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The ketolide antimicrobial agent HMR-3004 inhibits neutrophil superoxide production by a membrane-stabilizing mechanism

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Abstract

We have investigated the membrane-stabilizing potential of the prototype ketolide antimicrobial agent, HMR-3004 (3.75–125 μM), as well as the effects of this agent on the production of superoxide by human neutrophils activated with FMLP, the calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA) or opsonized zymosan (OZ), each of which uses different transductional mechanisms to activate NADPH-oxidase. Membrane-stabilizing activity was investigated using a hemolytic procedure, while superoxide production was assayed by lucigenin-enhanced chemiluminescence. At concentrations of 3.75 μM and greater, HMR-3004 caused dose-related inhibition of superoxide production by neutrophils activated by all four stimuli of membrane-associated oxidative metabolism, which was not associated with cytotoxicity or superoxide-scavenging activity. At the same concentrations, HMR-3004 antagonized the hemolytic actions of the membrane-disruptive bioactive phospholipids, lysophosphatidylcholine (LPC), platelet-activating factor (PAF) and lyso-PAF. A mechanistic relationship between the membrane-stabilizing and the anti-oxidative properties of HMR-3004 was suggested by the observation that treatment of neutrophils with non-cytolytic concentrations of LPC antagonized the inhibitory effects of the ketolide on superoxide production by these cells. These membrane-stabilizing, anti-oxidative activities of HMR-3004 suggest that in addition to its antimicrobial properties, this agent possesses anti-inflammatory properties which are superior to those of the presently available macrolide and azalide agents. © 1999 International Society for Immunopharmacology. Published by Elsevier Science Ltd. All rights reserved.

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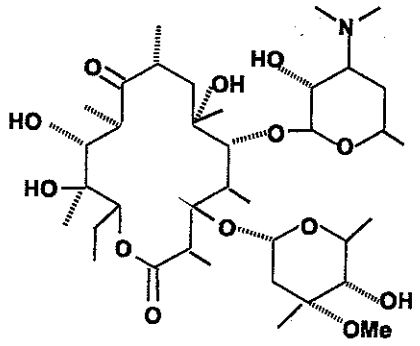
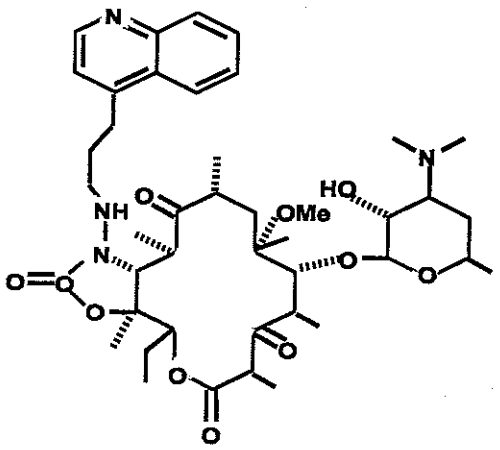
1. Introduction

Although they are primarily antimicrobial, macrolide antibiotics have been reported to possess anti-inflammatory properties [9], which may explain their usefulness in the treatment of a variety of acute [26] and chronic inflammatory disorders, including bronchial asthma [24,28], sub-acute sclerosing panbronchiolitis [20] and ischemic heart disease, including unstable angina and acute myocardial infarction [14].

Interference with the production of pro-inflammatory, tissue-damaging reactive oxidants appears to be the primary mechanism of macrolide-mediated anti-inflammatory activity [4,15,22]. These agents do not possess superoxide-scavenging properties and apparently inhibit the assembly and/or activity of the superoxide-generating NADPH-oxidase complex in the cell membrane of activated phagocytes [4,33]. Macrolide-mediated inhibition of NADPH-oxidase



Erythromycin A



HMR- 3004

Fig. 1. Molecular structures of HMR-3004 and erythromycin A.

activity has been attributed to two different, but possibly related mechanisms. Firstly, these agents have been reported to inhibit the activity of the phospholipase D-phosphatidic acid phosphohydrolase transduction pathway in activated neutrophils [2,25], and secondly, macrolides have been reported to possess membrane-stabilizing properties, which may affect the protein motions, both lateral and rotational, that must take place in the assembly and functioning of the multicomponent NADPH-oxidase [5].

