Inhibitory effect of roxithromycin on adhesion of *Klebsiella pneumoniae* strains 3051, CF504 and LM21

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The effect of subinhibitory concentrations of roxithromycin on the adhesion of three strains of *Klebsiella pneumoniae* to Int-407 cells was studied. Adherence was markedly inhibited and the effect was increased when roxithromycin was added to the cell culture medium rather than to the bacterial growth medium. Several assays were performed in order to understand the mechanism by which roxithromycin exerted this inhibitory effect. The greatest effect was obtained when roxithromycin was concentrated in the extracellular compartment; when roxithromycin was concentrated in the intracellular compartment, the inhibitory effect was reduced. The analysis of adhesion factors of bacteria showed that exposure to roxithromycin did not alter their apparent structure or quantity. Roxithromycin appears to interfere in the interaction between bacteria and eukaryotic receptors.

**Introduction**

*Klebsiella pneumoniae* accounts for 10% of nosocomial infections caused by Enterobacteriaceae in intensive care units, notably sepsicaemia, urinary and respiratory tract infections and meningitis. Epidemiological studies have shown that infection is preceded by intestinal colonization. Moreover, translocation through the intestinal mucosa has been demonstrated in mice.

Mucosal colonization by bacteria is always linked to adhesion processes. The adhesion of *K. pneumoniae* to intestinal mucous membranes is a necessary stage in the establishment of nosocomial infections in vivo. It is possible to mimic in-vivo interactions of bacteria and mucous membranes by means of cultures of intestinal tissue cells in vitro. Indeed, strains of *K. pneumoniae* involved in nosocomial infections adhere in vitro to Caco-2 and Int-407 cells. Two adherence patterns have been observed: a diffuse pattern in which bacteria are scattered on the intestinal cell surface, and an aggregative adherence pattern where bacteria are piled up on the surface of the cells. Ninety percent of clinical isolates adhere in an aggregative pattern.

Roxithromycin is a macrolide antibiotic with a broad spectrum of antibacterial activity, but excluding many Gram-negative bacilli. We previously examined the effect of subinhibitory concentrations of roxithromycin on the adhesion of *K. pneumoniae* strains to Caco-2 cells.

Adhesion of *K. pneumoniae* strain CF504, which harbours the adhesin CF29K, to Caco-2 cells was diminished by >50% at concentrations of roxithromycin ranging from MIC/2 to MIC/8. Inhibition of adhesion was more effective when roxithromycin was added to the culture medium of the Caco-2 cells than when it was added to the growth medium of the bacteria. Further work with Int-407 cells has shown that addition of roxithromycin to the bacterial culture medium of *K. pneumoniae* strains CF504 and LM21 inhibits adherence of bacteria by >50% for a concentration of MIC/2 (32 mg/L). Roxithromycin added to the cell culture medium of the Int-407 cells inhibited adhesion of *K. pneumoniae* CF504 by 80% at a concentration of MIC/2 and by 46.5% at a concentration of MIC/8, and by 96.7% at MIC/2 and 42% at MIC/8 with strain LM21.

The present study was designed to extend this work and examine the effect of subinhibitory concentrations of roxithromycin on *K. pneumoniae* strain 3051, and to examine the mechanism by which roxithromycin exerts its inhibitory effect.

**Materials and methods**

**Bacterial strains**

*Klebsiella pneumoniae* strains CF504, LM21 and 3051 were obtained as clinical isolates from nosocomial
infections. Strain CF504 presents a diffuse pattern of adherence; the protein, adhesin CF29K, is involved in adherence. Strain LM21 adheres with an aggregative pattern, and a capsule-like material seems to be involved in this adhesion phenotype. Strain 3051 adheres in a diffuse manner; the type 1 fimbriae are responsible for this adhesion.

Transconjugant *Escherichia coli* strain CF604 carries a 185 kb plasmid from *K. pneumoniae* CF504 and over-expresses the adhesin CF29K. The MIC of roxithromycin (Roussel-Uclaf, Paris, France) was 64 mg/L for these four strains as determined by dilution on Mueller–Hinton broth and agar.

