

Another point of interest emerging from these experiments is that furosemide administered by aerosol in combination with the anti-inflammatory compounds here considered turned out in a positive interaction in protecting guinea-pigs against anaphylactic respiratory crisis. In particular, for each combination (FRU + NIM; FRU + ASA; FRU + INDO) the ratio between the expected ED_{175} (calculated under the hypothesis of additivity) and the observed ED_{175} resulted > 1 , indicating a synergistic interaction between the two compounds present in the mixture. This could be explained primarily by a better control of anaphylactic mediator release and by a sustained interruption of the amplification mechanism brought about by histamine and TXA_2 during the respiratory crisis. Taken together, these results emphasize that NSAIDs given by aerosol may represent a valuable approach in the treatment of acute immune bronchoconstriction of the guinea-pig, and support the encouraging clinical findings of Bianco et al. [18]. It so happens that furosemide and acetylsalicylic acid given in combination by inhalation were reported to be effective in an additive way against acute bronchoconstriction induced in man by hypertonic saline and allergen [20]. The question of the effectiveness of both furosemide and NSAIDs in the anaphylactic bronchoobstructive phenomenon is rather stimulating and justifies further investigations.

5. References

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Differential Inhibition of Superoxide, Hydroxyl and Peroxyl Radicals by Nimesulide and Its Main Metabolite 4-Hydroxynimesulide

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Summary

The superoxide and hydroxyl radical scavenging activities of nimesulide (CAS 51803-78-2) and its main metabolite 4-hydroxynimesulide (CAS 109032-22-6) were investigated by Electron Spin Resonance (ESR) spectroscopy, with the spin trapping technique. Hydroxynimesulide is a good scavenger of both $O_2^{\bullet-}$ ($IC_{50} = 40 \mu\text{mol/l}$) and $HO^{\bullet}$ ($IC_{50} = 54.8 \mu\text{mol/l}$) radicals, and its high reactivity towards $HO^{\bullet}$ was confirmed by the rate constant for reaction with $HO^{\bullet}$ ($K = 8.9 \times 10^{10} \text{ mol}^{-1}$

l s^{-1}) determined by competition kinetic studies with 5,5-dimethyl-1-pyrroline-N-oxide. Nimesulide, which has been shown by ESR to be inactive as a superoxide quencher, has a rate constant of reaction with $HO^{\bullet}$ slightly greater than that of its metabolite ($3.3 \times 10^{11} \text{ mol}^{-1} \text{ l s}^{-1}$). In the $HO^{\bullet}$ -induced peroxidation of phosphatidylcholine (PC) liposomes, both compounds act as potent preventive antioxidants, but the $HO^{\bullet}$ entrapping capacity of the parent drug was again greater than that of hydroxynimesulide (IC_{50} 2.12 vs 3.84 $\mu\text{mol/l}$). The

metabolite is also a potent scavenger of the peroxy radical (ROO°) in the propagation phase of PC peroxidation (marker conjugated dienes), with an $\text{IC}_{50} = 2.67 \mu\text{mol/l}$: at $5 \mu\text{mol/l}$ it induces a lag time in the decomposition of PC hydroperoxides (PC-OOH) into aldehydic products of 40 h longer than in the controls (markers: conjugated dienes and total carbonyl functions). In PC liposomes, in the presence of preformed PC-OOH, the metabolite prevents PC peroxidation stimulated by $5 \mu\text{mol/l}$ Fe^{2+} , via the Fenton reaction (marker: conjugated dienes), at the micromolar level ($\text{IC}_{50} = 17 \mu\text{mol/l}$) through an anti-free radical activity and a free iron chelating mechanism. Hydroxynimesulide in fact interacts with Fe^{2+} ions, giving rise to a strong complex, with a stability constant ($\log K$) estimated to be around 8/9.

In addition, hydroxynimesulide efficiently protects *ex vivo* synovial fluid lipids from the oxidative stress induced by Fe^{2+} /ascorbate, already at $10 \mu\text{mol/l}$ (marker: thiobarbituric acid reactive substances). These results provide evidence for strong antioxidant and iron-chelating properties of 4-hydroxynimesulide, which can act synergistically with the specific HO° scavenging activity of the parent drug in preventing and limiting *in vivo* the free radical-mediated tissue damage in both acute and chronic inflammatory situations.

Zusammenfassung

Hemmung von Superoxid-, Hydroxyl- und Peroxyl-Radikalen durch Nimesulid und seinen Hauptmetaboliten 4-Hydroxynimesulid

Die Eigenschaften von Nimesulid (CAS 51803-78-2) und seinem wichtigsten Metaboliten 4-Hydroxynimesulid (CAS 109032-22-6) als Radikalfänger von Superoxid- und Hydroxyl-Radikalen wurden mit Hilfe der Elektronen-Spin-Resonanz (ESR)-Spektroskopie unter Verwendung der Spin-Trapping-Technik untersucht. Hydroxynimesulid ist ein guter Radikalfänger sowohl von $\text{O}_2^{\circ-}$ -Radikalen (Hemmkonzentration $\text{HK}_{50} = 40 \mu\text{mol/l}$) als auch von HO° -Radikalen ($\text{HK}_{50} = 54,8 \mu\text{mol/l}$). Seine starke Reaktivität bei HO° wurde durch die Geschwindigkeitskonstante der Reaktion mit HO° ($K = 8, 9 \times 10^{10} \text{ mol}^{-1} \text{ l s}^{-1}$), die unter Verwendung von 5,5-Dimethyl-1-pyrrolin-N-oxid bestimmt wurde, bestätigt. Nime-

sulid, das bei Superoxid keine Radikalfängerwirkung zeigte, wie ESR-Untersuchungen ergaben, weist bei HO° eine Reaktionsgeschwindigkeitskonstante auf, die etwas größer als die seines Metaboliten ist ($3,3 \times 10^{11} \text{ mol}^{-1} \text{ l s}^{-1}$). Bei der HO° -induzierten Peroxidation von Phosphatidylcholin (PC)-Liposomen erwiesen sich beide Verbindungen als effektive Antioxidantien, die Ausgangssubstanz erwies sich jedoch wieder als der bessere Radikalfänger im Vergleich zu Hydroxynimesulid ($\text{HK}_{50} 2,12$ gegenüber $3,84 \mu\text{mol/l}$). Der Metabolit ist auch beim Peroxylradikal (ROO°) in der Propagationsphase der PC-Peroxidation (Marker: konjugierte Diene) ein stark wirksamer Radikalfänger, mit einer $\text{HK}_{50} = 2,67 \mu\text{mol/l}$: Bei $5 \mu\text{mol/l}$ bewirkt es eine Verzögerungsphase beim Abbau von PC-Hydroperoxiden (PC-OOH) zu Aldehydverbindungen, die dann 40 h länger dauert als bei den Kontrollen (Marker: konjugierte Diene und gesamte Carbonylfunktionen). Bei PC-Liposomen verhindert der Metabolit bei Vorliegen vorgebildeter PC-OOH eine über die Fenton-Reaktion mit $5 \mu\text{mol/l}$ Fe^{2+} stimulierte PC-Peroxidation (Marker: konjugierte Diene) im mikromolaren Bereich ($\text{HK}_{50} = 17 \mu\text{mol/l}$) durch eine radikalbindende Aktivität und die Bildung freier Eisenchelatkomplexe. Hydroxynimesulid reagiert dabei mit dem Fe^{2+} -Ion unter Bildung eines starken Komplexes mit einer Stabilitätskonstante ($\log K$) von etwa 8/9.

