

Interestingly nimesulide and its derivatives are also potent inhibitors of superoxide anion release in polymorphonuclear leukocytes [11], and a recent work underlines the link between NADPH-oxidase inhibition and COX-2 expression [22]. Further work is necessary to establish if the COX-2-selective inhibition, NADPH-oxidase inhibition and other cAMP-mediated effects in human polymorphonuclear leukocytes [23, 24] are related phenomena.

5. References

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Protective Effect of Furosemide Combined with Non-steroidal Anti-inflammatory Drugs Administered by Inhalation Route on Guinea-pigs Anaphylaxis Model

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Summary

The exposure of ovalbumin sensitized guinea-pig to an aerosol of the specific antigen causes a respiratory crisis in approximately 100 s (dyspnoea time) associated with a substantial increase in blood concentration of both histamine (from 27.5 ± 1.8 ng/ml to 1570 ± 26 ng/ml; $n = 8$) and thromboxane B_2 (TXB₂, from 0.52 ± 0.03 ng/ml to 18.1 ± 0.6 ng/ml; $n = 8$).

The aerosol treatment of the animals (20 min) with furosemide (CAS 54-31-9, frusemide, FRU), nimesulide (CAS 51803-78-2, NIM), acetylsalicylic acid (CAS 50-78-2, ASA) and indometacin (CAS 53-86-1, INDO) at the concentrations of 1-3-10 and 30 mg/ml,

before ovalbumin challenge, brought about an attenuation of anaphylactic response. The rank order of potency for the prolongation of dyspnoea time was FRU > NIM > ASA > INDO. In these experiments blood evaluation performed at the peak of the dyspnoea time for histamine concentration in the treated animals indicated that whereas FRU ($ED_{25} = 2.14$ mg/ml (1.97–2.38)) and NIM ($ED_{25} = 2.74$ mg/ml (2.37–3.19)) were equiactive in reducing the release of histamine, ASA and INDO were devoid of this activity. On the contrary, the results obtained with ASA and INDO indicated a greater intrinsic activity in antagonizing TXB₂ formation than that shown by the log-dose response curves of NIM and FRU.

In another series of experiments, the interaction of FRU with the other anti-inflammatory drugs in protecting guinea-pig from immune bronchoconstriction has been evaluated using the combination of two equiactive doses. The mixtures considered were FRU + NIM, FRU + INDO and FRU + ASA. The results obtained indicated that FRU interacts positively with the three non-steroidal anti-inflammatory drugs in delaying the onset of the dyspnoeic crisis in guinea-pig. However, when FRU was combined with NIM the gain obtained (209 %) appeared superior to that reached when FRU was combined with ASA (180 %) or INDO (126 %).

Taken together, these results suggest that non-steroidal anti-inflammatory compounds given by aerosol may represent a valid pharmacological intervention in protecting guinea-pig from anaphylactic bronchoconstriction.

Zusammenfassung

Protektive Wirkung von Furosemid in Kombination mit nichtsteroidalen entzündungshemmenden Substanzen nach inhalativer Verabreichung im Anaphylaxie-Modell des Meerschweinchens

Die Behandlung von Ovalbumin-sensibilisierten Meerschweinchen mit einem Aerosol, das das spezifische Antigen enthält, verursacht nach ca. 100 s eine respiratorische Krise (Dyspnoe-Zeit). Gleichzeitig kommt es zu einer wesentlichen Steigerung der Plasmaspiegel sowohl von Histamin (von $27,5 \pm 1,8$ ng/ml auf 1570 ± 26 ng/ml; $n = 8$) als auch von Thromboxan B_2 (TXB₂, von $0,52 \pm 0,03$ ng/ml auf $18,1 \pm 0,6$ ng/ml; $n = 8$).

Die Aerosolbehandlung der Tiere (20 min) mit Furosemid (CAS 54-31-9, Frusemid, FRU), Nimesulid (CAS 51803-78-2, NIM), Acetylsalicylsäure (CAS 50-78-2, ASS) und Indometacin (CAS 53-86-1, INDO) bei Konzentrationen von 1, 3, 10 und 30 mg/ml vor der Ovalbumin-Exposition ergab eine Abschwächung der

anaphylaktischen Reaktion. Die Verlängerung der Dyspnoe-Zeit erfolgte in der Reihenfolge FRU > NIM > ASS > INDO. In diesen Versuchen wurden bei den Versuchstieren im Maximum der Dyspnoe-Zeit Blutuntersuchungen durchgeführt und die jeweiligen Histaminkonzentrationen ermittelt. Es ergaben sich Hinweise darauf, daß FRU ($ED_{25} = 2,14$ mg/ml (1,97–2,38)) und NIM ($ED_{25} = 2,74$ mg/ml (2,37–3,19)) gleich stark die Freisetzung von Histamin hemmen, während ASS und INDO keine solche Wirkung aufwiesen. Im Gegensatz dazu wiesen die bei ASS und INDO erhaltenen Ergebnisse auf stärkere hemmende Eigenschaften bei der Bildung von TXB₂ hin, als sie die log-Dosis-Reaktionskurven von NIM und FRU zeigten.

