INTRODUCTION

Dipyrone (metamizol), aminopyrine (4-dimethylaminoantipyrine), isopropylantipyrine and antipyrine (Fig. 1) are non-steroidal anti-inflammatory drugs (NSAIDs) pertaining to the first group of compounds used as analgesic, antipyretic and anti-inflammatory therapeutic drugs,1–3 with antipyrine being first prepared in 1883.1 Chemically, these compounds are pyrazolone derivatives. Isopropylantipyrine was obtained from antipyrine by introducing an isopropyl group on C-4 in order to increase its potency. This modification improved the antipyretic and analgesic properties while maintaining the anti-inflammatory activity.1 The introduction of a dimethylamino group on C-4 of the antipyrine molecule resulted in aminopyrine (4-dimethylaminoantipyrine).1 A disadvantage of aminopyrine is its relative insolubility in water. The search for more soluble compounds led to the production of the sodium salt of antipyrinyl methylaminomethanesulphonic acid (dipyrone).1 The pharmacological activities of pyrazolones are not fully understood but it is known that they involve the inhibition of cyclooxygenase isoenzymes, platelet thromboxane synthesis and prostanoid synthesis.4–6 Notably, other putative therapeutic effects have been found for this family of

Dipyrone and aminopyrine are effective scavengers of reactive nitrogen species

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Reactive nitrogen species (RNS), namely nitric oxide (NO•) and peroxynitrite (ONOO−) are produced in the inflammatory sites and may contribute to the deleterious effects of inflammation. The aim of the present study was to evaluate the putative scavenging effect of a particular group of non-steroidal anti-inflammatory drugs (NSAIDs), the pyrazolone derivatives dipyrone, aminopyrine, isopropylantipyrine, and antipyrine against RNS, using in vitro non-cellular screening systems. The results obtained showed that dipyrone and aminopyrine were highly potent scavengers of NO• and ONOO− while antipyrine exerted little effect and isopropylantipyrine no effect whatsoever against these two RNS and that, in the presence of bicarbonate, the scavenging potencies of both dipyrone and aminopyrine were slightly decreased. It could thus be inferred that the observed scavenging effects may be of therapeutic benefit for patients under anti-inflammatory treatment with dipyrone and aminopyrine in the case of overproduction of RNS. On the other hand, the possible depletion of physiological NO• concentrations, namely at the gastrointestinal tract as well as the formation of reactive derivatives of aminopyrine and/or dipyrone, resulting from their reaction with RNS, may otherwise be harmful for these patients.

Keywords: Dipyrone, aminopyrine, isopropylantipyrine, antipyrine, reactive nitrogen species, nitric oxide, peroxynitrite

Received 17 January 2006
Revised 27 May 2006
Accepted 28 May 2006
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Redox Report, Vol. 11, No. 3, 2006
DOI 10.1179/13510006X116637 © W. S. Maney & Son Ltd
NSAIDs, related to the prevention of deleterious effects mediated by reactive oxygen species (ROS). ROS are generated during inflammation, following the activation of mast cells, macrophages, eosinophils and neutrophils and may subsequently provoke or exacerbate damage at the inflammatory site. It was demonstrated that aminopyrine and dipyrone prevent phorbol myristate acetate (PMA)-induced neutrophil burst with a high degree of efficiency, while isopropylantipyrine exerted little effect and antipyrine no effect whatsoever. It was also observed that dipyrone and aminopyrine are highly potent scavengers of the hydroxyl radical (HO•) and hypochlorous acid (HOCl) while, in accordance with the neutrophil burst results, isopropylantipyrine showed little effect and antipyrine no effect whatsoever against these two ROS. None of the studied pyrazolones was capable of scavenging the superoxide radical (O2•-) or hydrogen peroxide (H2O2) and dipyrone was shown to be the most reactive against the peroxyl radical (ROO•). Significantly, aminopyrine is a strong myeloperoxidase inhibitor whereas antipyrine showed no effect against this enzyme. Thus, the inhibition of the neutrophil oxidative burst by pyrazolone derivatives together with the reported ROS scavenging effects may contribute to their therapeutic efficacy.