Ketolides are a new class of semi-synthetic 14-membered ring macrolides, which differ structurally from erythromycin A by having a 3-keto group instead of an L-cladinose at position three on the erythronolide A ring [3]. Relative to conventional macrolides, the principle advantages of this novel group of antimicrobial agents are their increased potency and broadened spectrum, including activity against erythromycin A-resistant Gram-positive bacteria [27], as well as considerably improved accumulation in tissues and phagocytes [22]. However, relatively little is known about the anti-oxidative, anti-inflammatory interactions of ketolides with human neutrophils.

In the current study we have investigated the effects of the prototype ketolide antimicrobial agent, HMR-3004 (formerly known as RU-64004), on reactive oxidant production by stimulated human neutrophils in vitro, as well as the membrane-stabilizing potential of this agent and its relationship to anti-inflammatory activity.

2. Experimental procedures

2.1. HMR-3004

The structure of this ketolide antimicrobial agent relative to that of erythromycin is shown in Fig. 1. The most notable differences are the 3-keto group and the L-cladinose at position three on the erythronolide A ring of HMR-3004 and erythromycin respectively, as well as the quinoline group of HMR-3004, which is linked to the 11–12 position by a cyclic hydrazano-carbamate function. The ketolide antimicrobial agent, as well as roxithromycin, a semi-synthetic macrolide which was used in a limited series of experiments, were provided by Dr A Bryskier, Hoechst Marion Roussel, Paris, France and dissolved in 0.025 N HCl/0.15 M phosphate-buffered saline (PBS) to give a stock solution of 10 mM. Subsequent dilutions were made in Hanks' balanced salt solution (HBSS, pH 7.4) and the antimicrobial agent used at a final concentration of 3.75–125 μM for studies of neutrophil superoxide production and for membrane-stabilizing experiments.

2.2. Other chemicals and reagents

Unless stated these were obtained from the Sigma Chemical Co (St Louis, MO, USA).

2.3. Neutrophils

Human neutrophils were obtained from heparinized (5 units of preservative-free heparin/ml) venous blood of healthy adult volunteers and separated from mononuclear leukocytes by

centrifugation on Ficoll–Paque (Pharmacia Fine Chemicals, Uppsala, Sweden)-metrizoate (Nyegaard, Oslo, Norway) cushions at 400 *g* for 25 min at room temperature. The resultant pellet was suspended in PBS (0.15 M, pH 7.4) and sedimented with 3% gelatin for 15 min at 37°C to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity and viability were resuspended to 1×10^7 ml in PBS and held on ice until used.

2.4. Superoxide generation

This was measured using a lucigenin (bis-*N*-methyl-acridinium nitrate)-enhanced chemiluminescence (LECL) method [23]. Neutrophils were pre-incubated for 15 min at room temperature in 900 μl HBSS containing 0.2 mM lucigenin in the presence and absence of HMR-3004 (3.75–125 μM) followed by 15 min at 37°C. Spontaneous and stimulus-activated LECL responses were then recorded in an LKB Wallac 1251 chemiluminometer (Turku, Finland) after the addition of 100 μl of the following stimuli of neutrophil membrane-associated oxidative metabolism: the synthetic chemotactic tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP; 1 μM), the calcium ionophore A23187 (1 μM), phorbol 12-myristate 13-acetate (PMA; 25 ng/ml) and opsonized zymosan (0.5 mg/ml). LECL readings were integrated for 5 s intervals and recorded as millivolts × seconds$^{\times 1}$ (mV s^{-1}). Additional experiments were performed to investigate the effects of low, non-cytotoxic concentrations of the membrane-destabilizing phospholipid, lysophosphatidylcholine (LPC), added 11 min (2 μM) and 6 min (2 μM) before HMR-3004 (15.5 μM) on the anti-oxidative interactions of HMR-3004 with PMA-activated neutrophils.

Superoxide-scavenging activity of HMR-3004 was investigated by LECL using a cell-free xanthine (1 mM)-xanthine oxidase (70 milliunits/ml) superoxide-generating system.

