysed according to the Perrin equation which relates measured anisotropy with the rotational relaxation time \((\theta)\) of the
fluorophore [25]. Although this equation applies only to isotropic rotation of a fluorophore and is not strictly applicable to the anisotropic rotation of the probes in lipid bilayers, it is possible to offer some correction for the hindered motions of the probe if the appropriate experimental conditions are used [18,25,38,39].

Changes in anisotropy with increasing concentration of the drug can also result in changes in the excited state lifetime of the fluorophore \( (\tau') \) [25]. This latter effect needs to be eliminated by the use of corrected anisotropy values \( (r') \) given by equation (3) [18,25,38]:

\[
\frac{r'}{r_s} = \frac{\theta + \tau}{\theta + \tau'} r_s
\]

where \( \theta \) and \( \tau \) values are characteristic of the probe (\( \theta = 1.75 \text{ ns} \) and \( \tau = 7.9 \text{ ns} \)) [40-43]; \( r_s \) were measured values of steady-state anisotropy and \( \tau' \) values are calculated by means of fluorescence quenching using equation (4). Indeed, in collisional quenching there is an equivalent decrease in fluorescence intensity and in lifetime; that is expressed by equation (4) [25]:

\[
\frac{I_0}{I} = \frac{\tau}{\tau'}
\]

where \( I_0/\tau' \) and \( I/\tau \) are, respectively, the corrected fluorescence intensity/lifetime of the fluorophores (probe) in the absence and presence of the drug. The decrease in \( \tau' \) occurs because quenching is an additional rate process that depopulates the excited state. The decrease in \( I \) occurs because quenching depopulates the excited state without fluorescence emission [25].

Figure 3A shows a comparison of the \( r' \) values and the experimental \( r_s \) values obtained for sulindac. The difference between \( r_s \) and \( r' \) (Figure 3B) is a measure of the fluidizing effect of the drugs. Sulindac sulphide has shown a similar profile to sulindac.

Sulindac and sulindac sulphone induce a membrane fluidization in a concentration dependent manner once the resultant anisotropy \( (r_s - r') \) decreases with increasing concentrations of the drugs. To compare the efficacy of these compounds to increase membrane fluidity an IC\(_{25}\) value was calculated. The determination of IC\(_{25}\) values was obtained from the plot (linear part) of the percentage of increase of membrane fluidity as a function of drug concentration (in \( \mu \text{M} \)). IC\(_{25}\) is thus defined as the concentration of each compound (in \( \mu \text{M} \)) that is required to increase membrane fluidity ratio \( [(r' - r_s)/r_s] \times 100 \) in 25%. IC\(_{25}\) values were reached with 110 + 20 \( \mu \text{M} \) and 115 + 30 \( \mu \text{M} \) for sulindac and sulindac sulphone, respectively.

Contrastingly, the values of resultant anisotropy \( (r_s - r') \) obtained with increasing concentrations of sulindac sulphide are constant, meaning that this drug has no visible influence in membrane fluidity (data not shown). To understand these different effects it is important to analyse it according to the drug interactions with the membrane. Sulindac and its metabolites are amphiphilic compounds which possess identical stereochemical configuration (Figure 1). However, it must be pointed out that the polarity of their substituents in the sulphur atoms varies considerably and it is possible to distinguish two groups according their interactions with the membrane [10]. Sulindac and sulindac sulphone which have the same hydrophobic benzylidenylindene structure present a similar behaviour and have shown smaller penetration in the membrane than sulindac sulphide [10,16]. However the presence of the drugs in the membrane, even in small amounts, can have a perturbing effect in lipid packing which is consistent with the changes in membrane fluidity observed in the present studies. On the other hand, although sulindac sulphide partitions deeply into the

![Figure 3](image-url)
Interactions of sulindac and its metabolites with phospholipid membranes