In einer weiteren Versuchsreihe wurde die Wechselwirkung von FRU mit anderen entzündungshemmenden Substanzen bei der Schutzwirkung gegen immunvermittelte Bronchokonstriktion beim Meerschweinchen bewertet. Folgende Kombinationen wurden in jeweils gleich stark wirksamen Dosen untersucht: FRU + NIM, FRU + INDO und FRU + ASS. Es zeigte sich, daß FRU mit den drei nichtsteroidalen entzündungshemmenden Substanzen bei der Verzögerung des Beginns der Dyspnoe-Krise beim Meerschweinchen eine positive Interaktion aufweist. Die Kombination von FRU mit NIM erzielte dabei ein besseres Ergebnis (209 %) als die Kombination von FRU mit ASS (180 %) oder INDO (126 %).

Mit Aerosol applizierte nichtsteroidale entzündungshemmende Substanzen könnten eine wirkungsvolle pharmakologische Behandlung zum Schutz von Meerschweinchen vor anaphylaktischer Bronchokonstriktion darstellen.

Key words: Anaphylaxis · Anti-inflammatories, combination with furosemide, non-steroidal · CAS 54-31-9 · Furosemide, combination with NSAID

1. Introduction

Previous studies [1] demonstrated that furosemide (CAS 54-31-9, frusemid, FRU) only when given by inhalation to actively sensitized guinea-pigs protects these animals from acute immune bronchoconstriction. On this basis, and since furosemide is neither a bronchodilator nor a receptor antagonist, it has been proved that the reduction of the severe anaphylactic bronchial reaction is associated with the inhibition of both histamine and thromboxane B_2 (TXB₂) release in the circulating blood of these animals.

The relevance of these pharmacological studies is reinforced by the clinical findings concerning the beneficial effects of furosemide in various bronchospastic phenomena. In fact it has been shown that the protective efficacy of inhaled furosemide is not restricted only to the attenuation of the bronchospastic response to physical stimuli [2] but it is also extended to atopic asthmatics where the acute and late phase of the reaction to inhaled allergen is significantly reduced [3].

The non-steroidal anti-inflammatory compound nimesulide¹⁾ (CAS 51803-78-2, N-(4-nitro-2-phenoxyphenyl)-

methanesulfonamide, NIM) [4], in spite of its different mechanism of action, appears to share with furosemide antianaphylactic activity in guinea pigs [5]. This compound inhibits bronchial response to histamine in this animal species and reduces the contraction caused by the autacoid in human bronchial strips [6]. Moreover, in perfused sensitized guinea-pig lungs, nimesulide reduced the anaphylactic release of histamine in a concentration dependent way, whereas indomethacin (CAS 53-86-1, INDO), which is more potent in reducing thromboxane A_2 (TXA₂) formation, potentiated the immune release of histamine [5].

All these observations prompted us to investigate the possible interaction of furosemide and nimesulide given by aerosol in the control of the systemic anaphylactic crisis induced in actively sensitized guinea-pigs. The interaction of furosemide with acetylsalicylic acid (CAS 50-78-2, ASA) and indomethacin was also investigated in light of the central role of the inflammatory process in the pathogenesis of the bronchoobstructive phenomenon. In fact several reports have already indicated that oral ASA and other non-steroidal anti-inflammatory compounds (NSAIDs) can be effective in the treatment of bronchial asthma, and cytological studies, performed by bronchoalveolar lavage, have clearly demonstrated eosinophilic airway inflammation not only in patients affected by severe asthma but also in patients with mild asthma and no current symptoms.

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

2. Material and methods

2.1. Animals and sensitization procedure

Male Hartley strain guinea-pigs (300–350 g), obtained from BMG-Allevamento (Civitate al Piano, BG, Italy) were actively sensitized to ovalbumin (100 mg/kg i.p. plus 100 mg/kg s.c.) according to the procedure originally described by Piper and Vane [7].