2.5. Cellular ATP levels

After 30 min incubation with HMR-3004 (15.5–125 μM) at 37°C in HBSS, neutrophil ATP levels were measured in the lysates of control and drug-treated cells (10^6) using a luciferin/luciferase chemiluminescence method [16].

2.6. Membrane stabilization

The membrane-stabilizing potential of HMR-3004 was measured using a hemolytic assay. Sheep erythrocytes were washed three times and resuspended to 5% in HBSS. The erythrocytes (final concentration of 0.5%) were then coincubated with HMR-3004 (15.5–125 μM) for 30 min at 37°C followed by the addition of the membrane-destabilizing bioactive phospholipids, LPC, platelet-activating factor (PAF) and lyso-PAF (LPAF) at concentrations (5–8 μM) which caused partial hemolysis. After 5 min, intact erythrocytes were removed by centrifugation and the supernatants assayed spectrophotometrically at 405 nm for hemoglobin content.

In an additional series of experiments the membrane-stabilizing potential of HMR-3004 was compared with that of roxithromycin.

2.7. Interactions of HMR-3004 with LPC

The ultraviolet absorption spectra of mixtures of HMR-3004 (62.5 μM) and LPC (2 mM) relative to identical concentrations of the individual agents were measured using a Pye Unicam SP 1700 double-beam UV spectrophotometer.

2.8. Statistical analysis

The results of each series of experiments are expressed as the mean values ± the standard error of the mean (S.E.M.). Where appropriate, levels of statistical significance were calculated by Student's *t*-test.

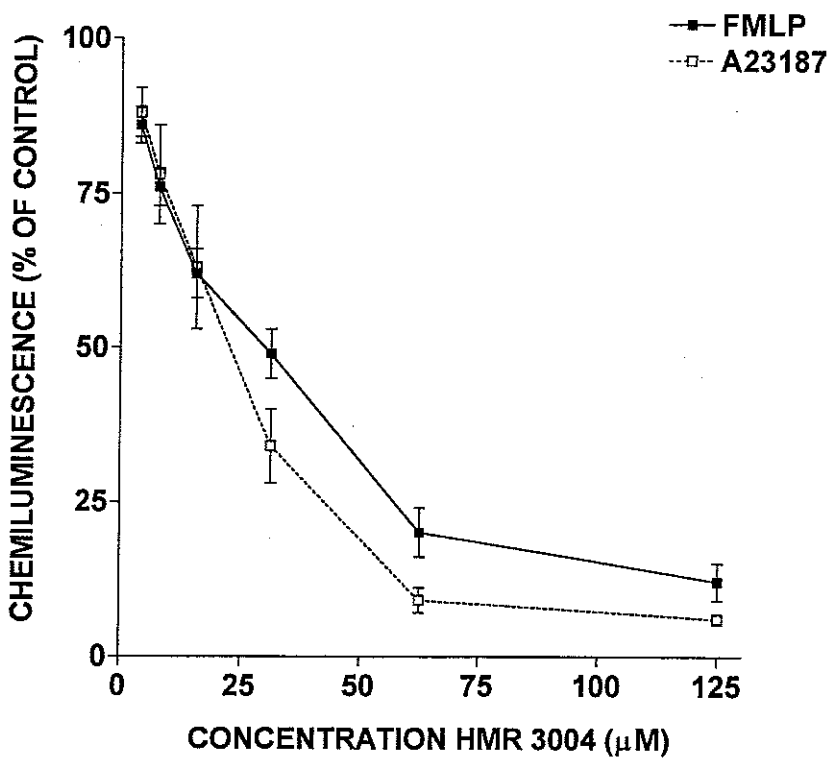


Fig. 2. The effects of HMR-3004 on superoxide production by neutrophils activated with FMLP (■——■) or the calcium ionophore, A23187 (□---□). The results of 16 experiments are presented as the mean peak lucigenin-enhanced chemiluminescence responses ±S.E.M.s. The absolute values for the drug-free, control systems stimulated with FMLP and A23187 were 1154 ± 107 and 912 ± 128 mV s^{-1} respectively. The corresponding value for unstimulated neutrophils was 228 ± 25 mV s^{-1}.