It is also a molecule with an extremely negative potential near the carboxylate group and near the sulphur, in contrast to the rest of the methylthiophenyl moiety for which the potential approaches zero [44]. Therefore, sulindac sulphide penetrates into the membrane, as shown by its quenching efficiency, with its ionized carboxyl group positioned near the polar surface of the bilayer [10]. The interaction between the negative charge of this drug and the polar headgroups of the membrane bilayer might have a consequent stabilizing effect of the membrane surface and for that reason the membrane remains intact with no visible changes in its fluidity. Additionally, the absence of interaction between sulindac and sulindac sulphone and the polar headgroups can justify their effect in membrane fluidity in opposition to the effect of sulindac sulphone, which stabilizes the membrane. This hypothesis is corroborated by zeta-potential measurements (Figure 4) performed in the presence of increasing concentrations of sulindac sulphone, once the potential of the membrane surface was altered, becoming increasingly negative, with the addition of sulindac sulphone. Since EPC is zwitterionic the liposomes in the absence of the drug present a zeta-potential value of \( \sim 0 \) mV which then decreases down to \( \sim -18 \) mV in the presence of sulindac sulphide. The membrane zeta-potential values measured with similar concentrations of sulindac and sulindac sulphone remained unchanged, confirming the fact that these drugs do not interact with the polar headgroups of membrane phospholipids.

**Evaluation of antioxidant activity**

Drugs antioxidant efficiency against lipoperoxidation is related to their scavenging capacity and to their ability to interact with and/or penetrate the lipid bilayers. Indeed, the scavenging depends on the chemical structure of the inhibitor, but it may also be promoted by concentrating the scavenger in the neighbourhood of the target lipid. Therefore, the efficiency of sulindac and its metabolites as protectors against peroxidation processes was evaluated using probes with different lipophilic properties and consequently with different locations on lipid bilayers.

**Evaluation of antioxidant activity with fluorescein probe.**

Peroxidation of the hydrophilic fluorescein probe induced by peroxyl radicals was studied both in aqueous and liposome media in an attempt to explore possible factors that may change the antioxidant effect when the drug is not homogeneously distributed in an aqueous solution. In aqueous buffer solution at physiological pH, the scavenging activity of sulindac and its metabolites against peroxyl radicals was evaluated by the ORAC method [22]. The ORAC method described in the literature for aqueous buffer solutions was further adapted in the current work to assays performed in liposomes. Data obtained for fluorescence decay studies and the corresponding linear fits are depicted in Figure 5 with increasing concentrations of sulindac. In both media (liposomes and aqueous media) a good linear fit was obtained in all cases \( (R > 0.988) \). The linear fits obtained for each assay permitted a better evaluation of the ability of the compounds studied to act as antioxidants by determining their IC\(_{50}\) values. Table I shows the peroxyl scavenger efficiency of sulindac and its metabolites reflected in their IC\(_{50}\) values which were calculated as the concentration (in \( \mu M \)) of each compound required to obtain 50% of the ratio \( \frac{\text{AUC}_{\text{Drug}}/\text{Trolox} - \text{AUC}_{\text{blank}}}{\text{AUC}_{\text{blank}}} \) where AUC is the area under curve obtained from the fluorescence decay of the probe in the absence (blank) or in presence of drug or Trolox used as reference [22].

From the observation of IC\(_{50}\) values obtained it is possible to conclude that sulindac and its metabolites have similar peroxyl radical scavenger capacity when the assays are performed in aqueous media.

In the assays carried out using fluorescein in the presence of liposomes as membrane models, similar values of IC\(_{50}\) were obtained for sulindac and sulindac sulphone, yet for sulindac sulphide the IC\(_{50}\) values were higher than the values obtained in the absence of liposomes (Table I). A brief comment regarding the interaction of the drugs with model membranes is needed to analyse the results, since this interaction may be related to the discrepancy in the values obtained. In fact, the peroxyl radical is generated in the aqueous media where the target probe for peroxidation (fluorescein) is also located [45]. In this case, the efficiency of an antioxidant to interrupt the oxidative sequence will be greater when located in the vicinity of the target probe. The difference in IC\(_{50}\) values obtained for sulindac