Zusätzlich schützt Hydroxynimesulid schon bei $10 \mu\text{mol/l}$ (Marker: Thiobarbitursäure-reaktive Substanzen) *ex vivo* Lipide der Synovialflüssigkeit vor Fe^{2+} /Ascorbat-induziertem Oxidationsstress.

Diese Ergebnisse lassen darauf schließen, daß 4-Hydroxynimesulid stark antioxidativ und eisenchelatbildend wirkt. Dabei können sich *in vivo* gemeinsam mit der Ausgangssubstanz aufgrund deren spezifischer Radikalfängeraktivität für HO° -Radikale synergistische Wirkungen hinsichtlich der Vermeidung oder Begrenzung radikalvermittelter Gewebsschädigungen sowohl bei akuten als auch chronischen Entzündungszuständen ergeben.

Key words: Anti-inflammatories, non-steroidal · Aulin® · CAS 51803-78-2 · 4-Hydroxynimesulide, electron spin resonance studies · Mesulid® · Nimed® · Nimesulide, metabolite · Nisulid® · Radical scavengers

1. Introduction

Nimesulide¹⁾ (CAS 51803-78-2, N-(4-nitro-2-phenoxyphenyl)-methanesulfonamide) is a non-steroidal anti-inflammatory drug (NSAID) with potent anti-inflammatory, antipyretic and analgesic properties which is well tolerated gastrointestinally [1] because of its weak inhibition of prostaglandin synthesis in gastric mucosa [2]. Various non prostaglandin mechanisms have been proposed to explain its mode of action: inhibition of a) histamine release and activity [3–5], b) cytokine release [6], c) platelet aggregation [7], d) platelet-activating factor (PAF) synthesis [8], e) phosphodiesterase type IV activity [9], f) neutrophil myeloperoxidase activity [10].

The ability of nimesulide to interfere with the release of oxidants (superoxide anion, hypochlorous acid) from activated neutrophils [11–14] has been recently corroborated by studies from our laboratory [15–17] which show that the drug has a direct free radical scavenging activity. In two different cell free systems it acts as a preventive antioxidant by specifically quenching the HO° radical, the highly reactive species which, by promoting hydrogen abstraction from polyunsaturated fatty acids (PUFA), leads on one side to activation of prostaglandins biosynthesis, and on the other to cellular damage mediated by peroxidation of membrane phospholipids. Its main metabolite, 4-hydroxynimesulide [15, 16] also appreciably inhibits formation of thiobarbituric acid reactive substances (TBARS) in metal ion-dependent lipid peroxidation of microsomal membrane, thus indicating antilipoperoxidant intervention.

Since there might be a synergistic effect of nimesulide with its metabolite in suppressing reactive oxygen species (ROS) involved in the inflammatory reactions, we have

¹⁾ Manufacturer: Helsinn Healthcare SA, Pambio-Noranco, Lugano (Switzerland). Trademarks: Aulin® (I, CH, IRL, P, F), Mesulid® (B, GR, I), Nimed® (P), Nisulid® (CH).

investigated by Electron Spin Resonance (ESR) spectroscopy the scavenging activity of 4-hydroxynimesulide against $O_2^{\cdot-}$ and $HO^{\cdot}$ radicals, the promoters of lipid peroxidation, and using the well established membrane model system of phosphatidylcholine liposomes, the scavenging activity against chain-carrying lipid peroxy radicals ($ROO^{\cdot}$).

In addition, since it is well known that transition metals catalyze lipid peroxidation and free iron is decompartmentalized from ferritin during the inflammatory response, in the last part of the study we evaluated a) the protective effect of hydroxynimesulide on phosphatidylcholine hydroperoxides/ Fe^{2+} -promoted lipid peroxidation and b) its chelating properties for free ferrous ions.

2. Materials and methods

2.1. Chemicals

The organic solvents used were of analytical grade (Carlo Erba, Milan, Italy). Nimesulide and 4-hydroxynimesulide were kindly provided by Helsinn Healthcare SA (Pambio-Noranco, Lugano, Switzerland). Soybean phosphatidylcholine (PC), 2,4-dinitrophenylhydrazine, (+)- α -tocopherol and ascorbic acid were purchased from Fluka Chimica (Milan, Italy); hypoxanthine, 2-thiobarbituric acid (TBA), nitylotriacetic acid (NTA) and diethylenetriaminepentaacetic acid (DETAPAC) from Sigma Chemical Co. (Trimital, Milan, Italy); allopurinol from Janssen (Inalco, Milan, Italy); 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) from Aldrich (Trimital, Milan, Italy); mannitol from Carlo Erba (Milan, Italy). Xanthine oxidase (20 U/ml) and superoxide dismutase (5000 U/mg) were purchased from Boehringer Mannheim Italia (Milan, Italy).

The synovial fluid used in these experiments, kindly provided by Istituto Ortopedico G. Pini (Milan, Italy), was a small fraction of samples (white cell count: $2-3 \times 10^4$ cells/mm³) drawn from patients with rheumatoid arthritis (RA) for authentic medical purposes.

2.2. Apparatus

Spectrophotometric studies were carried out in a computer-aided Perkin Elmer Lambda 16 spectrophotometer (Perkin Elmer, Monza, Italy). Ultrasound irradiation of water was carried out with a Microson ultrasonic cell disruptor Heat System Ultrasonic Inc. (New Highway, Farmingdale, NY, USA) working with a type P1 sonicator probe. Electron Spin Resonance (ESR) spectroscopy studies were performed on a Varian E-Line Century Series instrument (Varian, Milan, Italy) (X-band, 100 kHz field modulation) operating under the following conditions: magnetic field 335 ± 10 mT; microwave power 30–40 mW; gain $4-6.3 \times 10^4$; modulation amplitude 1 mT; time constant 0.250 s; scanning time 2 min.

2.3. ESR studies

2.3.1. $HO^{\cdot}$ scavenging activity

$HO^{\cdot}$ radicals, generated by ultrasound irradiation (23 kHz; 15 min in a dark room at 37 ± 1 °C) of deionized water were detected by ESR spectroscopy, using 10 mmol/l DMPO as spin trapping agent. The hyperfine coupling constants (hfsc) for the DMPO-OH adduct were measured by Mn^{2+} in MgO, as a reference. The $HO^{\cdot}$ entrapping capacities of nimesulide and hydroxynimesulide were evaluated by taking up different concentrations (1–100 μ mol/l) of the drugs ($CHCl_3$ solutions, dried under nitrogen) into 1.5 ml of 10 mmol/l DMPO aqueous solutions. After ultrasound irradiation, the samples were immediately transferred into quartz tubes for ESR analysis. The IC_{50} values for nimesulide and hydroxynimesulide were determined by measuring the relative signal intensities of the radical adduct (the height of the second peak of the quartet) in the presence and absence of the drugs.