**Cell cultures**

The cell line Int–407 (ATCC CCL6), derived from human embryonic jejunum and ileum, was used. The cells were cultured in Eagle’s minimum essential medium (EMEM; Polylabo, Strasbourg, France), supplemented with 10% heat-inactivated fetal calf serum (Polylabo), 1% L-glutamine (Boehringer-Mannheim, Meylan, France), 1% non-essential amino acids (Boehringer-Mannheim), penicillin 10,000 U/mL, streptomycin 10 μg/mL, and amphotericin B (ATGC Biotechnologie, Noisy Le Grand, France) 25 μg/mL in an atmosphere of 5% carbon dioxide.

For adhesion assays, cells were cultured in 24-well tissue culture plates (ATGC Biotechnologie) to confluency.

**Adhesion assays**

All bacterial strains, except *K. pneumoniae* 3051, were suspended in EMEM containing 2% D-mannose (Sigma-Aldrich, L’Isle d’Abeau, France). Because of the mannose-sensitivity of the adhesion mediated by the type 1 fimbriae of *K. pneumoniae* 3051, this strain was suspended in EMEM without D-mannose. Following suspension, 1 × 10⁸ bacteria were added to confluent monolayers of Int-407 cells and incubated for 3 h at 37°C in a 5% carbon dioxide atmosphere. After incubation, each well was rinsed three times in phosphate-buffered saline (PBS) pH 7.4 (Boehringer-Mannheim, Strasbourg, France) to remove the non-adherent bacteria. A solution of 0.5% Triton X-100 was then added to each well to release cells. Adherent bacteria were quantified by plating appropriate dilutions on Luria–Bertani (Difco, Paris, France) agar medium. Adherence was expressed in cfu/cm² of tissue culture well.

**The inhibitory effect of roxithromycin**

The effect of subinhibitory concentrations of roxithromycin was studied by adding roxithromycin at the dilution required to obtain the concentrations MIC/2 to MIC/32 in 1 mL of the culture medium of the Int-407 cells. The test strains of bacteria were suspended in cell culture medium containing the subinhibitory concentrations of roxithromycin and added to the Int-407 cells. Adhesion assays were performed as described above.

**Effect on adhesion of exposure to roxithromycin before and after adhesion**

Effect of high intracellular concentration of roxithromycin. Roxithromycin was concentrated within Int-407 cells using sodium bicarbonate (2.2 g/L in Mueller–Hinton medium) or pre-incubation for 1 h before adding bacteria. Appropriate dilutions of roxithromycin were added in order to obtain MIC/2 to MIC/32 in 1 mL of the cell culture medium. We have demonstrated previously that the MIC of roxithromycin was not changed in the presence of bicarbonates (unpublished data).

Effect of roxithromycin on adhering bacteria. After an adhesion step of 2 h, the supernatant was removed from each well. EMEM containing roxithromycin (MIC/4 and MIC/8) was added to the wells for 1 h. A control assay was performed with EMEM without roxithromycin.

Effect of roxithromycin on adhesin CF29K or on the capsule of *K. pneumoniae* LM21

Adhesin CF29K is overexpressed in *E. coli* strain CF604. *E. coli* CF604 were grown on Mueller–Hinton agar, then suspended in EMEM containing subinhibitory concentrations of roxithromycin (MIC/2 and MIC/4). Controls were carried out in parallel without antibiotic. Suspensions were incubated for 3 h at 37°C, after which the bacteria were harvested by centrifugation (15,000g, 4°C) and the surface proteins extracted as described below.

Similarly, *K. pneumoniae* LM21 was grown overnight on Mueller–Hinton agar plates, suspended in EMEM containing roxithromycin (MIC/2, MIC/4 and MIC/8) and incubated for 3 h at 37°C. After incubation, capsule extraction was performed as described below.

**Extraction and analysis of surface proteins of *E. coli* CF604**

Extraction of bacterial surface proteins was performed as described by Stirm et al. Surface bacterial proteins were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), using 12% (w/v) acrylamide and 0.8% (w/v) bisacrylamide for the separation gel. Samples were denatured in 1.5% (v/v) SDS–1.5% (v/v) 2-mercaptoethanol in 0.5 M Tris (pH 6.8) for 5 min at 100°C, before being loaded on to the gel. Electrophoresis was performed in a solution of 0.025 M Tris, 0.28 M glycine, and 0.1 (w/v) SDS (pH 8.6) and the gel was stained with Coomassie blue (2.5 g/L). The protein CF29K was identified by its molecular weight. The molecular weight standards (Pharmacia Biotech, Saclay, France) were phos-
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Analysis and quantification of the capsular polysaccharides of K. pneumoniae LM21