![Figure 4. Effect of increasing concentration of sulindac sulphide in zeta-potential values obtained for liposomes (400 \( \mu M \)) at pH 7.4.](image-url)
sulphide in the presence and in the absence of liposomes can be due to the interaction between the drug and lipid membranes. Indeed, as discussed previously, despite the similarity of the chemical structures of sulindac and its metabolites, the polarity of their sulphur atom substituent, CH$_3$S (sulindac sulphide) vs CH$_3$S$^-$O (sulindac) vs CH$_3$SO$_2$ (sulindac sulphone) varies considerably [10]. Therefore, sulindac sulphide possesses a hydrophobic sulphide substituent with a smaller dipole moment, which allows its penetration into lipid bilayers. The capacity of sulindac sulphide to penetrate into the membranes was also confirmed by the location studies previously described in this work, where this drug revealed the highest quenching efficiency of the probe inserted in the membrane. Furthermore, it has been reported in the literature that when sulindac sulphide is located in the membrane it leaves the ionized carboxyl group near the polar surface of the membrane [10]. This behaviour was once more supported by zeta-potential measurements (Figure 4) aforementioned. Besides the interaction with the membrane surface, the reported partition of sulindac sulphide also suggests that this drug is deeply inserted into the hydrocarbon

**Table I.** IC50 (± standard deviation calculated from at least three independent experiments performed in quadruplicate) for sulindac and its metabolites obtained with fluorescein (in the absence or in the presence of liposomes), HDAF and DPH-PA as fluorescent probes in liposomes. IC values were calculated from the linear fit of percentage ((AUC$_{drug}$-trolox - AUC$_{blank}$)/AUC$_{blank}$) vs concentration of drug (μM).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fluorescein (Buffer)</th>
<th>Fluorescein (LUVs)</th>
<th>HDAF (LUVs)</th>
<th>DPH-PA* (LUVs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulindac</td>
<td>10.0 ± 3.3</td>
<td>10.0 ± 2.1</td>
<td>27.5 ± 1.9</td>
<td>n.r.</td>
</tr>
<tr>
<td>Sulindac sulphide</td>
<td>12.0 ± 2.7</td>
<td>26.1 ± 1.5</td>
<td>18.2 ± 0.8</td>
<td>25.6 ± 1.0</td>
</tr>
<tr>
<td>Sulindac sulphone</td>
<td>13.5 ± 4.3</td>
<td>11.4 ± 4.0</td>
<td>28.9 ± 3.2</td>
<td>n.r.</td>
</tr>
</tbody>
</table>

* mean values calculated from the results obtained by fluorescence and anisotropy measurements. n.r. IC$_{50}$ was not reached.

Figure 5. Relative fluorescence intensity of fluorescein obtained with the oxidative system AAPH and different concentrations of sulindac sulphide in buffer (A) (0; 1.01; 2.03; 3.00; 4.11; 5.15; 10.00; 15.10; 20.08 μM) and in the presence of EPC liposomes (B) (0; 1.33; 2.67; 3.33; 6.67; 10.00; 13.30 μM). Corresponding ROO$^*$ scavenging activity of sulindac expressed as percentage oxidation in buffer (C) and in liposomes (D). Each point represents the values obtained for three experiments, performed in quadruplicate (mean ± standard error).
region [10,16]. It thus seems necessary to add higher concentrations of sulindac sulphide in the presence of LUV to obtain the same antioxidant activity observed in the assay with fluorescein in aqueous buffer because there is still part of the antioxidant molecules studied which are inserted in the membrane hydrocarbon region and interacting with membrane surface and consequently are not available in the aqueous solution to protect fluorescein from the oxidation induced by radicals.