After 21 days, the sensitized animals were placed in a plexiglass box (25 × 14 × 14 cm) and exposed to ovalbumin aerosol (1 % solution in saline) at the flow rate of 1 ml/min by means of an ultrasonic nebuliser (De Vilbiss, Ultra Neb 99, Somerset, PA, USA).

The guinea-pigs were observed by an examiner unaware of the treatment protocol until the appearance of signs of immunological respiratory crisis (dyspnoea time) and of leaning on their side or until 8 min had elapsed, at which time the observation period was terminated. Then, the animals were rapidly removed from the box and arterial blood was drawn for the determination of histamine and TXB₂ concentrations as described below. Furosemide, nimesulide, acetylsalicylic acid, and indomethacin dissolved in saline at various doses (1, 3, 10 and 30 mg/ml) were administered by aerosol for 20 min to the sensitized animals before exposure to ovalbumin challenge. The single doses of the drugs under examination were given randomly in groups of 8 guinea-pigs each.

A control group of 8 sensitized animals was also planned and treated for 20 min with an aerosol of saline before antigen challenge.

The time (in s) of appearance of the respiratory crisis of the guinea-pigs subjected to the various treatments was recorded and from the dose-response curve the effective dose of each drug capable to delay the onset of the dyspnoeic phenomenon up to 175 s (ED₁₇₅) was evaluated.

2.2. Furosemide – non-steroidal anti-inflammatory drugs interactions

In another series of experiments the interaction of furosemide with the other NSAIDs in protecting guinea-pigs from the immunological bronchoconstriction was evaluated. Using the combination of two equiactive doses the mixtures were tested by aerosol at the level of two doses given to groups of 10 animals each as describe above. The mixtures considered were according to the following proportions: FRU + NIM (0.44–0.56); FRU + ASA (0.10–0.90); FRU + INDO (0.03–0.97).

2.3. TXB₂ and histamine assays

Samples of arterial blood (7–8 ml) were rapidly collected from the abdominal aorta in siliconized glass containing indomethacin (5 µg/ml). 1 ml of blood sample was quickly treated with excess of methanol (25 ml) in order to convert TXA₂ into its mono-O-methyl-TXB₂ derivative and centrifuged at 800 g. The supernatant was removed and stored at –20 °C. TXB₂ was evaluated by radioimmunoassay (RIA) according to Granström and Kindahl [8]. These determinations have previously been validated [9].

Another aliquot of blood (5 ml) was haemolysed by the addition of 4.5 ml of water and 0.5 ml of 12 N perchloric acid. Histamine was extracted from the supernatant with n-butanol, according to the fluorimetric method of Shore et al. [10]. Aliquots of histamine standard were processed at the same time. The recovery were always in the range of 80–90 %.

The interference of the different treatments with the immunological release of both TXB₂ and histamine in the circulation of the guinea-pigs has been expressed as inhibition percent of that found in control animals.

2.4. Statistical analysis

The data obtained by the present experiments were processed as follows. Since the results concerning the time (in s) of appearance of the respiratory crisis showed heterogeneity of variance in different groups of treatment, the weighted least-square method was adopted in order to calculate the parameters of the log-dose effect curves necessary to interpolate the ED₁₇₅ value for each compound. The reciprocal of variance according to Miller and Miller [11] was used as weight. However, this ratio, since it has been evaluated utilizing only the 50 : 50 mixtures, should not be generalized but considered as indicative of the phenomenon (gain).

Using the combination of two equiactive doses, three mixtures of two drugs were prepared and tested in order to obtain the log-dose response curves and the observed ED₁₇₅ by interpolation.

For each mixture, the ED₁₇₅, obtained with the drugs administered alone, were utilized to calculate the expected ED₁₇₅ under the hypothesis of additivity according to the relation of Finney [12].

The ratio between the expected ED₁₇₅ and the observed ED₁₇₅ were determined and the values were taken as an indication of the potency ratio (PR) among the mixtures.

2.5. Drugs

The following drugs were used: furosemide, nimesulide, acetylsalicylic acid, indomethacin and ovalbumin grade V (Sigma Chemical Co., St. Louis, MO, USA), [³H]thromboxane B₂ RIA-Kit (New England Nuclear, Boston, MA, USA).

3. Results

The exposure of sensitized guinea pig to an aerosol of the specific antigen (ovalbumin) causes a progressive increase in breathing rate that within few seconds becomes irregular culminating in approximately 100 s in a severe dyspnoea and reclinacion of the animals on their sides (dyspnoea time). This respiratory crisis is associated with a remarkable increase of both histamine (from 27.5 ± 1.8 ng/ml to 1570 ± 26 ng/ml; n = 8) and TXB₂ (from 0.52 ± 0.03 ng/ml to 18.1 ± 0.6 ng/ml; n = 8) in the circulating blood.