3. Results

3.1. Superoxide generation

The effects of HMR-3004 on the production of superoxide by neutrophils activated with FMLP, calcium ionophore, PMA and opsonized zymosan are shown in Figs. 2 and 3. At concentrations of 3.75 μM and upwards, HMR-3004 caused dose-related inhibition of the peak LECL responses of neutrophils activated with FMLP and calcium ionophore. These inhibitory effects of HMR-3004 achieved statistical significance at concentrations of 3.75 μM and upwards ($P = 0.0003$ and $P = 0.04$ for FMLP and calcium ionophore respectively). In the case of opsonized zymosan-activated neutrophils, inhibition of superoxide generation was observed at 8 μM HMR-3004 ($P = 0.0025$) and upwards and at 15.5 μM ($P < 0.0001$) with PMA-stimulated cells.

The effects of pre-treatment of neutrophils with 4 μM LPC, administered as 2 × 2 μM exposures 5 min apart, on HMR-3004 (15.5 μM)-mediated inhibition of superoxide production

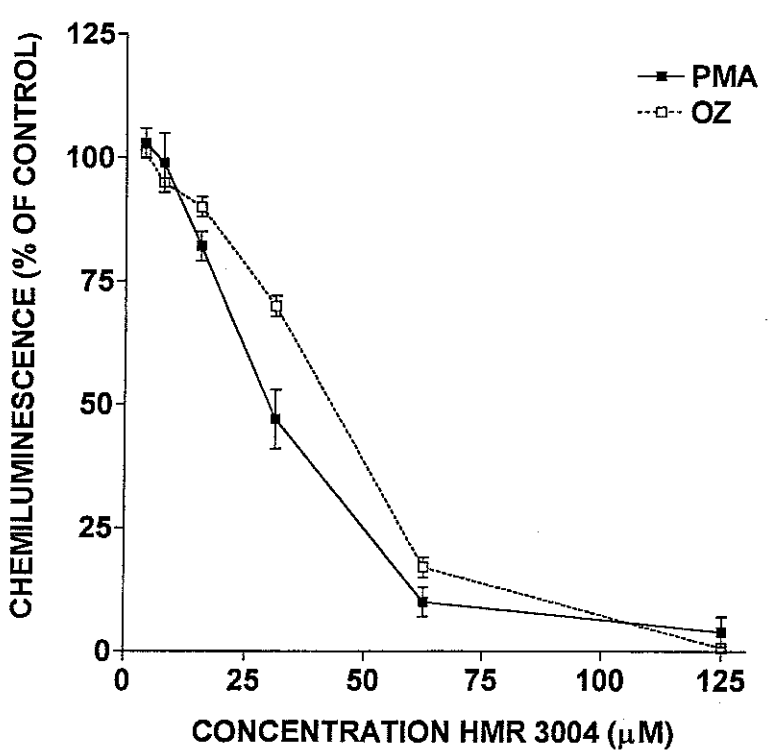


Fig. 3. The effects of HMR-3004 on superoxide production by neutrophils activated with opsonized zymosan (□——□) or phorbol myristate acetate (■——■). The results of 6–16 experiments are presented as the mean peak lucigenin-enhanced chemiluminescence responses ±S.E.M.s. The absolute values for the drug-free, control systems stimulated with opsonized zymosan and PMA were 3779 ± 384 and 3037 ± 471 mV s^{-1} respectively. The corresponding value for unstimulated neutrophils was 230±31 mV s^{-1}.

