Clindamycin, Erythromycin, and Roxithromycin Inhibit the Proinflammatory Interactions of 
Pseudomonas aeruginosa 
Pigments with Human Neutrophils In Vitro

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Received 5 December 1991/Accepted 10 April 1992

The Pseudomonas aeruginosa-derived phenazine pigments pyocyanin and 1-hydroxyphenazine (1-hp) prime 
human neutrophils for enhanced, stimulus-activated release of superoxide and myeloperoxidase (MPO), 
respectively. In the present study, the modulatory potentials of the antimicrobial agents clindamycin, 
erthyromycin, and roxithromycin, and pyocyanin and 1-hp (10 and 20 µg/ml) on the prooxidative interactions of pyocyanin and 1-hp 
(12.5 µM) with human neutrophils have been investigated. Clindamycin, erythromycin, and especially 
roxithromycin caused dose-related inhibition of the generation of superoxide by both untreated and 
pyocyanin-treated neutrophils during activation with either the synthetic chemotactic tripeptide N-formyl-
leucyl-1-phenylalanine (FMLP) or the calcium ionophore A23187. The antimicrobial agents also 
inhibited the generation of reactive oxidants by the MPO-H2O2-halide system during activation of both 
untreated and 1-hp-treated neutrophils by FMLP. These effects appeared to be due to drug-related interference 
with membrane-associated oxidative metabolism, since none of the antimicrobial agents inhibited the release of 
MPO by activated neutrophils, nor did they possess oxidant-scavenging properties. These data demonstrate 
that clindamycin, erythromycin, and especially roxithromycin antagonize the proinflammatory interactions of 
pyocyanin and 1-hp with neutrophils and indicate a possible therapeutic role for these antimicrobial agents in 
the prevention of tissue damage in diseases characterized by P. aeruginosa infection.

The Pseudomonas aeruginosa-derived phenazine pigment pyocyanin, at pathologically relevant concentrations, primes 
human neutrophils for increased oxygen consumption and increased generation of superoxide when the cells are sub-
sequently exposed to stimuli of membrane-associated oxidative 
metabolism (25, 31). The prooxidative potential of pyocyanin is amplified by its degradation product, 1-hydroxy-
phenazine (1-hp), which is also synthesized by P. aeruginosa 
and which primes neutrophils for enhanced release of myelo-
peroxidase (MPO) (31). Pigment-neutrophil interaction during P. aeruginosa infection may cause reactive oxidant-
damaged to host tissues, particularly during chronic 
colonization of the bronchial tree in bronchiectasis. In 
this situation, damage to the bronchial epithelium may be in-
flicted by prolonged exposure to oxidants and proteases 
released by chronically activated phagocytes (6, 7, 21). 
Moreover, in patients with P. aeruginosa pneumonia, pul-
monary parenchymal destruction might also be related to the 
release of oxidants and granule enzymes by pigment-primed 
hyperactive neutrophils (9, 17, 28).

Broad-spectrum antibiotic treatment with currently avail-
able agents is insufficient to eradicate P. aeruginosa from the 
bronchial tree of patients with bronchiectasis and cystic 
fibrosis once colonization has occurred (19). Inhibition of the 
reactive-oxidant component of neutrophil-induced chronic 
bronchial inflammation might therefore introduce a new 
strategy to prevent progressive tissue damage in bronchiect-
asis.

The lysosomotropic, weakly basic antimicrobial agents 
clindamycin, erythromycin, and roxithromycin, which accu-
mulate in phagocytes, have previously been shown to inhibit 
superoxide generation by activated neutrophils at therapeutic 
and supratherapeutic concentrations in vitro (1, 16, 18, 
32). In the present study, we have investigated the effects of 
these agents on the potentially harmful prooxidative inter-
actions of pyocyanin and 1-hp with human neutrophils in 
vitro.