The aerosol treatment of the animals for 20 min before antigen challenge with graded doses of furosemide, nimesulide, acetylsalicylic acid and indomethacin brought about a clear-cut attenuation of the anaphylactic response marked by a delayed onset of the dyspnoeic phenomenon (which was less pronounced), and by a significant reduction in blood concentration of both histamine and TXB₂. The protecting activity of the compounds under examination is dose-dependent and concerning the dyspnoea time the rank order of potency is FRU > NIM > ASA > INDO (Fig. 1).

Considering the interference of the drugs under evaluation with the immunological release of histamine, furosemide and nimesulide are almost equiactive in reducing the concentration of the autacoid in the blood (FRU: ED₂₅ = 2.14 mg/ml (1.97–2.38); NIM: ED₂₅ = 2.74 mg/ml (2.37–3.19)) whereas acetylsalicylic acid and indomethacin are almost devoid of this activity. In fact, while the log-dose response curves of both furosemide and ni-

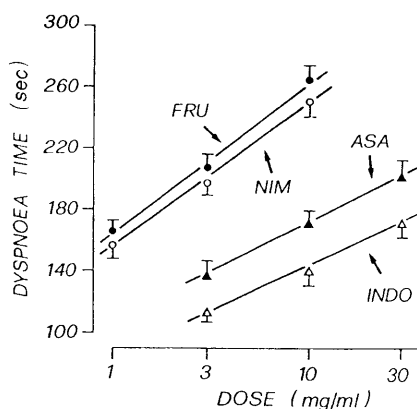


Fig. 1: The aerosol administration of furosemide (FRU), nimesulide (NIM), acetylsalicylic acid (ASA) and indomethacin (INDO) to conscious sensitized guinea-pigs delays the onset of the respiratory crisis induced by the specific antigen. Each point of the dose response curves is a mean value of 8 animals and vertical bars are SEM. The ED₁₇₅ (confidence lim. = 95 %) values for each treatment are: FRU, 1.25 mg/ml (1.08–1.49); NIM, 1.59 mg/ml (1.31–1.87); ASA, 11.32 mg/ml (10.25–12.47); INDO, 40.02 mg/ml (37.24–43.13). The dyspnoea time in saline treated guinea-pigs is 98.6 ± 5.3 s (n = 8).

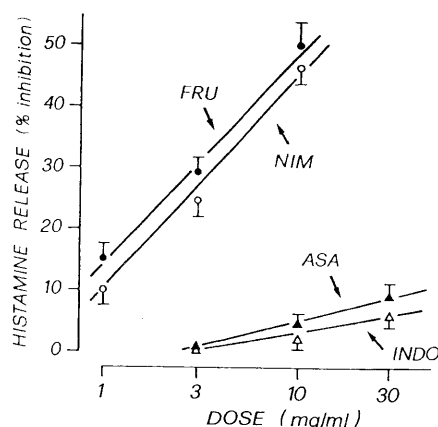


Fig. 2: Interference of furosemide (FRU), nimesulide (NIM), acetylsalicylic acid (ASA) and indomethacin (INDO) with the histamine release in the circulation of sensitized guinea-pigs during the anaphylactic pulmonary response. Each point of the dose response curves represents mean value and vertical bars the SEM of 8 animals. Drugs were given by aerosol 20 min before ovalbumin challenge and blood was drawn at the dyspnoea time. The concentration of histamine in the blood measured at the dyspnoea time of untreated (control) animals was 1570 ± 26 ng/ml ($n = 8$).

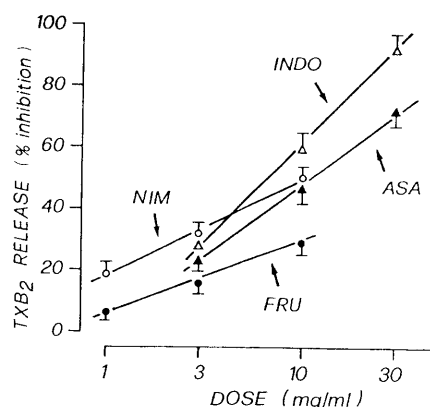


Fig. 3: Interference of furosemide (FRU), nimesulide (NIM), acetylsalicylic acid (ASA) and indomethacin (INDO) with TXB₂ release in the circulation of sensitized guinea-pigs during the anaphylactic pulmonary response. Each point of the dose response curves represents mean value and vertical bars the SEM of 8 animals. Drugs were given by aerosol 20 min before antigen challenge and blood was drawn at the dyspnoea time.

mesulide are characterized by a steep slope, those of acetylsalicylic acid and indomethacin present a slight slope. Only at the dose of 30 mg/ml the inhibitory activity of the latter two compounds is valuable in the range of 9% (Fig. 2).