The rates of reaction of hydroxyl radicals with nimesulide and hydroxynimesulide were determined by following the kinetic competition between the drugs and DMPO, according to the following protocol: different concentrations of the drugs dissolved in 0.01N NaOH (100–300 μ mol/l) were added to 10 mmol/l DMPO in water (final volume 1.5 ml; final pH 6). The

development of the DMPO-OH spin adduct was studied over a time period of 30 min exposure of the samples to ultrasound with the signal intensity of the second peak of the DMPO-OH adduct recorded at 5 min intervals. The peak heights were plotted to obtain the kinetics of formation of the DMPO-OH adduct (the slopes of the curves represent the reaction rates).

The rate constants were calculated as follows:

$$R/r - 1 = \frac{K_{\text{scav}} / HO^{\cdot}}{K_{\text{DMPO}} / HO^{\cdot}} \times \frac{[\text{scav}]}{[\text{DMPO}]}$$

in which r and R are the initial rates of DMPO-OH production in the presence and absence of the scavenger, and $K_{\text{scav}}/HO^{\cdot}$ and $K_{\text{DMPO}}/HO^{\cdot}$ the apparent bimolecular rate constants for the reaction of hydroxyl radicals with the scavenger (nimesulide or hydroxynimesulide) and DMPO, respectively; $[\text{scav}]$ and $[\text{DMPO}]$ represent the concentrations of the scavengers and of DMPO in the reaction mixture. R and r were determined from the initial rate measurements of the linear slopes of formation of DMPO-OH spin adduct, between $t = 0$ and $t = 30'$. The $K_{\text{DMPO-OH}}$ rate constant was $3.4 \times 10^9 \text{ mol}^{-1} \text{ s}^{-1}$ [18]. Three independent values of r were obtained with 100–300 μ mol/l concentrations of nimesulide or hydroxynimesulide and 10 mmol/l of DMPO.

2.3.2. Superoxide anion scavenging activity

50 μ l of hypoxanthine (10 mmol/l in 0.01 N NaOH), 50 μ l of DETAPAC (2 mmol/l in 0.01 N NaOH), 8.6 μ l of DMPO 8.7 mol/l, 50 μ l of freshly prepared xanthine oxidase suspension (1 U/ml in phosphate buffer, pH 7.4) were diluted with phosphate buffer (final volume 0.5 ml) and mixed for 10 s. ESR measurements were taken after exactly 80 s incubation at 37 °C. Different concentrations of inhibitors (allopurinol 1 mmol/l, SOD 0.625–0.0625 mg/ml, nimesulide and hydroxynimesulide 1–200 μ mol/l) were added to the incubation mixture before starting the reaction by the addition of xanthine oxidase. The superoxide scavenging activity of the compounds was determined as % inhibition of the signal height intensity of the second peak of the quartet of the DMPO- OOH spin adduct, taking as 100 % the control signal intensity, and expressed as IC_{50} values (mean $\pm$ S.D. of 5 determinations).

2.4. Radical scavenging activity in phosphatidylcholine liposomes (PCL)

PC [17, 19] were exposed for 15 min to a flux of $HO^{\cdot}$ radicals generated by water sonolysis (induction phase) and the reaction was left to proceed under aerobic conditions for 96 h. Induction, propagation ($R^{\cdot}$ and $ROO^{\cdot}$ radicals) and breakdown ($RO^{\cdot}$) phases were monitored by measuring conjugated dienes (absorbance and second derivative UV spectroscopy at 233 nm) and formation of breakdown products (total carbonyl functions as 2,4-dinitrophenylhydrazones) [17, 19]. Hydroxynimesulide, nimesulide (final concentrations 0.5–10 μ mol/l) and α -tocopherol (5 μ mol/l) were incorporated into PCL at the beginning of the induction and propagation phases of lipid peroxidation, and conjugated dienes and total carbonyl functions determined at different times, according to the protocol already published [17, 19]. The antioxidant activity was expressed as percentage inhibition of formation of conjugated dienes (induction, propagation phases) and/or 2,4-dinitrophenylhydrazones (breakdown phase) in respect to the controls (without the inhibitors). All data were analyzed by the Student t-test and expressed as means $\pm$ SD of 5 determinations ($p < 0.001$ was considered significant).

2.4.1. Free iron (II)-dependent lipid peroxidation

The PCL suspension was sonicated until the conjugated dienes reached 0.7 ± 0.048 absorbance units (a.u.). 10 μ l of aqueous 0.5 mmol/l $FeSO_4$ solution (final concentration 5 μ mol/l) were added to 1 ml of PCL suspension and the increase in conjugated dienes was monitored spectrophotometrically for 10 min at 37 °C. Inhibitors (nimesulide, hydroxynimesulide dissolved in methanol and EDTA dissolved in water) were incorporated immediately before the addition of iron.

2.5. Iron(II)-hydroxynimesulide interaction

The interaction between hydroxynimesulide and iron and the stoichiometry of the corresponding complex was determined spectrophotometrically by the Job method [20]. Briefly, different aliquots of an aqueous solution of $FeSO_4 \times 7H_2O$ (1 mmol/l)

and hydroxynimesulide (1 mmol/l in 3 mmol/l NaOH) were mixed and diluted with 10 mmol/l TRIS buffer, pH 7.4, to a final volume of 25 ml.

The concentration ratio of hydroxynimesulide/iron ranged from 9 : 1 to 1 : 9 and the sum of the concentrations was always 0.4 mmol/l. The stability of the Fe(II)-hydroxynimesulide complex (0.4 mmol/l at the stoichiometric ratio) was tested by titration with known amounts (75–525 $\mu\text{mol/l}$) of the chelating agents NTA and EDTA, according to Malkiel et al. [21], and monitoring the optical density at the absorption maximum (298 nm) after each addition (10 μl aliquots) of the ligands.

2.6. Ascorbate/ Fe^{2+} -induced peroxidation of lipid liposomes from synovial fluid

Lipid extracts from synovial fluid were prepared as described by Lunec and Dormandy [22]. Aliquots of the chloroform phase (each corresponding to 3.5 mg of lipids) were dried under nitrogen and the lipid films were resuspended in 0.9 ml of de-ionized water by anaerobic sonication for 15 min.

The liposomal suspensions were incubated for 24 h at 37 °C in the presence of FeSO_4 (100 $\mu\text{mol/l}$) and ascorbic acid (1 mmol/l) and different concentrations of nimesulide and hydroxynimesulide (2.5–50 $\mu\text{mol/l}$; final volume 1 ml). The reaction was stopped by addition of 500 μl of blocking solution (7.5 g TCA and 0.185 g TBA in 25 ml of 0.25 N HCl). The tubes were tightly capped and heated at 100 °C for 15 min. After cooling and centrifugation at 5000 rpm for 10 min, the amount of thiobarbituric acid-reactive substances (TBARS) in the clear supernatant was determined spectrophotometrically at 532 nm, using an extinction coefficient of $1.56 \times 10^5 \text{ mol}^{-1} \text{ cm}^{-1}$. The inhibitory effect was expressed as % inhibition of TBARS formation, setting the control values as 100 % activation (mean values of 5 determinations were used for calculation).