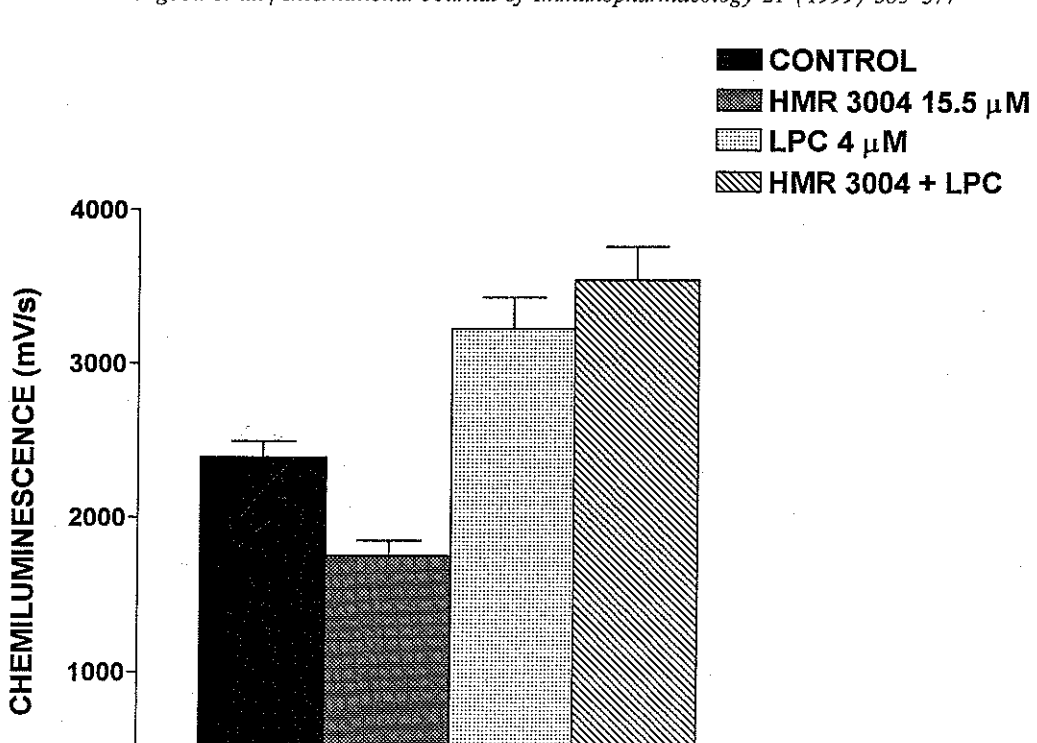


Fig. 4. Effects of pretreatment of neutrophils with lysophosphatidylcholine on the peak lucigenin-enhanced chemiluminescence responses of neutrophils activated with phorbol myristate acetate in the presence and absence of HMR-3004. The results are expressed as the mean values ± S.E.M.s.

by PMA-activated neutrophils are shown in Fig. 4. LPC completely neutralized the inhibitory effects of HMR-3004 ($P = 0.0001$) on neutrophil superoxide production.

The effects of HMR-3004 on the activity of the cell-free, xanthine-xanthine oxidase-superoxide-generating system are shown in Table 1. HMR-3004 did not possess detectable superoxide-scavenging activity at concentrations of up to 62.5 μM.

3.2. ATP levels

These results are shown in Table 2. Cellular ATP levels were unaffected by coincubation of neutrophils with HMR-3004 at concentrations of up to 125 μM.

3.3. Membrane-stabilization

The effects of HMR-3004 on the lysis of sheep erythrocytes mediated by the bioactive phospholipids LPC, PAF and LPAF are shown in Fig. 5. At the concentrations tested (15.5–

Table 1
Effects of HMR-3004 on superoxide production by the cell-free Xanthine–Xanthine oxidase system[a]

Concentration of HMR 3004	Peak LECL responses (mV s^{-1})	*P*-values
Control	1066±78	
15.5 μM	1108±86	$P=0.72$
31.25 μM	1023±86	$P=0.74$
62.5 μM	1014±68	$P=0.60$
125 μM	906±62	$P=0.06$

[a] Results of 14–23 experiments are presented as the mean values±S.E.M.s.

125 μM), HMR-3004 caused statistically significant ($P < 0.0006$–$P = 0.0001$) protection of the erythrocytes against hemolysis induced by all three membrane-destabilizing phospholipids. These results demonstrate that HMR-3004 is a potent membrane-stabilizing agent.

In a single experiment (two replicates in each system) designed to compare the relative efficiency of HMR-3004 and roxithromycin in protecting erythrocytes against LPC (6.5 μM)-induced hemolysis, the extent of hemolysis in the drug-free, LPC-treated control system was 46 and 24, 14, 10 and 13% in systems pre-treated with HMR-3004 at concentrations of 15.5, 31.25, 62.5 and 125 μM respectively. The corresponding values for systems pre-treated with roxithromycin prior to the addition of LPC were 44, 29, 25 and 23%. These results demonstrate that HMR-3004 is a more potent membrane-stabilizing agent than roxithromycin.

