Singlet oxygen scavenging activity of non-steroidal anti-inflammatory drugs

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It has long been known that singlet oxygen (\(^{1}\text{O}_2\)) is generated during inflammatory processes. Once formed in substantial amounts, \(^{1}\text{O}_2\) may have an important role in mediating the destruction of infectious agents during host defense. On the other hand, \(^{1}\text{O}_2\) is capable of damaging almost all biological molecules and is particularly genotoxic, which gives a special relevance to the scavenging of this ROS throughout anti-inflammatory treatments. Considering that the use of non-steroidal anti-inflammatory drugs (NSAIDs) constitutes a first approach in the treatment of persistent inflammatory processes (due to their ability to inhibit cyclooxygenase), a putative scavenging activity of NSAIDs for \(^{1}\text{O}_2\) would also represent a significant component of their therapeutic effect. The aim of the present study was to evaluate the scavenging activity for \(^{1}\text{O}_2\) by several chemical families of NSAIDs. The results suggested that the pyrazole derivatives (dipyrone and aminopyrine) are, by far, the most potent scavengers of \(^{1}\text{O}_2\) (much more potent compared to the other tested NSAIDs), displaying IC\(_{50}\)-values in the low micromolar range. There was a lack of activity for most of the arylpropionic acid derivatives tested, with only naproxen and indoprofen displaying residual activities, as for the oxazole derivative, oxaprozin. On the other hand, the pyrrole derivatives (tolmetin and ketorolac), the indolacetic acid derivatives (indomethacin, and etodolac), as well as sulindac and its metabolites (sulindac sulfide and sulindac sulfone) displayed scavenging activity in the high micromolar range. Thus, the scavenging effect observed for dipyrone and aminopyrine will almost certainly contribute to their healing effect in the treatment of prolonged or chronic inflammation, while that of the other studied NSAIDs may have a lower contribution, though these assumptions still require further in vivo validation.

Keywords: NSAIDs, singlet oxygen, endoperoxide of disodium 3,3′-(1,4-naphthalene)bispropionate (NDPO\(_2\)), dihydrorhodamine 123, microplate screening assay

Introduction

In biological tissues, inflammatory processes involve complex physiological responses, resulting from interactions between cells and soluble factors, to infection, ischemia or injury. It is well known that a few mediators play a prominent role in the inflammatory cascade, namely arachidonic acid-derived lipid compounds (i.e. eicosanoids) and cytokines. On the other hand, several lines of evidence indicate that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a pivotal role in cellular damage often observed in many inflammatory conditions.\(^1\)\(^-\)\(^3\) Accordingly, it has been shown that several synthetic and/or natural antioxidants are endowed with potent anti-inflammatory activity.\(^4\)
Furthermore, it has been extensively demonstrated that several non-steroidal anti-inflammatory drugs (NSAIDs) are effective scavengers of ROS and RNS, which probably contributes to their final therapeutic activity. In this respect, the most studied reactive species have been the ROS superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO$^-$), peroxyl radical (ROO$^-$) and hypochlorous acid (HOCl), and the RNS nitric oxide (NO) and peroxynitrite anion (ONOO$^-$).