**Evaluation of antioxidant activity with HDAF probe.**

The peroxyl radical scavenger activity of sulindac and its metabolites against HDAF peroxidation is very much reduced when compared to their effect as antioxidants of fluorescein oxidation (Table I). These results may be related with the position of the probe in the lipid membrane which is less accessible for the oxidation by the aqueous radical initiator AAPH. In fact, HDAF probe, which is an analogue of fluorescein but with an additional lipophilic chain, is located in the lipid bilayer with the hydrophobic chain inserted into the apolar chains of phospholipids and the polar oxidable portion near the polar head groups of phospholipids and consequently less exposed to the aqueous media [12,21]. This makes oxidation of the HDAF probe more difficult and higher concentrations of the radical initiator AAPH were needed. It is then conceivable that the increase of radical initiator has cost a higher radical generation and consequently concentrations of sulindac and its metabolites required to scavenge the formed radicals have increased. Furthermore the different IC50 values obtained for sulindac sulphide and the inactive forms sulindac and sulindac sulphone reflect once more the different behaviour of these compounds towards the lipid membranes. Hence, sulindac sulphide partitions deeply into the hydrocarbon region of the bilayer and interacts with the membrane surface being more accessible to protect the probe HDAF also located in the membrane. Accordingly the IC50 value of sulindac sulphide is significantly smaller compared to the IC50 of the other two compounds studied, meaning that it needed a smaller concentration of sulindac sulphide to reach the same level of protection against the peroxidation of HDAF probe. Sulindac and sulindac sulphone are devoid of any significant membrane interactions within the concentration range tested and are then less accessible to the lipophilic probe HDAF. Consequently their IC50 values were considerably higher, reflecting the need of superior concentrations to have the same protective effect against the peroxidation of HDAF probe.

**Evaluation of antioxidant activity with DPH-PA probe.**

Comparing the results obtained for peroxyl radical scavenger activity of sulindac and its metabolites against DPH-PA peroxidation, one can see that sulindac sulphide was the only effective drug (Table I). This is again a matter of understanding the interactions between the drugs and the membrane. As aforesaid, the partition of sulindac and its metabolites evaluated in previous studies [10] showed that sulindac and sulindac sulphone have a very small partition while sulindac sulphide, the active metabolite of sulindac, possesses a hydrophobic substituent and can penetrate into the lipid bilayer [10]. Consequently, at the concentration range tested, sulindac sulphone and sulindac were not in a high enough available quantity to protect the oxidizable portion of the probe which is inserted in the bilayer, whereas for the same concentration range sulindac sulphide was able to trap peroxyl radicals by an electrostatic adsorption to the zwitterionic head of phosphatidylcholine.

DPH-PA peroxidation can be assessed not only by the fluorescence decay of the probe but also by fluorescence anisotropy measurements, which report to fluidity changes. Indeed, the influence of antioxidants in membrane biophysics has been used to explain the mechanisms of the antioxidant actions [14,46,47] once the antioxidants can act either by scavenging free radicals or by modifying their propagation across cell membranes, namely by inducing membrane fluidity changes. In agreement with that suggested in the literature [13,48], a relationship between membrane fluidity and rate of peroxidation of lipidic bilayers can also be observed and, as a consequence, the rate of peroxidation was monitored simultaneously by a decrease in fluorescence intensity and an increase in fluorescence anisotropy as a function of time. From Figure 6 it is possible to conclude that the addition of sulindac sulphide induces a stabilization of the membrane as previously mentioned and reduces the relative anisotropy increase in
a concentration-dependent manner and thus modifies the propagation rate of the peroxyl radicals.

**Conclusions**

The rate of scavenging depends on the chemical structure of the inhibitor, but it may also be promoted by concentrating the scavenger in the neighbourhood of the target lipid. It is for the latter reason that many of the currently available antioxidant drugs are lipophilic and their precise positioning and orientation within the membrane, in relation to the unsaturated fatty acyl chains of membrane phospholipids, is also an important factor contributing to their scavenger effectiveness.

In the particular case of the work here described three chemical related compounds (sulindac, sulindac sulphide and sulindac sulphone) were analysed in regard to their interactions with lipid membrane and their consequent antioxidant effect. Despite the similarity between the compounds studied, sulindac sulphide is the only active drug and possesses an anti-inflammatory action. In comparison with the other compounds studied, sulindac sulphide also has higher hydrophobicity, which allows it to insert deeply into the hydrocarbon region of the bilayer besides binding to the membrane surface with its ionized carboxyl group. In contrast, the other two inactive compounds do not present significant membrane effects. From these considerations the apparent difference of antioxidant effects obtained with different probes in these considerations the apparent difference of anti-inflammatory action. In conclusion, the results obtained in this study demonstrate that a better understanding of drug effects on macromolecular targets such as membranes and of their ability to interfere with free radicals is fundamental to developing more efficient anti-inflammatory drugs and possibly to understanding the NSAID prevention of colonic carcinogenesis.

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Interactions of sulindac and its metabolites with phospholipid membranes


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