3. Results

3.1. ESR studies

3.1.1. $\text{HO}^\bullet$ scavenging activity

The direct $\text{HO}^\bullet$ scavenging activity of nimesulide and hydroxynimesulide was determined by ESR studies. The $\text{HO}^\bullet$ radical generated by water sonolysis reacted with DMPO to yield the typical DMPO-OH spin adduct spectrum (1 : 2 : 2 : 1 quartet), with identical hyperfine splitting constants $a_N = a_H = 14.9 \text{ G}$ [23]. The addition of nimesulide (Fig. 1) dose-dependently inhibited DMPO-OH formation: the control signal height intensity was reduced by $38 \pm 2.1\%$ at 1 $\mu\text{mol/l}$, $56 \pm 2.9\%$ at 10 $\mu\text{mol/l}$, $80 \pm 3.5\%$ at 50 $\mu\text{mol/l}$ and was practically negligible (100 % inhibition) at 100 $\mu\text{mol/l}$ ($\text{IC}_{50} = 8.3 \mu\text{mol/l}$; correlation coefficient = 0.9650). Hydroxynimesulide was less active than the parent drug in entrapping $\text{HO}^\bullet$ radicals, since the concentration required for 50 % quenching of the DMPO-OH spin adduct was 7 times greater ($\text{IC}_{50} = 54.8 \mu\text{mol/l}$; correlation coefficient = 0.9969), the minimal effective concentration being 25 $\mu\text{mol/l}$ (21.8 % inhibition) (data not shown).

The rates of reaction of nimesulide or hydroxynimesulide with $\text{HO}^\bullet$ were established by competition studies with the DMPO spin trap; the bimolecular rate constants for the reactions were determined from the initial rates of DMPO-OH adduct formation in the presence and the absence of the drugs, by competitive kinetic analysis (see experimental).

As shown in Fig. 2, competition plots were linear with both the compounds, and the rate constants calculated from their slopes:

$$k_{\text{nimesulide}}/\text{HO}^\bullet: 3.3 \pm 0.6 \times 10^{11} \text{ mol}^{-1} \text{ s}^{-1};$$

$$k_{\text{hydroxynimesulide}}/\text{HO}^\bullet: 8.9 \pm 2.1 \times 10^{10} \text{ mol}^{-1} \text{ s}^{-1}$$

indicate that both the compounds react with $\text{HO}^\bullet$ in free solution at a diffusion-controlled rate and that low concentrations (0.1 mmol/l) compete successfully with a very high concentration of the spin trap (10 mmol/l); the $\text{HO}^\bullet$ scavenging potency of nimesulide is slightly greater than that of its metabolite.

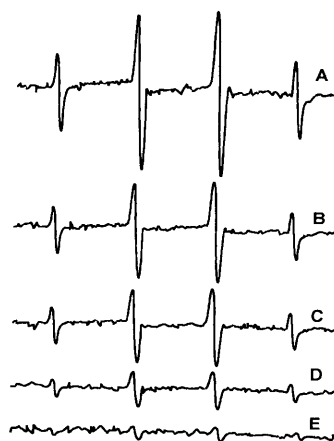


Fig. 1: Hydroxyl radical scavenging activity of nimesulide. The ESR spectra of the DMPO-OH spin adduct in the absence (A) and in the presence of different concentrations of nimesulide (B = 1 $\mu\text{mol/l}$; C = 10 $\mu\text{mol/l}$; D = 50 $\mu\text{mol/l}$; E = 100 $\mu\text{mol/l}$) were recorded after 15 min of ultrasound irradiation.

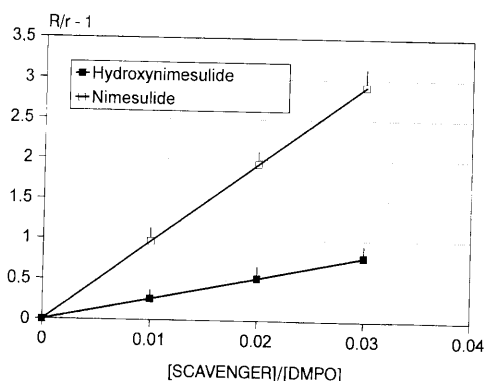


Fig. 2: Competition kinetic plots for the reaction of hydroxyl radicals with DMPO and nimesulide and hydroxynimesulide. R and r are the initial rates of DMPO-OH formation in the absence and the presence of different concentrations of the two compounds. Data are the means $\pm$ S.D. of 5 determinations.

3.1.2. Superoxide anion scavenging activity

The $\text{O}_2^{\bullet-}$ generated by the xanthine-xanthine oxidase system (chosen because the rate of superoxide production is unaffected by the spin-trap concentration) reacted with DMPO to yield DMPO-OOH spin adduct, recognizable by the characteristic hyperfine splitting constants ($a_N = 14.3 \text{ G}$, $a^{\beta}H = 11.7 \text{ G}$, $a^{\gamma}H = 1.25 \text{ G}$) [23] (Fig. 3). This was further confirmed by the fact that DMPO-OOH production was totally prevented by the addition of well known inhibitors, SOD or allopurinol, to the incubation medium (data not shown). As shown in Fig. 3, hydroxynimesulide inhibited the DMPO-OOH adduct formation dose-dependently: the percentage quenchings of the spin adduct were $17.1 \pm 1.2\%$ at 1 $\mu\text{mol/l}$, $27.3 \pm 1.8\%$ at 10 $\mu\text{mol/l}$, $41.0 \pm 2.9\%$ at 50 $\mu\text{mol/l}$ and $60.5 \pm 4.3\%$ at 100 $\mu\text{mol/l}$. At 200 $\mu\text{mol/l}$ the signal was completely quenched ($98 \pm 5.8\%$) ($\text{IC}_{50} = 40 \mu\text{mol/l}$). On the contrary, nimesulide was ineffective over this concentration range. DMPO-OOH quenching by hydroxynimesulide is due to a direct scavenging effect against the superoxide anion and not to an inhibitory effect on the promoter xanthine oxidase, since (data not shown) uric acid formation was not affected by the compound at any concentration tested (1–200 $\mu\text{mol/l}$).

The evaluation of the rate constant of reaction of hydroxynimesulide with superoxide by kinetic competition studies was impossible because of the great instability of the DMPO-OOH adduct, which spontaneously decays (half-life 91 s at pH 5) into a non-radical species and DMPO-OH [23].

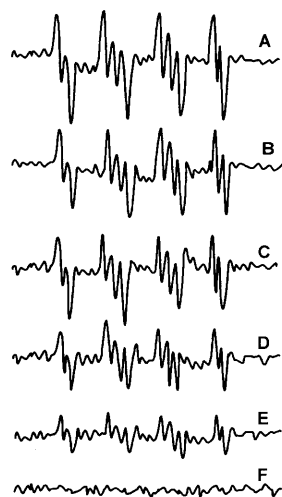


Fig. 3: Superoxide anion scavenging activity of hydroxynimesulide. A = DMPO-OOH spin adduct (control); B = 1 $\mu\text{mol/l}$ hydroxynimesulide; C = 10 $\mu\text{mol/l}$; D = 50 $\mu\text{mol/l}$; E = 100 $\mu\text{mol/l}$; F = 200 $\mu\text{mol/l}$.