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals and reagents 
were obtained from Sigma Chemical Co. (St. Louis, Mo.). 
Antimicrobial agents. Roxithromycin was provided by 
Roussel UCLAF (Paris, France), and erythromycin was 
provided by Abbott Laboratories (North Chicago, Ill.). Both 
agents (8 mg) were dissolved in 2 ml of 30% aqueous ethanol 
and diluted in distilled water. Clindamycin was provided by 
Upjohn International Inc. (Kalamazoo, Mich.) and was 
dissolved in distilled water. Stock solutions of all agents at 
200 µg/ml were diluted to final concentrations of 10 and 20 
µg/ml in the assay systems, comparable to therapeutic and 
supratherapeutic concentrations in plasma of these agents 
(11, 14, 30). Solvent control systems were included in all 
experiments.

Preparation of pyocyanin and 1-hp. Pyocyanin was pre-

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pared by photolysis of phenazine methosulfate (Aldrich Chemicals, Milwaukee, Wis.) by the method of Knight et al. (22), 1-hp was prepared by the method of Flood et al. (13). Each pigment was solubilized in sterile water to a stock concentration of 250 μg/ml and subsequently diluted in indicator-free HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered Hanks' balanced salt solution (HBSS, pH 7.4) to the concentrations required.

**Neutrophil preparation.** Neutrophils were obtained from heparinized (5 U of preservative-free heparin per ml) venous blood of healthy volunteers and separated from mononuclear leukocytes by centrifugation on 400-g Ficoll-Hypaque (Pharmacy Fine Chemicals, Uppsala, Sweden)-metrizoate cushions (Nyegaard, Oslo, Norway) for 25 min at room temperature. After sedimentation of erythrocytes in 3% gelatin for 15 min at 37°C, the neutrophil-rich supernatant was removed and pelleted. Residual erythrocytes were removed by selective lysis with 0.83% ammonium chloride for 4 min at 10°C. Neutrophils were washed once and found to be >90% viable as determined by trypsin blue (0.1%) dye exclusion before they were resuspended to a concentration of 107 cells per ml in HBSS, pH 7.4.

**Superoxide generation by neutrophils.** Superoxide was determined by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome c in a final reaction volume of 1 ml (4). Neutrophils (106/ml) were preincubated for 45 min with or without roxithromycin, erythromycin, and clindamycin (10 and 20 μg/ml) at room temperature. This procedure was included to prevent the loss of responsiveness of neutrophils to the N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated superoxide generation which occurs during prolonged preincubation of the cells at 37°C (1). Pyocyanin (12.5 μM) or an equal volume of HBSS was added, the reaction mixture was incubated for 5 min at 37°C, and 0.1 mM cytochrome c (type VI from horse heart) was added. Superoxide generation was initiated by the addition of the stimulant FMLP (1 μM) or calcium ionophore A23187 (1 μM) and was stopped after 5 min with 4 ml of 1% TCA in phosphate-buffered saline. Neutrophils were pelleted by centrifugation, and the optical density of the supernatant fluid was measured at 550 nm in a UV spectrophotometer (model SP1700; Pye Unicam, Cambridge, United Kingdom). The amount of reduced cytochrome c was calculated by using a molar extinction coefficient of 2.11 × 104 cm²/mmol (33). Superoxide-mediated reduction of cytochrome c was calculated as the difference in optical density between reaction mixtures with and without superoxide dismutase, and the results are expressed as nanomoles of reduced cytochrome c per 10⁶ neutrophils. We have previously reported that pyocyanin and 1-hp (12.5 μM), as well as erythromycin and roxithromycin at concentrations of up to 20 μg/ml, do not scavenge superoxide (1, 31). In the present study, the effects of clindamycin (20 μg/ml) on the generation of superoxide by a cell-free hypoxanthine (500 μM) and xanthine oxidase (50 mM/ml) were investigated to control for possible scavenging of superoxide by the antimicrobial agent.

**Oxygen consumption by human neutrophils.** Oxygen consumption was measured with a three-channel oxygen electrode (model DW1; Hansatech, Norfolk, United Kingdom). Neutrophils (2 × 10⁶) in 1 ml of HBSS were preincubated with or without roxithromycin (20 μg/ml) for 45 min at room temperature. Neutrophils were then brought to 37°C, and pyocyanin (25 μM) or an equal volume of HBSS was added. Baseline oxygen consumption was measured for 5 min prior to the addition of FMLP (1 μM) and for 20 min after the addition of the stimulus. Results are expressed as the total consumption of O₂ (in nanomoles) by neutrophils at 10 and 20 min after the addition of FMLP.