Nevertheless, another ROS, singlet oxygen (O$_2^*$), in addition to its well-known formation during photosensitization processes, has also been shown to have an important role in the inflammatory process. Figure 1 summarizes the production of ROS, including O$_2^*$ and RNS during inflammatory processes as reported elsewhere. Beyond its possible contribution to tissue healing during acute inflammation, O$_2^*$-mediated tissue damage in sustained or chronic inflammatory states, suggests that a potential effect against this ROS would be important for the therapeutic effect of anti-inflammatory drugs. Notwithstanding this rationale, the potential scavenging activity of NSAIDs for O$_2^*$ remains to be evaluated. The aim of the present study was to evaluate the scavenging activity for O$_2^*$ by several chemical families.
of NSAIDs – the pyrazole derivatives antipyrine, propyphenazone, dipyrone, and aminopyrine, sulindac and its metabolites sulindac sulfide and sulindac sulfone, the oxazole derivative oxaprozin, the pyrrole derivatives ketorolac and tolmetin, the indolacetic acid derivatives etodolac, indomethacin, and acemetacin, as well as the arylopropionic acid derivatives indoprofen, naproxen, ketoprofen, fenoprofen, flurbiprofen, ibuprofen, and fenbufen
fenbufen (Fig. 2). For this purpose, a previously developed non-cellular screening system was used,22 in which \( {^{1}\text{O}_2} \) was generated by thermal dissociation of the water-soluble endoperoxide of disodium 3,3′-(1,4-naphthalene)bispropionate (NDPO₂) and was detected by monitoring the \( {^{1}\text{O}_2} \)-induced oxidation of non-fluorescent dihydro-rhodamine 123 (DHR) to fluorescent rhodamine 123 (RH). This system allowed a rapid and thorough comparison of \( {^{1}\text{O}_2} \) scavenging potencies among the studied NSAIDs.

**Materials and methods**

**Materials**

All the chemicals and reagents were of analytical grade. Indomethacin, acemetacin, etodolac, tolmetin, ketorolac, flurbiprofen, fenoprofen, fenbufen, ketoprofen, naproxen, indoprofen, ibuprofen, dipyrone, aminopyrine, antipyrine, dihydrorhodamine 123 (DHR), ascorbic acid, and dimethylformamide were obtained from Sigma-Aldrich (St Louis, MO, USA). Oxaprozin was a gift from Helsinn Healthcare SA, Switzerland. Sulindac sulfide and sulindac sulfone were a gift from Merck Research Laboratories, USA. Propyphenazone was a gift from Novartis International Pharmaceutical Ltd (Ireland). Histidine was obtained from Fluka Chemie GmbH (Steinheim, Germany). NDPO₂ was obtained by synthesis as reported previously.22 All other reagents were obtained from Merck (Darmstadt, Germany).

**Generation of \( {^{1}\text{O}_2} \) by thermodissociation of NDPO₂**

\( {^{1}\text{O}_2} \) was generated by the thermal dissociation of water-soluble endoperoxide (NDPO₂) to 3,3′-(1,4-naphthalene)bispropionic acid (NDP) at 37°C, as described before.22

**\( {^{1}\text{O}_2} \) scavenging assay**

The \( {^{1}\text{O}_2} \) scavenging activity assay was performed by monitoring the \( {^{1}\text{O}_2} \)-induced oxidation of non-fluorescent DHR to fluorescent RH. The fluorimetric determinations were performed in a microplate reader (BIO-TEK, Synergy HT). A stock solution of 2.89 mM DHR in dimethylformamide was purged with nitrogen and stored at −20°C. Working solutions of DHR, histidine and NDPO₂ were prepared immediately before the assays, with phosphate buffer 100 mM, pH 7.4 placed on ice and protected from light. The use of histidine is of utmost importance for the sensitivity of the assay. This amino-acid reacts with \( {^{1}\text{O}_2} \) to form a trans-annular peroxide of the histidine imidazole ring moiety.22 Significantly, the reactivity of trans-annular endoperoxides of imidazole compounds with \( {^{1}\text{O}_2} \) probes, specially \( \text{N,N-dimethyl-4-nitrosoaniline} \), proceeds with much faster rates than by the direct oxidation through light-excited dyes or by \( {^{1}\text{O}_2} \).22 Reaction mixtures in the sample wells contained the following reagents at the indicated final concentrations (in a final volume of 250 µl): DHR (50 µM), histidine (10 mM), the tested NSAIDs at various concentrations (dissolved in 100 mM phosphate buffer, pH 7.4, or DMSO) and NDPO₂ (1 mM). The assays were performed at 37°C. No interference was found for DMSO under these assay conditions. The fluorescence signal was collected after a 30 min incubation period at the emission wavelength 528 nm with excitation at 485 nm. The effects are expressed as the percentage inhibition of the \( {^{1}\text{O}_2} \)-induced oxidation of DHR. Each study corresponds to four experiments, performed in triplicate. The following equation was used for calculating \( {^{1}\text{O}_2} \) scavenging activity, where \( F \) is the fluorescence intensity (arbitrary units):