3.2. Radical scavenging activity in the PC liposomes

As previously demonstrated [17], in the $\text{HO}^\bullet$ -mediated initiation of PCL peroxidation, nimesulide significantly protects phospholipids from free radical attack (preven-

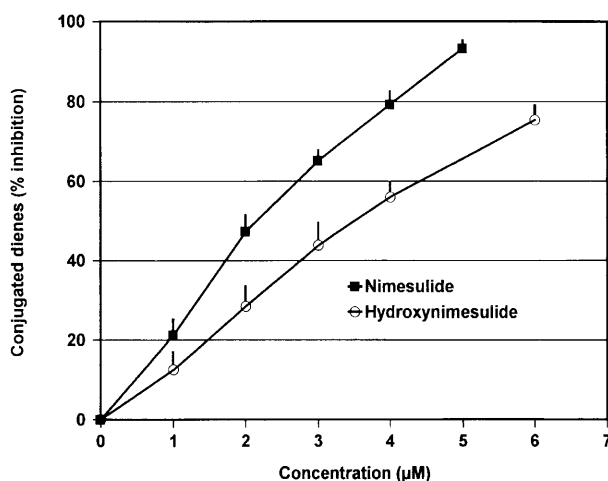


Fig. 4: Preventive antioxidant activities of nimesulide and hydroxynimesulide in PCL ($\text{HO}^\bullet$ scavenging activity). All values (means $\pm$ S.D. of 5 determinations) were statistically different from the controls (28.70 ± 0.7 nmol/ml) ($p < 0.001$).

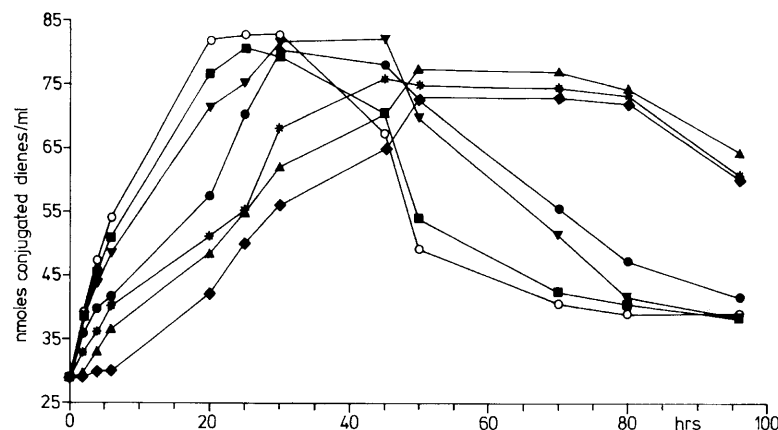


Fig. 5: Time-course of conjugated dienes in the propagation and breakdown phases of lipid peroxidation in PCL: chain-breaking antioxidant activity of hydroxynimesulide. Hydroxynimesulide and α -tocopherol were added to PCL at the starting of the propagation phase. Values are the means $\pm$ S.D. of 5 determinations (standard deviations in the text). $\circ$ Control, $\blacksquare$ 0.5, $\blacktriangledown$ 1.0, $\bullet$ 2.5, $\ast$ 5.0, $\blacktriangle$ 10.0 $\mu\text{mol/l}$; $\blacklozenge$ vitamin E.

tive antioxidant), by dose-dependently inhibiting conjugated diene formation ($\text{IC}_{50} = 1.85$ $\mu\text{mol/l}$). By contrast, the drug was ineffective as a chain-breaking antioxidant, since there was no inhibition of conjugated diene increase during the propagation phase nor inhibition of total carbonyl function formation at the end of the breakdown phase (main species involved lipid oxy radicals) at any concentration (1–200 $\mu\text{mol/l}$).

This was confirmed in the present study, in which the antioxidant activity of the drug was compared to that of 4-hydroxynimesulide, using a higher concentration of phosphatidylcholine (0.256 mmol/l instead of 0.064 mmol/l), to achieve a drug/phospholipid ratio closer to the *in vivo* situation.

In the initiation phase, in which the $\text{HO}^\bullet$ radical is the main species involved [19] (Fig. 4), hydroxynimesulide was less effective than nimesulide in entrapping hydroxyl radicals (IC_{50} 3.84 vs 2.12 $\mu\text{mol/l}$): at 1 $\mu\text{mol/l}$ conjugated diene formation was inhibited by $12.5 \pm 0.6\%$ and $21.2 \pm 0.5\%$ in comparison with the controls (28.70 ± 0.7 nmol/ml conjugated dienes), by $28.4 \pm 2.6\%$ and $47.3 \pm 1.9\%$ at 2 $\mu\text{mol/l}$, and by $43.8 \pm 2.7\%$ and $65.2 \pm 3.2\%$ at 3 $\mu\text{mol/l}$. The inhibitory activity of nimesulide was maximal ($93.2 \pm 3.7\%$) at 5 $\mu\text{mol/l}$ and for the metabolite ($75.4 \pm 3.4\%$) at 6 $\mu\text{mol/l}$.

In the post-initiation phase, the liposomal suspension was kept under aerobic conditions at 37°C for 96 h. The increase of conjugated dienes (Fig. 5) was linear until the 6th h after t_0 ($t_0 = 28.70 \pm 0.7$ nmol/ml, $t_{2h} = 39.24 \pm 0.9$ nmol/ml; $t_{4h} = 47.23 \pm 1.4$ nmol/ml; $t_{6h} = 54.14 \pm 2.2$ nmol/ml), with 4.24 ± 0.35 nmol/ml/h being the average rate of peroxidation. Afterwards the process was slower and the maximal absorbance value was obtained at the 20th h (82.03 ± 2.9 nmol/ml; $t_0 \text{-----} t_{20h} = 53.33 \pm 1.6$ nmol/ml), and then plateaued until the 30th h.

The decomposition of PC hydroperoxides (breakdown phase), which leads via the formation of alkoxyl radicals to secondary aldehydic products, began after 30 h and the absorption value had fallen to 1.82 a. u. at the 45th h (67.47 ± 2.4 nmol/ml); the loss of conjugated dienes was fast until the 50th h (49.19 ± 0.9 nmol/ml) and then became slower, reaching the minimal value of 38.76 ± 1.7 nmol/ml at the 96th h (Fig. 5). Hydroxynimesulide, added at the beginning of the propagation phase ($t = 0$), inhibited the increase of conjugated dienes dose-dependently: the lowest concentration 0.5 $\mu\text{mol/l}$ prevented formation of conjugated dienes (t_{20h}) by 10.03% (76.68 ± 2.8 nmol/ml), 1 $\mu\text{mol/l}$ by 20.10% (71.31 ± 1.9 nmol/ml), 2.5 $\mu\text{mol/l}$ by 46.10% (57.44 ± 0.9) and 5.0 $\mu\text{mol/l}$ by 58.15% (51.02 ± 1.4 nmol/ml), with an $\text{IC}_{50} = 2.67$ $\mu\text{mol/l}$. 10 $\mu\text{mol/l}$ did not produce significantly more inhibition (63.1% ; 48.38 ± 1.5 nmol/ml).