**MPO-mediated iodination.** Neutrophils (10⁶/ml) were preincubated with or without roxithromycin, erythromycin, and clindamycin (10 and 20 μg/ml) for 45 min at room temperature. 1-hp (12.5 μM) was added to appropriate tubes, and the cells were incubated for 5 min at 37°C in HBSS containing 0.6 μCi of Na¹²⁵I (specific activity, 17 Ci/mg) and 40 nmol of carrier NaI in 900 μl of HBSS. MPO-mediated iodination was then activated by the addition of FMLP (1 μM). After 15 min of incubation at 37°C, the reactions were terminated by the addition of 10% trichloroacetic acid. After three washes with trichloroacetic acid, the amount of protein-bound ¹²⁵I was determined with a solid-state gamma counter. The results are expressed as nanomoles of ¹²⁵I per 10⁶ neutrophils. To control for possible oxidant-scavenging properties of the antimicrobial agents, a fixed concentration (20 μg/ml) of each agent was used to investigate their effects on iodination of bovine serum albumin (1 mg/ml) by a cell-free oxidizing system of glucose (5 mM), glucose oxidase (200 μU), horseradish peroxidase (200 μU), and Na¹²⁵I (0.6 μCi/ml).

**Neutrophil degranulation.** Neutrophils were incubated at a concentration of 2 × 10⁶/ml in HBSS in the presence or absence of the antimicrobial agents for 45 min at room temperature. 1-hp (12.5 μM) was added to appropriate tubes, the cells were incubated for 5 min at 37°C, and FMLP (1 μM) combined with cytochalasin B (CB, 0.25 μg/ml) was added. The tubes were incubated for 15 min at 37°C. To stop the reaction, tubes were transferred to ice for 10 min. Samples were centrifuged, and cell-free supernatants were assayed for MPO activity by a colorimetric assay (27). The results are expressed as the percentage of the total cellular MPO content.

**Statistical analysis.** The results of each series of experiments, with the exception of those for oxygen uptake, are expressed as the mean percentages of inhibition, ± the standard errors of the mean, of drug-treated control systems relative to those of the corresponding drug-free control systems. The statistical significance of data was established at P < 0.05 by the Wilcoxon rank sum test and a paired, two-sided Student’s t test.

**RESULTS**

**Effects of the antimicrobial agents on superoxide generation by untreated neutrophils and neutrophils treated with pyocyanin and activated with FMLP or calcium ionophore.** Clindamycin and erythromycin at 20 μg/ml and roxithromycin at 10 and 20 μg/ml significantly inhibited superoxide generation by neutrophils activated with FMLP in the presence of 12.5 μM pyocyanin (Table 1). Similar effects were observed when calcium ionophore was used as the stimulus of superoxide generation, and significant inhibition was observed with 10 and 20 μg of roxithromycin per ml. The order of potency with respect to drug-mediated inhibition of stimulus-activated superoxide generation by both untreated and pyocyanin-primed neutrophils was roxithromycin > erythromycin and roxithromycin > clindamycin. However, the only significant difference between antibiotics was observed with roxithromycin and erythromycin (10 and 20 μg/ml) (P < 0.05).

As with erythromycin and roxithromycin (1), clindamycin (20 μg/ml) did not interfere with the generation of superoxide.
TABLE 1. Effects of clindamycin, erythromycin, and roxithromycin on superoxide generation by FMLP- or calcium ionophore-activated neutrophils in the presence or absence of pyocyanin

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Superoxide generation by neutrophils treated with*:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No antibiotic</td>
</tr>
<tr>
<td>FMLP (1 μM)</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>FMLP (1 μM) + pyocyanin (12.5 μM)</td>
<td>12.8 ± 1</td>
</tr>
<tr>
<td>Calcium ionophore (1 μM)</td>
<td>10.1 ± 2</td>
</tr>
<tr>
<td>Calcium ionophore (1 μM) + pyocyanin (12.5 μM)</td>
<td>13.7 ± 2</td>
</tr>
</tbody>
</table>

*a* Results are expressed as nanomoles of reduced cytochrome c per 10^6 neutrophils and show the means ± standard errors of the mean for five to eight different experiments. The absolute value for unstimulated neutrophils in the absence of pyocyanin was 4.7 ± 1.