However, the picture remained incomplete since reactive nitrogen species (RNS), namely nitric oxide (NO•) and peroxynitrite (ONOO•) are also produced in the inflammatory sites and may contribute to the deleterious effects of inflammation. Indeed, NO• is produced by a family of isoenzymes, termed nitric oxide synthases (NOSs) which are typically induced during inflammation. Possible pro-inflammatory effects of NO• include augmentation of vascular permeability of inflamed tissues, the generation of other destructive free radicals (namely ONOO•) by reaction with O2•-, the induction of cyclooxygenase as well as angiogenic and inflammatory cytokines, activation of matrix metalloprotease and induction of chondrocyte apoptosis. ONOO• itself is a relatively long-lived cytotoxicant with strong oxidizing properties towards various cellular constituents, including sulphhydrils, lipids, amino acids and nucleotides.

Therefore, considering the possible importance of RNS in the pathophysiology of inflammation, indicative of beneficial effects by RNS scavengers, the aim of the present study was to evaluate the putative inhibitory effect of the pyrazolones dipyrone, aminopyrine, isopropylantipyrine and antipyrine against RNS, using in vitro non-cellular screening systems.

**MATERIALS AND METHODS**

**Materials**

All chemicals and reagents were of analytical grade. Dipyrone, aminopyrine, antipyrine, dihydrorhodamine 123 (DHR 123), diethylenetriaminepentaacetic acid (DTPA), 4,5-diaminofluorescein (DAF-2), sodium nitroprusside dihydrate, ebselen, carboxy-PTIO and manganese dioxide, were obtained from Sigma Chemical Co. (St Louis, MO, USA). Potassium chloride was obtained from Fluka Chemie GmbH (Steinheim, Germany). Hydrogen peroxide (30% solution), sodium bicarbonate, sodium nitrite, sodium hydroxide, hydrochloric acid and sodium chloride were obtained from Merck (Darmstadt, Germany). Isopropylantipyrine was kindly supplied by Novartis International Pharmaceutical Ltd, Ireland and ONOO• was obtained by synthesis (see below).

**Synthesis of ONOO•**

Synthesis of ONOO• was essentially performed as described before. Briefly, an acidic solution (0.7 M HCl) of 0.6 M H2O2 was mixed with 0.66 M NaNO2 on ice for 1 s and the reaction quenched with ice-cold 3 M NaOH. Residual H2O2 was removed by mixing with granular MnO2 pre-washed with 3 M NaOH. The stock ONOO• solution was filtered, then frozen (~20°C) and the top layer of the solution collected for the experiment. ONOO• concentration was determined by measuring absorbance at 302 nm (ε = 1670 M−1 cm−1). The typical yield of freshly prepared ONOO• ranged from 60–80 mM. Higher concentrations (> 200 mM) of ONOO• can be obtained by freeze fractionation. However, in the present study, only freshly prepared ONOO• solution was used in an effort to minimise nitrite ion contamination. Prior to each study, the concentration of the ONOO• stock was determined spectrophotometrically in 0.1 M NaOH.
ONOO\textsuperscript{−} scavenging activity was measured by fluorimetry, through monitoring the oxidation of non-fluorescent dihydorhodamine 123 (DHR 123) to the fluorescent rhodamine 123 by ONOO\textsuperscript{−} according to a described procedure\textsuperscript{15} with modifications. A stock solution of 2.89 mM DHR 123 in dimethylformamide was purged with nitrogen and stored at −20°C. Working solutions of DHR 123 diluted from the stock solution were placed on ice under darkness immediately before the determinations. Buffer (90 mM NaCl, 50 mM Na\textsubscript{2}PO\textsubscript{4}, and 5 mM KCl, pH 7.4) was purged with nitrogen and placed on ice before use. At the outset of the experiments, 100 μM DTPA was added to the buffer. Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 300 μl): DHR 123 (5 μM), tested compounds dissolved in DMSO, at various concentrations (0–10 μM for dipyrone and aminopyrine and 0–5 mM for antipyrine and isopropylantipyrine) and ONOO\textsuperscript{−} (600 nM). The mixtures were incubated in a microplate reader (Synergy HT, BIO-TEK) for 5 min at 37°C. The fluorescence signal induced by DHR 123 reacting with ONOO\textsuperscript{−} was measured using the microplate reader with excitation and emission wavelengths of 485 ± 20 nm and 528 ± 20 nm, respectively. Ebselen was used as a positive control. Effects are expressed as the percentage inhibition of the ONOO\textsuperscript{−}-induced DHR 123 oxidation. In a parallel set of experiments, the assays were performed in the presence of 25 mM NaHCO\textsubscript{3} in order to simulate the physiological conditions with high CO\textsubscript{2} concentrations \textit{in vivo}. This evaluation is important because, under physiological conditions, the reaction of ONOO\textsuperscript{−} with CO\textsubscript{2} predominates, with the rate constant of reaction of CO\textsubscript{2} with ONOO\textsuperscript{−} being very rapid (k\textsubscript{2} = 3–5.8 × 10\textsuperscript{4} M\textsuperscript{−1} s\textsuperscript{−1}).\textsuperscript{17} Thus, the reactivity of the putative scavengers for ONOO\textsuperscript{−} should be able to match or exceed that of bicarbonate. Each study corresponds to four experiments, performed in triplicate. The following formula was used for calculating ONOO\textsuperscript{−} scavenging activity:

\[
\text{ONOO}^\text{−} \text{scavenging activity (\%) = } \frac{F_{\text{control}} - F_{\text{blank}}}{F_{\text{sample}} - F_{\text{blank}}} \times 100\% \quad \text{Eq. 1}
\]

where F is fluorescence intensity in arbitrary units.

\textbf{Results}

\textit{NO}^\text{•} scavenging activity

Figure 2 shows the results obtained from the NO\textsuperscript{•} scavenging assay for dipyrone and aminopyrine. These compounds were strong inhibitors of the NO\textsuperscript{•}-elicited oxidation of DAF-2 to triazolofluorescein in a concentration-dependent manner with dipyrone demonstrating itself to be the most active, while antipyrine and iso-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Compound} & \textbf{IC}_{50} (μM) & \textbf{IC}_{50} (μM) & \textbf{IC}_{50} (μM) \\
\hline
Dipyrone & 0.54 ± 0.04 & 1.8 ± 0.2 & 4.8 ± 0.7 \\
Aminopyrine & 4.3 ± 0.8 & 1.5 ± 0.1 & 3.5 ± 0.6 \\
Isopropylantipyrine & > 5000 & NA & NA \\
Antipyrine & > 5000 & > 5000 & > 5000 \\
Carboxy-PTIO & 1.8 ± 0.4 & – & – \\
Ebselen & – & 2.5 ± 0.1 & 16 ± 1 \\
\hline
\end{tabular}
\caption{Scavenging activities (IC\textsubscript{50} mean ± SEM) of dipyrone, aminopyrine, isopropylantipyrine, antipyrine, carboxy-PTIO, and ebselen against NO\textsuperscript{•} and ONOO\textsuperscript{−} (in the absence or presence of NaHCO\textsubscript{3}).}
\end{table}
propylantipyrine were only shown to exert weak activity against this RNS (data not shown). The resulting IC_{50} values were 0.54 ± 0.04, 4.3 ± 0.8, > 5000, > 5000 µM (mean ± SEM) for dipyrone, aminopyrine, isopropylantipyrine and antipyrine, respectively (Table 1). The positive carboxy-PTIO control gave rise to an IC_{50} of 1.8 ± 0.4 µM (mean ± SEM; Table 1).

**ONO'O' scavenging activity**

Figure 3 shows the results obtained from the ONOO' scavenging assay for dipyrone and aminopyrine in both the absence and presence of 25 mM NaHCO₃. In the absence of NaHCO₃, these compounds were shown to be strong inhibitors of the ONOO' elicited oxidation of DHR 123 in a concentration-dependent manner and at a rather similar potency. Isopropylantipyrine was devoid of any activity, while antipyrine was shown to be only a weak inhibitor (data not shown). In the presence of NaHCO₃, the inhibitor activities of both dipyrone and aminopyrine were slightly decreased. In the absence of NaHCO₃, the resulting IC_{50} values were 1.8 ± 0.2, 1.5 ± 0.1 and > 5000 µM (mean ± SEM) for dipyrone, aminopyrine and antipyrine, respectively (Table 1). In the presence of NaHCO₃, the resulting IC_{50} values were 4.8 ± 0.7, 3.5 ± 0.6 and > 5000 µM (mean ± SEM) for dipyrone, aminopyrine and antipyrine, respectively (Table 1). The positive control ebselen gave rise to an IC_{50} of 2.5 ± 0.1 and 16 ± 1 µM (mean ± SEM) in both the absence and presence of 25 mM NaHCO₃, respectively (Table 1).