In all the samples protected with hydroxynimesulide the plateau of conjugated dienes was reached later than the control ($t = 25$ h for $0.5 \mu\text{mol/l}$, $t = 30$ h for 1 and $2.5 \mu\text{mol/l}$ vs $t = 20$ h for the control) and the effect was much more pronounced with the highest concentrations (5 and $10 \mu\text{mol/l}$), with which the conjugated dienes plateaued only after 45 h (Fig. 5).

The same was observed in the breakdown phase: hydroxynimesulide (1 – $10 \mu\text{mol/l}$) significantly delayed the beginning of phosphatidylcholine hydroperoxide decomposition (controls $t = 30$ h): by 15 h ($t = 45$ h) at 1 and $2.5 \mu\text{mol/l}$ and by 40 h ($t = 70$ h) at the highest concentrations.

At the last observation time ($t = 96$ h), absorbance for conjugated dienes fell to control levels in the 0.5 – $2.5 \mu\text{mol/l}$ protected samples, while it was still higher in the presence of 5 or $10 \mu\text{mol/l}$ hydroxynimesulide (60.38 ± 3.1 and 64.21 ± 3.5 vs 38.76 ± 1.7 nmol/ml). In accordance with these findings, hydroxynimesulide inhibited the decomposition of peroxidized phosphatidylcholine (determined as total carbonyl functions) only at 5 and $10 \mu\text{mol/l}$: total carbonyl functions were 30.4% and 33.8% lower than in the controls (47.15 ± 2.7 and 44.87 ± 3.1 vs 67.78 ± 3.4 nmol dinitrophenylhydrazones/ml).

At equimolecular concentrations ($5 \mu\text{mol/l}$), the typical chain-breaking antioxidant α -tocopherol was slightly more potent than hydroxynimesulide in restraining both the propagation and breakdown phases of PC peroxidation (Fig. 5).

3.3. Inhibition by hydroxynimesulide of iron(II)/PC-OOH-amplified lipid peroxidation in PCL

Catalytic iron concentrations stimulate lipid peroxidation by decomposition of lipid hydroperoxides to alkoxyl radicals, through a Fenton-like reaction $\text{ROOH} + \text{Fe}^{2+} \rightarrow \text{RO}^\bullet + \text{Fe}^{3+} + \text{HO}^-$ [24], in which alkoxyl radicals are the main species involved. However, recent studies [25, 26] indicate that during the iron-induced decomposition of lipid hydroperoxides, hydroxyl radicals are also generated: this was evident from our ESR experiments. As shown in Fig. 6 (A), the addition of free iron ($5 \mu\text{mol/l}$ FeSO_4) to phosphatidylcholine hydroperoxides (obtained by exposure of PCL to ultrasound irradiation until the formation of 25.92 ± 1.78 nmol conjugated dienes/ml), resulted in the formation of the typical DMPO-OH radical spin adduct. When PCL hydroperoxides or Fe^{2+} ions were omitted, no DMPO-OH radical adducts were detected. No adduct formation was observed when native instead of peroxidized PC was treated with iron and when Fe^{3+} instead of Fe^{2+} was used as catalyst (Fig. 6, B–C). This last evidence indicates that the DMPO-OH adduct formation was not due to a nucleophilic attack of water on the Fe^{3+} -DMPO complex (Fe^{3+} arises from the reduction of hydroperoxides by Fe^{2+}) [27]. Generation of hydroxyl radicals was further confirmed by the addition of typical $\text{HO}^\bullet$ scavengers (Fig. 6, D–E): 100 mmol/l mannitol completely eradicated the DMPO-OH signals; in the presence of 100 mmol/l CH_3OH the four-line spectrum of DMPO-OH disappeared, and the typical six-line spectrum ($a_N = 15.8$; $a_H = 22.86$ G) of the 2-hydroxymethyl-5,5-dimethyl-1-pyrrolidinyloxy (DMPO- CH_2OH) radical adduct was formed [26].

As shown in Fig. 7, the iron-induced decomposition of hydroperoxide, via generation of $\text{RO}^\bullet$ (main)/ $\text{HO}^\bullet$ (minor) radical species, greatly stimulated the burst of PC peroxidation. The kinetics of the increase of conjugated dienes proceeded with two different rates: the first is fast (0.57 ± 0.11 a. u. / min, corresponding to the formation of 21.06 ± 0.76 nmol/ml/min), since it involves the massive formation of $\text{R}^\bullet$ (and then of $\text{ROO}^\bullet$ species) from the reaction of $\text{RO}^\bullet/\text{HO}^\bullet$ radicals with RH (= native PC):

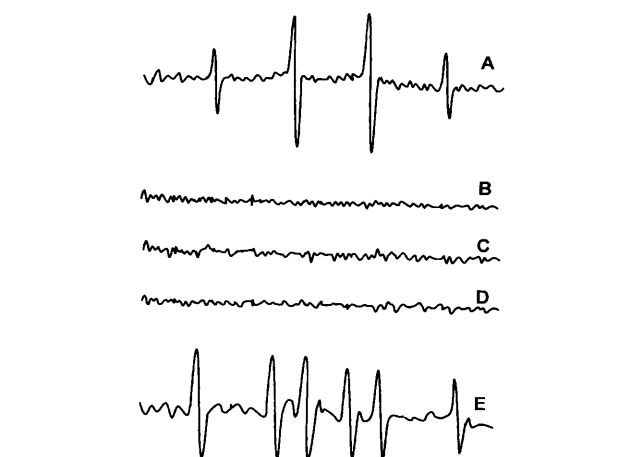


Fig. 6: ESR spectra of DMPO radical spin adducts obtained by reaction of PC-OOH with iron. A = PC-OOH + FeSO_4 ($5 \mu\text{mol/l}$); B = PC + FeSO_4 ; C = PC-OOH + FeCl_3 ($5 \mu\text{mol/l}$); D = PC-OOH + FeSO_4 + mannitol (100 mmol/l); E = PC-OOH + FeSO_4 + CH_3OH (100 mmol/l).

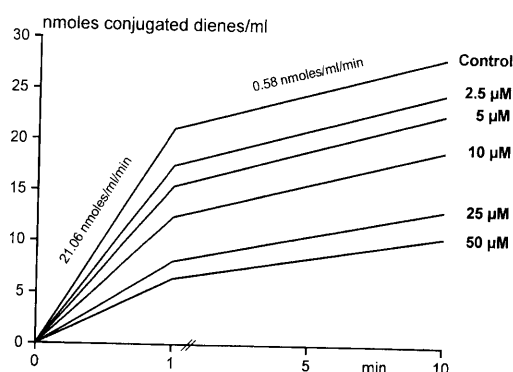


Fig. 7: Kinetics of iron/PC-OOH-amplified lipid peroxidation in PCL: protective effect of hydroxynimesulide. Hydroxynimesulide was incorporated in PCL immediately before the addition of FeSO_4 ($5 \mu\text{mol/l}$).