*b* P < 0.01.

*c* P < 0.05.

by the xanthine oxidase-hypoxanthine system (data not shown).

Effects of roxithromycin on oxygen consumption by control and pyocyanin-treated, FMLP-activated neutrophils. The effects of roxithromycin on pyocyanin-treated, FMLP-activated neutrophils are shown in Table 2. Roxithromycin at 20 μg/ml caused significant inhibition of oxygen consumption by neutrophils activated with FMLP in the presence of 25 μM pyocyanin at 10 and 20 min (P < 0.01) after the addition of FMLP.

Effects of the antimicrobial agents on MPO-mediated iodination by control and 1-hp-treated, FMLP-activated neutrophils. The effects of the antimicrobial agents on MPO-mediated iodination are shown in Table 3. Clindamycin, erythromycin, and roxithromycin at 10 and 20 μg/ml caused significant inhibition of MPO-mediated iodination of neutrophils activated with FMLP in the absence and presence of 12.5 μM 1-hp. The order of potency was roxithromycin > erythromycin (P < 0.05) and roxithromycin > clindamycin (P < 0.05). None of the test agents at a fixed concentration of 20 μg/ml inhibited the iodination of bovine serum albumin by a cell-free glucose-glucose oxidase-horseradish peroxidase system (data not shown).

Effects of roxithromycin on neutrophil degranulation by control and 1-hp-treated, FMLP-CB-activated neutrophils. Roxithromycin at 10 and 20 μg/ml had no significant effect on MPO release by neutrophils activated with FMLP-CB in the presence or absence of 12.5 μM 1-hp. The results for systems containing neutrophils only, FMLP-CB, and FMLP-CB plus 1-hp were 1.9% ± 1%, 17% ± 7%, and 35% ± 6% of total MPO release, respectively. The results for systems containing roxithromycin-CB and roxithromycin at 10 and 20 μg/ml were 16% ± 6% and 16% ± 6%. Results for a system containing (10 μg/ml), FMLP-CB, and 1-hp and a system containing roxithromycin (20 μg/ml), FMLP-CB, and 1-hp were 39% ± 8% and 39% ± 5%, respectively.

**DISCUSSION**

Excessive neutrophil migration into the bronchial tree is a feature of bronchiectasis (8), and neutrophils are present in increased numbers in purulent sputum obtained from patients with bronchiectasis. Pyocyanin and 1-hp are produced by P. aeruginosa strains which initially colonize the respiratory tract (35). Both pigments are present in sufficient concentrations in sputum to affect neutrophil oxidative metabolism and degranulation, thereby enhancing the proinflammatory activities of these cells and increasing the potential threat of oxidant-mediated toxicity to bystander cells and tissues (31, 34). Preventing either neutrophil migration into the bronchial tree or activation of these cells could modulate progression of bronchiectasis and other chronic inflammatory diseases associated with oxidant-mediated tissue damage.

It has previously been reported that erythromycin and clindamycin at supratherapeutic concentrations, as well as roxithromycin at both therapeutic and supratherapeutic concentrations, inhibit the generation of reactive oxidants by stimulus-activated neutrophils (1, 15, 18, 26, 32). The inhibitory effects of the macrolide antibiotics are apparently selective for relatively weak stimuli such as those of FMLP and the calcium ionophore A23187, whereas they have little effect on the corresponding responses activated by opsonized particulate stimuli and the tumor promoter phorbol myristate acetate (1, 18).

In this study, clindamycin, erythromycin (20 μg/ml), and roxithromycin (10 and 20 μg/ml) inhibited superoxide production by both pyocyanin-treated and untreated FMLP-activated neutrophils. Essentially similar effects were observed when calcium ionophore was used as the stimulus of membrane-associated oxidative metabolism. Roxithromycin was the most potent inhibitor of superoxide generation, probably as a consequence of the greater intraphagocytic accumulation of this agent relative to erythromycin and clindamycin (2, 16, 29). Although the precise biochemical mechanism by which clindamycin, erythromycin, and roxithromycin inhibit superoxide generation by activated neutrophils has not been elucidated, the presently available data indicate that these agents affect the activity of NADPH-