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\text{\( {^{1}\text{O}_2} \) scavenging activity (\%) = 100 - \frac{F_{\text{sample}} - F_{\text{blank}}}{F_{\text{control}} - F_{\text{blank}}} \times 100\ Eq. 1\n\]

**Results**

\( {^{1}\text{O}_2} \) scavenging activity for pyrazole derivatives

The pyrazole NSAIDs dipyrone and aminopyrine were shown to be potent scavengers of \( {^{1}\text{O}_2} \) in a...
concentration-dependent manner, dipyrone being the most potent (Fig. 3). IC$_{50}$ values were 18.3 ± 1.8 and 56.3 ± 2.3 µM, respectively (Table 1). Neither antipyrine nor propyphenazone displayed any scavenging activity for 1O$_2$, up to the concentration of 5 mM.

1O$_2$ scavenging activity for sulindac and sulindac metabolites
Sulindac and its two metabolites sulindac sulfide and sulindac sulfone were shown to be efficient scavengers of 1O$_2$ in a concentration-dependent manner, sulindac sulfide being the most potent (Fig. 4). IC$_{50}$ values were 1.9 ± 0.1, 1.0 ± 0.06 and 1.7 ± 0.08 mM, respectively (Table 1).

1O$_2$ scavenging activity for pyrrole derivatives
The pyrrole NSAIDs tolmetin and ketorolac were shown to be efficient scavengers of 1O$_2$ with similar potencies (Fig. 5). IC$_{50}$ values were 2.4 ± 0.3 and 3.0 ± 0.3 mM, respectively (Table 1).

1O$_2$ scavenging activity for indolacetic acid derivatives
The indolacetic acid NSAIDs indomethacin and etodolac were shown to be efficient scavengers of 1O$_2$ with similar potencies (Fig. 5). IC$_{50}$ values were 3.0 ± 0.1 and 3.4 ± 0.1 mM, respectively (Table 1). Acemetacin only displayed residual scavenging activity for 1O$_2$ up to the concentration of 5 mM (38 ± 3% of inhibition at 5 mM).
Oxaprozin only displayed residual scavenging activity for $1^\text{O}_2$ up to the concentration of 5 mM (30 ± 2% of inhibition at 5 mM; Fig. 5).

**Discussion**

It has long been known that $1^\text{O}_2$ is generated during inflammatory processes by means of the myeloperoxidase/H$_2$O$_2$/Cl$^-$ system, through the reaction between H$_2$O$_2$ and HOCl. This ROS may also be formed in inflammatory processes as a by-product resulting from the spontaneous dismutation of $\text{O}_2^\cdot$. Once formed, in substantial amounts, $1^\text{O}_2$ may have an important role in mediating the destruction of infectious agents during host defense. On the other hand, it is common knowledge that a sustained production of ROS in prolonged or chronic inflammation can lead to severe damage to surrounding tissues, with consequences like cardiovascular disease, multiple sclerosis, diabetes, cancer, and dementia. Importantly, $1^\text{O}_2$ is capable of damaging almost all biological molecules and is particularly genotoxic, which gives a special relevance to the scavenging of this ROS through anti-inflammatory treatments. Considering that the use of NSAIDs constitutes a first approach in the treatment of persistent inflammatory processes, due to their ability to inhibit cyclooxygenase, a putative scavenging activity of NSAIDs for $1^\text{O}_2$ would also be of high importance for their therapeutic effect. From the NSAIDs assayed in the present non-cellular in vitro screening system, the results indicate that the pyrazole derivatives dipyrone and aminopyrine are, by far, the most potent scavengers of $1^\text{O}_2$ (much more potent compared to the other tested NSAIDs), displaying IC$_{50}$-values in the low micromolar range, near ascorbic acid values. Certainly, such potent activity will have an impact on biological tissues where $1^\text{O}_2$ is being produced at a high rate. It is noteworthy that, at a similar concentration, these two compounds were previously reported to inhibit the oxidative burst in human neutrophils and to scavenge several other ROS (HOCI, HO$^\cdot$, ROO$^\cdot$) and RNS (•NO and ONOO$^-$), which corroborates the thesis that their antioxidant activity plays a crucial role in the respective anti-inflammatory activity. Dipyrone is itself a pro-drug that undergoes non-enzymatic hydrolysis in the stomach to form aminopyrine, which is rapidly and almost completely absorbed, meaning that only the antioxidant potency of the latter will be mandatory for the systemic therapeutic activity. The other tested pyrazole derivatives, antipyrine and propyphenazone, were devoid of any activity up to 5 mM concentration, which is also in accordance to previous results for other ROS and RNS. Accordingly, it may be considered that $1^\text{O}_2$ reacts with the secondary amine, present in the chemical structure of dipyrone and aminopyrine but absent in antipyrine and propyphenazone, in a similar way to what was already demonstrated for HOCl. If true, the presumed $1^\text{O}_2$-induced transformation of aminopyrine into the characteristic blue cation radical (APS$^+$), deserves further attention, since prolonged exposure to APS$^+$ may lead to agranulocytosis. Considering the other studied NSAIDs, there was an observed lack of activity for most of the arylpropionic acid derivatives, with naproxen and indoprofen only displaying residual activities, as happened for the oxazole derivative oxaprozin. On the other hand, the pyrrole derivatives tolmetin and ketorolac, and the indolacetic acid derivatives indomethacin and etodolac, displayed scavenging activity in the high micromolar range.