- $\text{RO}^\bullet + \text{RH} \rightarrow \text{R}^\bullet$
- $\text{HO}^\bullet + \text{RH} \rightarrow \text{R}^\bullet$
- $\text{R}^\bullet + \text{O}_2 \rightarrow \text{ROO}^\bullet$
- $\text{ROO}^\bullet + \text{RH} \rightarrow \text{R}^\bullet$

The second phase is slower (0.016 ± 0.004 a.u./min; 0.58 ± 0.15 nmol/ml/min) since $\text{R}^\bullet$ is produced only through c) and d) reactions. As shown in Fig. 7 and Table 1, hydroxynimesulide dose-dependently inhibited the iron-amplified lipid peroxidation: the rate of formation of conjugated dienes in the faster phase was lowered at the lowest concentrations (2.5 , $5 \mu\text{mol/l}$) by 17.2% and 26.9% (17.43 ± 0.56 and 15.39 ± 0.33 nmol/ml/min) and this effect was more pronounced at the higher concentrations: 41.2% at $10 \mu\text{mol/l}$ (12.38 ± 1.14 nmol/ml/min), 61.7% at $25 \mu\text{mol/l}$ (8.06 ± 0.97 nmol/ml/min), and plateaued (69.6%) at $50 \mu\text{mol/l}$ (6.40 ± 0.59 nmol/ml/min) ($\text{IC}_{50} = 17 \mu\text{mol/l}$). Because of the small amounts of $\text{HO}^\bullet$ species involved and the specific $\text{HO}^\bullet$ quenching capacity of nimesulide, the drug was active but less than its metabolite, approximately by one order of magnitude ($\text{IC}_{50} = 150 \mu\text{mol/l}$): minimal effective concentration (12% reduction) $50 \mu\text{mol/l}$ and maximal inhibition (77.3%) at $200 \mu\text{mol/l}$ (Table 1).

These findings clearly suggest that hydroxynimesulide protects lipid peroxides from Fe^{2+} stress, either through an $\text{RO}^\bullet/\text{HO}^\bullet$ and $\text{ROO}^\bullet$ scavenging effect or an iron chelating mechanism, or both [in this model system the potent iron chelating agent EDTA dose-dependently reduced the oxidation rate, by 18.7% at $5 \mu\text{mol/l}$ (17.12 ± 0.58 nmol/ml/min), 58.4% at $7.5 \mu\text{mol/l}$ (8.76 ± 0.5 nmol/ml/min) and by 96.5% at $10 \mu\text{mol/l}$ (0.74 ± 0.08 nmol/ml/min)].

Table 1: Effects of nimesulide, hydroxynimesulide and EDTA on iron/PC-OOH-dependent lipid peroxidation in PCL.

Drug concentration (μmol/l)	Oxidation rate (nmol/ml/min)
Control	21.06 ± 0.76
Nimesulide	
50	18.54 ± 1.59 (11.9)
100	14.85 ± 0.89 (29.5)
150	11.99 ± 0.81 (43.0)
200	4.78 ± 0.97 (77.3)
IC ₅₀ = 150	
Hydroxynimesulide	
2.5	17.43 ± 0.56 (17.2)
5	15.39 ± 0.33 (26.9)
10	12.38 ± 1.14 (41.2)
25	8.06 ± 0.97 (61.7)
50	6.40 ± 0.59 (69.6)
IC ₅₀ = 17	
EDTA	
5	17.12 ± 0.58 (18.7)
7.5	8.76 ± 0.50 (58.4)
10	0.74 ± 0.08 (96.5)

Values are the means ± S.D. of 5 determinations ($p < 0.001$). In brackets the percentage of inhibition values are shown.

3.4. Hydroxynimesulide/iron(II) complex

Fig. 8 shows the absorption spectra (200–450 nm) of the hydroxynimesulide/ Fe^{2+} complex obtained at different molar ratios of the compounds: the highest molar absorbance was observed at 298 nm and the maximal absorbance value at a ratio hydroxynimesulide/ Fe^{2+} 1 : 1.5 (160 μmol/l vs 240 μmol/l). The stability constant of the complex was determined by titrating it with different Fe^{2+} ligands: NTA was unable to displace hydroxynimesulide from the complex, since at 360 μmol/l, the concentration of the stoichiometric ratio NTA/ Fe^{2+} (1.5 : 1), the initial UV absorbance at 298 nm was reduced only by 11.6 %. EDTA reduced the absorbance of the complex hydroxynimesulide/ Fe^{2+} by 20 % at 75 μmol/l, by 58 % at 150 μmol/l and by 100 % at 240 μmol/l, the concentration of the stoichiometric ratio for the complex EDTA/ Fe^{2+} (1 : 1) (data not shown). Titration curves indicate that the stability constant of the hydroxynimesulide/ Fe^{2+} complex is intermediate between those of the NTA/ Fe^{2+} ($\log K = 6$) and the EDTA/ Fe^{2+} ($\log K = 11.1$) complexes and estimated at around $\log K = 8/9$ [both the values take into account the degree of dissociation of the ligands ($\log \alpha = 2.8$ for NTA; 3.3 for EDTA) at pH 7]. The favourable stoichiometric ratio of the hydroxynimesulide/ Fe^{2+} complex and the good affinity of the metabolite for iron confirm that hydroxynimesulide in this

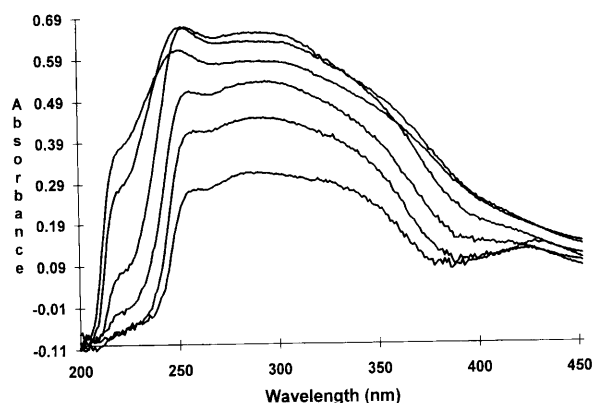


Fig. 8: Absorption spectra of the hydroxynimesulide/ Fe^{2+} complex at different molar ratios of the compounds. For details see Materials and methods.

model acts through a dual mechanism, by entrapping different radical species and by a Fe^{2+} chelating intervention.

3.5. Antioxidant effect of hydroxynimesulide and nimesulide on Fe^{2+} /ascorbate-induced peroxidation in lipid liposomes from synovial fluid

The time-course of accumulation of peroxidized products (TBARS) in synovial lipid liposomes from RA patients by radical generation shows an apparent maximum after 5 h, with formation of 5.81 ± 0.42 nmol/ml of TBARS (100 % activation). Incubation of liposomal suspensions after incorporation of increasing concentrations of nimesulide and hydroxynimesulide results in dose-dependent decreases in TBARS formation. Both compounds exhibited remarkable anti-lipoperoxidant activity (20.7 ± 1.4 % inhibition hydroxynimesulide; 17.4 ± 0.9 % nimesulide) already at 5 μmol/l. The reductions in TBARS formation were again of the same order of magnitude at 10 μmol/l (42.7 ± 1.9 % hydroxynimesulide; 43.3 ± 2.2 % nimesulide). At 25 μmol/l the effect was slightly higher for nimesulide (62.5 ± 2.7 % vs 53.8 ± 3.2 % hydroxynimesulide), and did not significantly increase further at higher concentrations. The amounts of TBARS were negligible when Fe^{2+} /ascorbate was omitted, in either the presence or the absence of drugs.