**TABLE 2. Effects of roxithromycin (20 μg/ml) on pyocyanin (25 μM)-enhanced oxygen consumption by FMLP (1 μM)-stimulated neutrophils**

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>FMLP-activated O₂ consumption (nmol/2 × 10^6 neutrophils) at:</th>
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<tbody>
<tr>
<td></td>
<td>10 min</td>
</tr>
<tr>
<td>FMLP + pyocyanin</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>FMLP + pyocyanin + roxithromycin</td>
<td>17 ± 1`</td>
</tr>
</tbody>
</table>

*a* Results are expressed as mean values ± standard errors of the mean for three separate experiments.

*b* Neutrophils only.

*c* P < 0.01 (FMLP + pyocyanin + roxithromycin versus FMLP + pyocy- anin only).
oxidase (1, 32). This is supported by the absence of superoxide-scavenging properties in the test antimicrobial agents (1, 32) and the observation in the present study that roxithromycin impairs oxygen consumption by FMLP-activated neutrophils.

Unlike pyocyanin, 1-hp does not prime neutrophils for increased production of superoxide (31). However, this agent causes potent enhancement of MPO-mediated iodination by priming neutrophils for increased release of primary granules following activation with FMLP. In the present study, the test antimicrobial agents did not interfere with the release of MPO by either untreated or 1-hp-primed neutrophils. However, these agents caused a dose-related inhibition of MPO-mediated iodination by neutrophils. The order of potency was roxithromycin > erythromycin and roxithromycin > clindamycin. These inhibitory effects of the macrolides and clindamycin on MPO-mediated iodinating activity of both control and 1-hp-primed neutrophils are probably due to interference with NADPH-oxidase activity, leading to inhibition of the generation of H2O2 and subsequent secondary inhibition of the MPO-H2O2-halide system.

Aerobic gram-negative bacilli are uniformly resistant to clindamycin and macrolide antimicrobial agents. Consequently, these agents have no primary value in the chemotherapy of P. aeruginosa infections. However, if the proinflammatory effects of pyocyanin and 1-hp (31) are operative in vivo, the data presented here indicate a possible secondary role for agents such as clindamycin, erythromycin, and especially roxithromycin in the prevention of tissue damage inflicted by pigment-primed, activated neutrophils during the colonization of the respiratory tract with P. aeruginosa. Although this suggestion is speculative and requires rigorous investigation using models of experimental chemotherapy, combinations of clindamycin or macrolides with standard anti-Pseudomonas chemotherapeutic agents appear to satisfy some critical preliminary prerequisites. First, the possibility that clindamycin or macrolides interfere with the direct action of anti-Pseudomonas antibiotics has been examined. The aminoglycosides tobramycin and amikacin and the beta-lactam antibiotics piperacillin, ceftazidime, and imipenem were tested by a checkerboard technique, with each antibiotic in combination with either clindamycin, erythromycin, or roxithromycin against a pyocyanin-producing clinical isolate of P. aeruginosa. At concentrations achievable in the blood, no significant antagonism or synergism was found (unpublished data). These findings are in keeping with those of Fass et al., who tested the combination clindamycin and gentamicin, and those of Zinner et al. and Leng et al., who tested clindamycin in combination with gentamicin or amikacin (10, 24, 36). Likewise, in assays of intraphagocytic killing of the same bacterial isolate, neither clindamycin, erythromycin, nor roxithromycin interfered with cooperative interactions between human blood neutrophils and the anti-Pseudomonas agents in the destruction of intracellular bacilli (our unpublished data). Moreover, some investigators have reported that in vitro exposure of Klebsiella pneumoniae and Escherichia coli to clindamycin (3, 5) or of P. aeruginosa to the macrolide agent josamycin (23) renders these microbial pathogens more susceptible to eradication by host defense mechanisms.

In conclusion, macrolide antibiotics such as roxithromycin with antioxidant properties may have useful modulatory effects on neutrophil-mediated tissue damage in diseases such as bronchiectasis. This destructive component of chronic inflammation in chronic bronchial sepsis might be reduced by inhibiting the generation of reactive oxidants by pyocyanin- and 1-hp-primed activated neutrophils in the bronchial tree.

**REFERENCES**