![Figure 6](image-url)
lack of $^{1}\text{O}_2$ scavenging activity of acemetacin compared to indomethacin is somewhat striking. Nevertheless, in vivo, acemetacin is rapidly converted into indomethacin,\textsuperscript{31} minimizing this way the differences between these two compounds. Similarly, sulindac is converted, in vivo, to the metabolites sulindac sulfide and sulindac sulfone.\textsuperscript{32,33} Sulindac sulfide, but not sulindac sulfone, blocks prostaglandin synthesis by non-selective inhibition of cyclooxygenase-1 and cyclooxygenase-2.\textsuperscript{34} Sulindac sulfide is the most active $^{1}\text{O}_2$ scavenger among these three compounds, which gives credibility to a possible contribution of $^{1}\text{O}_2$ scavenging activity for the final therapeutic activity of sulindac. It may be argued that the effects described for these NSAIDs occur at relatively high concentrations compared to those in plasma and synovial fluid of healthy humans.\textsuperscript{35,36} However, the lower part of the corresponding activity curves still lies within therapeutic concentrations. Considering that these NSAIDs are also scavengers of other ROS and RNS, as well as inhibitors of neutrophil oxidative burst under these concentrations,\textsuperscript{5–12} the sum of these activities will certainly have an impact on their final antioxidant effect. In addition, in inflamed tissues some NSAIDs were reported to accumulate leading, for example, to concentrations in the synovial fluid about 3–8 times higher than those in control joints.\textsuperscript{37} Furthermore, the scavenging effect of antioxidants is highly dependent on the amount of reactive species being produced, giving rise to completely different IC$_{50}$ values.\textsuperscript{38} Thus, it is possible that much lower concentrations of NSAIDs are active in vivo. Indeed, it has already been shown that indomethacin is a strong antioxidant in vivo,\textsuperscript{39} despite the high concentrations required in vitro for this NSAID in this and previous assays for scavenging ROS and RNS.

Conclusions

The results obtained in the present study showed that the pyrazole derivatives dipyrone and aminopyrine are potent scavengers of $^{1}\text{O}_2$ within therapeutic concentrations. Some of the other studied NSAIDs also display $^{1}\text{O}_2$ scavenging activity, though at much higher concentrations. Thus, the scavenging effect observed for pyrazole derivatives will almost certainly contribute to their healing effect in the treatment of prolonged or chronic inflammation, while that of the other studied NSAIDs may have a more limited contribution, though these assumptions still require further in vivo validation.

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