4. Discussion

This study is the first to our knowledge that describes the radical scavenging activity against oxy radicals of nimesulide and its main metabolite, 4-hydroxynimesulide, as detected by ESR spectroscopy. Using this unequivocal technique, it has been possible to demonstrate that hydroxynimesulide is a strong scavenger of both superoxide and hydroxyl radicals. There were significant decreases of the DMPO-OOH and DMPO-OH free radical spin adduct formation in the presence of micromolar concentrations of the compound. The high reactivity of hydroxynimesulide with $\text{HO}^\bullet$ radicals was further established by competition kinetics with DMPO. The competition plot was linear, and the rate constant calculated from its slope ($K = 8.9 \times 10^{10} \text{ mol}^{-1} \text{ l s}^{-1}$) indicates that the molecule reacts with $\text{HO}^\bullet$ at essentially a diffusion-controlled rate.

Nimesulide, which has been shown by ESR to be inactive as a superoxide quencher, has a rate constant for reaction with $\text{HO}^\bullet$ radicals ($3.3 \times 10^{11} \text{ mol}^{-1} \text{ l s}^{-1}$) higher than that of its metabolite. For comparison, the rate constants for reaction with $\text{HO}^\bullet$ of typical NSAIDs, such as indomethacin and diclofenac, determined by the deoxyribose assay (which gives results comparable to those obtained with pulse radiolysis) [28, 29], indicate that both these drugs react more slowly with $\text{HO}^\bullet$ (K values around $10^{10} \text{ mol}^{-1} \text{ l s}^{-1}$). Hence we can draw this order of potency as $\text{HO}^\bullet$ quenchers: nimesulide > hydroxynimesulide > indomethacin $\approx$ diclofenac.

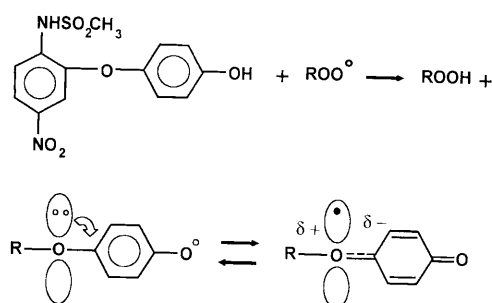
The greater activity of nimesulide than hydroxynimesulide towards $\text{HO}^\bullet$ radicals was also demonstrated in a phospholipid bilayer constituted by phosphatidylcholine, the most important functional lipid in cellular membrane, in which lipid peroxidation was induced by $\text{HO}^\bullet$ radicals generated by water sonolysis. Both the compounds act as preventive antioxidants, but the $\text{HO}^\bullet$ entrapping capacity of the parent drug is nearly double that of its metabolite ($\text{IC}_{50} = 2.12$ vs 3.84 μmol/l).

In addition, two other important features of hydroxynimesulide stem from PCL experiments:

1. Its high capacity for entrapping the peroxy radical $\text{ROO}^\bullet$, which is responsible for the spread of the peroxidation phenomenon. Already at 1 μmol/l the compound significantly reduced the rate of PC peroxidation and markedly delayed the onset of the breakdown phase

which leads, through decomposition of hydroperoxides to alkoxyl radicals, to the formation of the highly cytotoxic alkenals and hydroxyalkenals, which can act as chemoattractant factors in the inflammatory reaction [30].

Hydroxynimesulide, with its favourable lipophilicity ($\log P = 1.27$), easily locates within the PCL bilayer. Here it behaves as a typical intramembranous chain-breaking antioxidant because of the presence of a phenolic function which quenches $\text{ROO}^\bullet$ species by hydrogen transferring reactions: the phenoxyl radical formed is highly stabilized by conjugative electron delocalization, involving the ether oxygen that is para to the HO group (Scheme 1). This structural feature explains well why nimesulide is ineffective in quenching peroxy radicals during the propagation phase of PCL peroxidation. On the basis of the same mechanism, hydroxynimesulide, but not nimesulide, acts as an effective superoxide quencher.



Scheme 1: Chain-breaking antioxidant intervention of hydroxynimesulide.

2. Its ability to dose-dependently reduce the hydroperoxides/ Fe^{2+} -stimulated PC peroxidation, via Fenton reaction: stimulation which is appreciable even when the iron concentration is low, close to the one reached in vivo in the inflamed synovium [31]. This is not only through a radical scavenging action, but also a chelation mechanism, as clearly evidenced by the formation of a strong Fe^{2+} /hydroxynimesulide complex.

This aspect might be of clinical importance in those pathological situations in which iron is released in free form from ferritin and transferrin, such as in rheumatoid arthritis in which large numbers of neutrophils enter the joints and the resulting O_2^- production, together with the local decrease of pH, greatly contributes to iron decompartmentalization. The increased availability of iron as catalyst for decomposition of preformed hydroperoxides markedly exacerbates the formation in the synovium of secondary oxy radicals ($\text{HO}^\bullet$ and $\text{RO}^\bullet$ radicals, as evidenced by others and our ESR experiments on PC liposomes) which both directly, by release of lysosomal enzymes from activated neutrophils, and indirectly, by oxidative inactivation of protease inhibitors, cause deconstruction of macromolecular components. In this context, both nimesulide and hydroxynimesulide remarkably protect synovial fluid lipids obtained from inflamed joints (and for this reason containing lipid hydroperoxides) from Fe^{2+} /ascorbate degradation already at $10 \mu\text{mol/l}$, a concentration in the range of those detected in vivo in synovial fluids during chronic treatment. That both the compounds exhibited comparable antioxidant activity in this membrane model might be a consequence of multiple factors, such as partitioning and localization across the bilayer thickness, and the rapid conversion of nimesulide to hydroxynimesulide by $\text{HO}^\bullet$ radical attack [16].

This opens interesting perspectives for the use of nimesulide in rheumatoid arthritis, in which synovial lipid hydroperoxides provide a reservoir of potential radicals which can be mobilized by iron.

The results of this study raise the possibility that hydroxynimesulide, owing to its preventive antioxidant activity, might cooperate in vivo with the parent drug in reducing the rate of radical chain initiation in membrane PUFA. In addition, as a chain-breaking antioxidant and an iron-chelating agent, it should limit the extension of the peroxidative damage of phospholipids, even after initiation, thus delaying the macroscopic membrane derangement and the formation of cytotoxic alkenals.

The antioxidant activities of nimesulide and hydroxynimesulide, described here in detail, together with the results of other authors showing marked inhibitory effects for both the compounds on the respiratory burst of activated neutrophils [9–14], reinforce the view of a multifactorial intervention underlying the mechanism of the anti-inflammatory action of the drug.

It is currently under investigation whether the antioxidant effectiveness of hydroxynimesulide enhances the antioxidant capacity of vitamin E, presumably by regeneration of tocopheroxyl radical through hydrogen donating reaction.

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