3.4. Interactions of HMR-3004 with LPC

Spectrophotometric analysis of mixtures of HMR-3004 with LPC failed to reveal any interactions between these agents, demonstrating that the observed membrane-stabilizing

Table 2
Effects of exposure of neutrophils to HMR-3004 on cellular ATP levels[a]

Concentration of HMR-3004	Cellular ATP (nmoles/10^6 cells)	*P*-values
Control	25.4±4	
15.5 μM	23.7±3	$P=0.28$
31.25 μM	24.5±3	$P=0.91$
62.5 μM	20.5±3	$P=0.47$
125 μM	20.4±3	$P=0.41$

[a] Results of 9 different experiments are presented as the mean values±S.E.M.s.

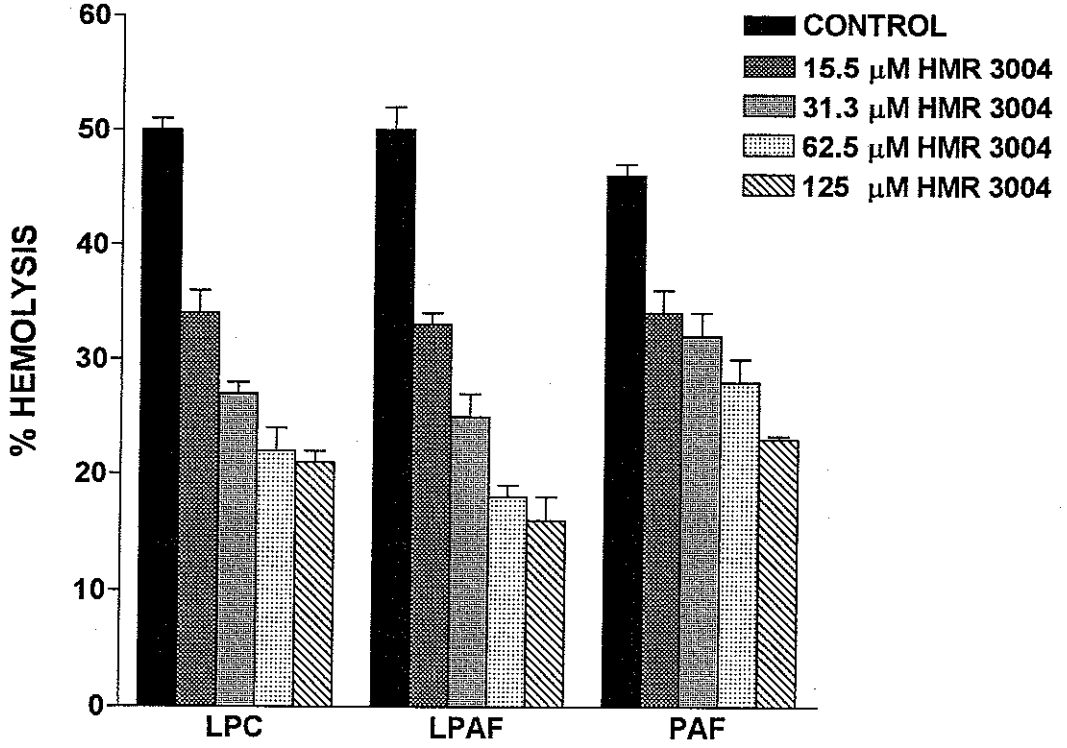


Fig. 5. The effects of HMR-3004 on the lysis of sheep erythrocytes mediated by lysophosphatidylcholine (LPC), platelet-activating factor (PAF) and LPAF. The results of 6–10 experiments are expressed as the mean percentages ± S.E.M.s of the corresponding LPC-, PAF- and LPAF-treated systems in the absence of HMR-3004. The respective absolute values for these systems were 50 ± 1%, 46 ± 1% and 50 ± 2% hemolysis respectively.

properties of HMR-3004 are not a secondary consequence of interactions of this agent with LPC.