Furthermore, considering the capacity of the four compounds to interfere with the immune release of TXB₂, the log-dose response curves of indomethacin and acetylsalicylic acid indicate a greater intrinsic activity than that shown by the log-dose response curves of nimesulide and furosemide at low concentration (Fig. 3).

3.1. Furosemide – non-steroidal anti-inflammatory drugs interactions

The results obtained with the three mixtures (FRU + NIM; FRU + INDO; FRU + ASA) clearly indicate that furosemide interacts positively with the three NSAID in delaying the onset of the dyspnoeic crisis induced by the anaphylactic response in guinea-pig. In fact, as shown in Table 1, the ratio between the expected and the observed ED₁₇₅ values of each mixture is > 1 . Since the ED₁₇₅ values were calculated under the hypothesis of additivity, it is reasonable to speculate that furosemide interacts in a synergistic way with each of the NSAID present in the mixture. Moreover when the diur-

Table 1: Furosemide (FRU) given by aerosol in combination with nimesulide (NIM), indomethacin (INDO) or acetylsalicylic acid (ASA) display a positive interaction in protecting guinea-pigs from anaphylactic pulmonary reaction.

Drug combination	ED ₁₇₅ expected (mg/ml)	ED ₁₇₅ (conf. lim. 95 %) observed (mg/ml)	Potency ratio
FRU + NIM	1.42	0.46 (0.36–0.58)	3.09
FRU + INDO	20.72	7.40 (3.22–13.10)	2.80
FRU + ASA	6.26	2.77 (1.44–5.63)	2.26

etic compound is combined with nimesulide the gain obtained (209%) appears to be superior to that reached when furosemide is combined with acetylsalicylic acid (180%) or indomethacin (126%).

4. Discussion

Non-steroidal anti-inflammatory drugs (NSAID), in spite of their well known antiprostaglandin action, have never been considered as potential antiasthmatic agents; these drugs with their antiprostaglandin activity are responsible for a number of unwanted side effects including severe bronchoconstriction in about 10% of predisposed patients. Moreover NSAIDs are ineffective against a variety of bronchoconstrictor stimuli including methacholine [13], histamine [14], leukotriene D₄ [15], adenosine [16] and exercise [17]. All these factors have hampered the inquiry of the clinical efficacy of these compounds in bronchoconstrictive diseases. However, the use of NSAIDs by inhalation that allows to obtain in the airways of atopic patients a higher local concentration of these agents with a reduced risk of adverse systemic effects appears to have some clinical relevance and may in the future represent an alternative approach in the treatment of this pulmonary disease [18].

With the present experiments performed in conscious guinea-pigs the evidence is provided that inhalation of NSAIDs alone or in combination with furosemide displays beneficial effects in immune dyspnoeic episodes. In fact, graded concentrations of nimesulide, acetylsalicylic acid, indomethacin and furosemide given alone by aerosol to ovalbumin-sensitized guinea-pigs protect these animals from the bronchopulmonary reaction induced by the specific antigen. This protective activity, on a shape of reduced severity and onset delay of the respiratory crisis, is linked to inhibition of anaphylactic mediator release, namely histamine and TXA₂. In this animal species, the immunological release of the above autacoids, which are detectable in the blood represents the major determinant of the dyspnoeic response. It is also known that histamine released by sensitized mastocytes upon antigen-antibody reaction increases airway resistance and stimulates the generation of bronchoactive and vasoactive TXA₂ in the lungs, setting up in this way a vicious circle difficult to break [19]. The protective activity of the four compounds above mentioned, in the light of their inhibitory effect on autacoid release, appears to originate from a different mechanism of action. In fact, both furosemide and nimesulide seem to be more effective in preventing the immune release of histamine, whereas acetylsalicylic acid and indomethacin, by virtue of their more pronounced ability to affect the activity of cyclooxygenase enzymes, show a better control of cyclooxygenase-derived metabolites of arachidonic acid, TXA₂ included. In this respect, inhaled furosemide has been already shown to inhibit the immune release of histamine from sensitized guinea-pigs and mast cells of the rat whereas nimesulide has been proved to possess antianaphylactic and antihistaminic activity in this animal species and to affect human neutrophil function *in vitro* [20].

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