**DISCUSSION**

The results obtained from the present study showed that dipyrone and aminopyrine were highly potent scavengers of NO' and ONOO', while antipyrine had little effect and isopropylantipyrine no effect whatsoever against these two RNS. In the presence of NaHCO₃, the inhibitor activities of both dipyrone and aminopyrine were slightly decreased. It has been reported that physiological
concentrations of CO\textsubscript{2} can modulate ONOO\textsuperscript{-} reactivity due to the rapid nature of the reaction between these two compounds.\textsuperscript{15} In fact, CO\textsubscript{2}, by competing with ONOO\textsuperscript{-}, significantly weakens the ability of several phenolic compounds such as caffeic acid, o- and p-coumaric acid, gallic acid and ferulic acid\textsuperscript{21} as well as Trolox, glutathione and uric acid.\textsuperscript{17} The effects observed in the present study are in line with those findings.

It is interesting to note that the secondary amine present in the chemical structure of aminopyrine (but absent from the chemical structure of isopropylantipyrine and antipyrine; Fig. 1) has previously been reported to be highly reactive with pro-oxidant compounds, namely ROS.\textsuperscript{9,22,23} Notably, the IC\textsubscript{50} values observed for the studied RNS were similar to those obtained before for HO\textsuperscript{•} and HOCl.\textsuperscript{9} Therefore, it is very much likely that its reactivity with RNS is similar to that of ROS. As noted by Halliwell and Gutteridge,\textsuperscript{24} an antioxidant is ‘any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents the oxidation of that substrate’. This implies that the scavenging activities for RNS and ROS for any antioxidant should be tested \textit{in vivo}, in the presence of endogenous antioxidants. Although this point still requires experimental clarification, under sustained overproduction of ROS and RNS, endogenous antioxidants may decrease substantially or become depleted, thereby increasing the importance of exogenous antioxidants. Significantly, aminopyrine was also previously shown to exhibit a strong inhibitor effect against myeloperoxidase within similar concentrations (IC\textsubscript{50} of 0.72 ± 0.09 \textmu M)\textsuperscript{10} which re-inforces the antioxidant profile of this drug. Dipyrone reactivity may also be explained by the presence of this secondary amine in its structure. However, when administered orally, dipyrone may itself be considered a pro-drug since it undergoes non-enzymatic hydrolysis in the stomach to form 4-methylaminoantipyrine, which is rapidly and almost completely absorbed.\textsuperscript{25} The maximum therapeutic concentrations of 4-methylaminoantipyrine reached after administration of a single oral dose of dipyrone is up to 36 \textmu M while that of aminopyrine is up to 160 \textmu M.\textsuperscript{24} Since the RNS scavenging effects observed \textit{in vitro} for dipyrone and aminopyrine were well within these therapeutic concentrations, it is highly likely that during therapy with these NSAIDs, NO\textsuperscript{•} and ONOO\textsuperscript{-} may be effectively scavenged \textit{in vivo}.

As explained in the introduction, these RNS may contribute to the pathophysiology of inflammation and, as such, it could be inferred that the scavenging effects observed for dipyrone and aminopyrine in the present study are of enormous benefit for the patient under treatment. However, these data should be interpreted with a degree of caution since the possible depletion of physiological NO\textsuperscript{•} concentrations may also be harmful to the patient. At physiological levels, NO\textsuperscript{•} is mainly involved in homeostatic biochemical and physiological processes such as signal transduction, neurotransmission, smooth muscle relaxation, peristalsis, gastroprotective effects, inhibition of platelet aggregation, blood pressure modulation, immune system control and learning and memory.\textsuperscript{26–28} Therefore, a possible variation of NO\textsuperscript{•} physiological levels by dipyrone and aminopyrine could potentially affect these physiological processes. The contribution of NO\textsuperscript{•} to maintaining blood pressure is paradigmatic. However, from looking at the literature it can be assumed that these NSAIDs do not affect or even lead to a small decrease in blood pressure.\textsuperscript{29,30} This apparent contradiction may be due to other dipyrone- and aminopyrine-related effects such as the scavenging effect of these compounds against ROS, ONOO\textsuperscript{-} or their neutrophil burst inhibitory activities.\textsuperscript{9} ROS are generated in the vasculature mainly by NAD(P)H oxidase in a mechanism that is angiotensin II-dependent. Activation of this enzyme leads to the production of O2\textsuperscript{•} and a decreased availability of NO\textsuperscript{•} while increasing the levels of tissue-damaging ONOO\textsuperscript{-} by virtue of the reaction between NO\textsuperscript{•} and O2\textsuperscript{•}\textsuperscript{-}.\textsuperscript{31} NAD(P)H-dependent ROS formation, could also contribute to vascular injury by sustaining NAD(P)H oxidase activation, promoting inflammatory gene expression, extracellular matrix reorganization and growth (hypertrophy/hyperplasia) of vascular smooth muscle cells.\textsuperscript{31} It seems, therefore, from the literature-derived data, that the ONOO\textsuperscript{-} and ROS scavenging effects of dipyrone and aminopyrine probably prevail over NO\textsuperscript{•} depleting effects.