4. Discussion

It is well-established that conventional macrolide antimicrobial agents such as erythromycin, clarithromycin and roxithromycin inhibit the superoxide-generating NADPH-oxidase complex of activated phagocytes [4,15,22], an activity which may explain the beneficial anti-inflammatory effects of these agents in acute and chronic inflammatory disorders [9,14,20,24,26,28]. This may not, however, be the only mechanism of macrolide-mediated anti-inflammatory activity, since these agents have been reported to inhibit the production of the pro-inflammatory cytokines interleukin-5 [18] and interleukin-8 [17], by activated Th2 lymphocytes and monocytes/macrophages respectively, and to accelerate apoptosis in neutrophils [7].

In the current study, we have investigated the effects of the novel, prototype ketolide, HMR-3004 on the production of superoxide by human neutrophils activated with FMLP, the calcium ionophore A23187, PMA and opsonized zymosan. At concentrations of 3.75 μM and upwards, HMR-3004 caused dose-related inhibition of the production of superoxide by neutrophils, which was stimulus non-specific, as previously reported in a preliminary study [1]. This is a noteworthy distinction between HMR-3004 and the conventional macrolides, the latter being less potent inhibitors of the pro-oxidative activities of stimulated neutrophils, especially with respect to superoxide production activated by PMA and opsonized zymosan [4–6]. This difference in the sensitivity of neutrophil superoxide production to the inhibitory effects of conventional macrolides and HMR-3004 may simply reflect the rate and extent of uptake of the ketolide by neutrophils relative to that of the macrolides. Uptake of HMR-3004 by neutrophils is rapid, being complete after only 5 min of exposure to the ketolide and reaching an average intracellular/ extracellular ratio of 461, which is considerably higher than that achieved by the conventional macrolides [34].

We have previously reported that macrolide antibiotics increase the resistance of sheep erythrocytes [5,6] and human ciliated respiratory epithelium [13] to the cytolytic effects of LPC, PAF and LPAF, indicating that these antimicrobial agents possess membrane-stabilizing properties. We proposed that membrane-stabilization is a possible mechanism of macrolide-mediated inhibition of superoxide production by activated neutrophils [5,6]. In the present study, we observed that HMR-3004, at essentially the same concentrations that inhibit superoxide production by activated neutrophils, protects sheep erythrocytes against the lytic effects of all three bioactive phospholipids, being considerably more potent in this respect than roxithromycin, which was previously found to be the top performing, membrane-stabilizing, anti-oxidative macrolide [5,6]. A mechanistic relationship between membrane-stabilization and inhibition of superoxide production by activated neutrophils is well-recognized [5,6,29], probably because increased membrane fluidity promotes superoxide production by facilitating the protein motions, both rotational and lateral, that are necessary for the assembly and function of the multicomponent NADPH-oxidase [21]. The proposed mechanistic relationship between the membrane-stabilizing and anti-oxidative properties of HMR-3004 is strengthened by the observation that pre-treatment of neutrophils with non-cytolytic concentrations of the membrane-disruptive agent, LPC, antagonized the inhibitory effects of the ketolide on superoxide production. A membrane-stabilizing mechanism would also explain the non-specific inhibitory effects of the ketolide on membrane-associated oxidative metabolism in neutrophils exposed to the four different stimuli used in the current study, all of which utilize different transductional mechanisms to activate NADPH-oxidase.

Although other investigators have proposed that macrolide-mediated inhibition of neutrophil superoxide production may be achieved by interference with phosphatidate phosphohydrolase [2,25], this mechanism and the membrane-stabilizing mechanism described by us are not mutually exclusive. This contention is based on two lines of evidence. Firstly, propranolol, an inhibitor of phosphatidate phosphohydrolase [30], like the macrolides, also possesses membrane-stabilizing properties [10] which are related to its inhibitory effects on superoxide production by activated neutrophils [5,6]. Secondly, diacylglycerol, the primary product of phosphatidate phosphohydrolase activity potentiates NADPH-oxidase activity [32]. This suggests that membrane-stabilizing agents such as macrolides and HMR-3004 not only affect