In the gastrointestinal (GI) tract, NO\textsuperscript{•} participates in the modulation of the smooth musculature tone, such as the regulation of intestinal peristalsis, gastric emptying and antral motor activity. It also regulates acid and gastric mucus secretion, alkali production and is involved in the maintenance of mucosal blood flow. Under physiological conditions, NO\textsuperscript{•} acts as an endogenous mediator, modulating both the repair and integrity of the tissues and exhibits gastroprotective properties against various types of aggressive agents. However, high NO\textsuperscript{•} concentrations are related to numerous pathological GI tract processes including peptic ulcer, chronic gastritis, gastrointestinal cancer, bacterial gastroenteritis, celiac or chronic inflammatory bowel diseases (for a review, see Martin \textit{et al.}\textsuperscript{26}). Thus, NSAIDs with NO\textsuperscript{•}-depleting activities may yet again have ambivalent damaging/protecting effects in the GI tract. Of note is that long-term NSAIDs use commonly associated with gastric erosions as well as a 2–5-fold increase in relative risk and 30% attributable risk of ulcer perforation, upper gastrointestinal bleeding and death.\textsuperscript{32} NSAIDs may also damage the small and large bowel. In regular users of NSAIDs, an 8–10% prevalence of small intestinal ulceration has been reported as well as an increased frequency of small and large bowel perforation.\textsuperscript{32}
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Significantly, apart from NO•, prostaglandins synthesised by cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) contribute strongly to the gastric defence system.33 Both COX-1 and COX-2 contribute to gastric mucosal defence. In normal gastric mucosa, the combined inhibition of both COX-1 and COX-2 is necessary to induce lesion formation while in the face of pending injury, such as the presence of exogenous acid in the gastric lumen, isolated inhibition of COX-1 is sufficient to damage the mucosa.32 Specific inhibition of COX-2 does not induce gastric injury either in normal mucosa or in the presence of exogenous intraluminal acid. However, severe injury develops during suppression of COX-2 when the function of one of the other factors involved in the complex system of mucosal defence such as NO• or afferent neurons is impaired,33 which may be the case during inflammation therapy with dipyrone and aminopyrine. In fact, dipyrone has been reported to induce gastric damage both in laboratory animals34 and humans.35 Worth mentioning from the Sanchez et al.34 study is that, in addition to inhibiting prostaglandin synthesis, gastric damage induced by the administration of dipyrone to rats was associated with an inhibition of the NO•cGMP pathway and eNOS activity, which is in agreement with the expected consequences, considering the NO• scavenging effects observed in the present study.

The use of pyrazolone NSAIDs, namely dipyrone and aminopyrine, has long been associated with a high incidence of agranulocytosis. Several studies found strong evidence indicating that the mechanism of aminopyrine-induced agranulocytosis involves a drug-dependent anti-neutrophil antibody that requires covalent binding of the drug or, most probably, a reactive derivative of the drug, to neutrophils.23 The generation of an aminopyrine reactive derivative can be accomplished through its oxidation. It was previously demonstrated that dipyrone and aminopyrine are highly reactive with HO•, ROO• and HOC1.9 Thus, it is probable that the reactivity of aminopyrine against NO• and ONOO– also leads to the formation of an aminopyrine reactive derivative capable of binding to neutrophils, although this has yet to be demonstrated.

CONCLUSIONS

These results have shown that dipyrone and aminopyrine were highly potent scavengers of NO• and ONOO–, while antipyrine had little effect and isopropylantipyrine no effect whatsoever against these two RNS and that in the presence of NaHCO3, the inhibitor activities of both dipyrone and aminopyrine were slightly decreased. These RNS may contribute to the pathophysiology of inflammation and, as such, it could be inferred that the observed scavenging effects are of therapeutic benefit for the patient under treatment. However, the possible depletion of physiological NO• concentrations, namely at the GI tract together with the formation of reactive derivatives of aminopyrine and/or dipyrone may also be harmful for the patient. Thus, more experimental data are required to evaluate the possible risk/benefit obtained by the RNS scavenging effects of dipyrone and aminopyrine.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support given by REQUIMTE (project REQEVA). David Costa thanks FCT and FSE for a PhD grant (SFRH/BD/10483/2002).

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