Extraction of capsular polysaccharides was performed as described by Linker & Jones. Quantification of the capsular polysaccharides was performed by measuring the uronic acid content. Briefly, the sample containing the capsular polysaccharides was layered on an ice-cold borate solution (1:40 (v/v) in concentrated H$_2$SO$_4$). A 0.1% (w/v) carbazole solution was added to the mixture and the sample was heated for 30 min at 55°C to develop the colour reaction. Absorbance at 530 nm is indicative of a positive uronic acid test. Uronic acid concentration was determined from a standard curve of D-mannuronic acid ranging from 10 to 1000 mg/L. Total cellular protein was measured by a Bio-Rad protein assay (Bio-Rad, Ivry-Sur-Seine, France).

Results

Inhibitory effect of roxithromycin on the adhesion of K. pneumoniae

Addition of roxithromycin to the cell culture medium of Int-407 cell line markedly inhibited the adhesive capacity of K. pneumoniae 3051 (Figure 1); this adhesive inhibitory effect ranged from 88.1% at MIC/2 to 67% at MIC/32, compared with controls.

Effect of intracellular concentration on the inhibitory effect of roxithromycin on adhesion

The intracellular concentration of roxithromycin induced by the addition of sodium bicarbonate reduced the inhibitory effect on adhesion (Figure 2a). At a sub-inhibitory concentration of MIC/2, adherence was reduced by only 38% for K. pneumoniae CF504 and by 40% for K. pneumoniae LM21, compared with values of almost 90% when roxithromycin was not concentrated intracellularly.

When intestinal cells were incubated with roxithromycin for 1 h before adding the bacteria, a weak inhibitory effect on adhesion was observed for both strains of K. pneumoniae (Figure 2b).

![Figure 1](image1.png)  ![Figure 2](image2.png)  ![Figure 3](image3.png)

**Figure 1.** Effect of roxithromycin concentration on K. pneumoniae 3051 adherence to Int-407 cells.

**Figure 2.** Adherence of K. pneumoniae CF504 (■) and K. pneumoniae LM21 (□) (a) in the presence of sodium bicarbonate and roxithromycin and (b) when Int-407 cells were preincubated for 1 h with roxithromycin.

**Figure 3.** Effect of roxithromycin concentration on adherent K. pneumoniae strains CF504 (■) and LM21 (□).
Figure 3 shows that adhesion was greatly reduced for \textit{K. pneumoniae} LM21 and clearly reduced for \textit{K. pneumoniae} CF504, with values of 80\% and 50.5\%, respectively of inhibition for a roxithromycin concentration of MIC/8.

Effect of roxithromycin on adhered bacteria

After migration on SDS–PAGE (Figure 4), no apparent change was observed for the adhesin CF29K overexpressed in \textit{E. coli} CF604 when bacteria were treated with subinhibitory concentrations of roxithromycin.

For \textit{K. pneumoniae} LM21, the uronic acid content (and hence the quantity of capsular polysaccharides) was not significantly increased in the presence of subinhibitory concentrations of roxithromycin: without roxithromycin the capsular polysaccharide extract contained 1.94 ± 0.08 ng of uronic acid/mg cellular protein; in the presence of roxithromycin this level was not significantly different (e.g. 2.06 ± 0.02 ng/mg cellular protein at MIC/4).

Discusssion

Previous work has shown an inhibitory effect of roxithromycin on the diffuse adhesion of \textit{K. pneumoniae} strain CF504 to Caco-2 cells.\textsuperscript{8} We have also shown an inhibitory effect on adhesion for subinhibitory concentrations of roxithromycin in two strains of \textit{K. pneumoniae} (CF504 and LM21), demonstrating diffuse and aggregative adhesion patterns, respectively.\textsuperscript{9} In this case, the cell line used was Int-407; these cells are derived from human embryonic intestine.

The present study extended this work by examining the effect of subinhibitory concentrations of roxithromycin on \textit{K. pneumoniae} strain 3051, which exhibits a diffuse adherence phenotype and has type 1 fimbriae thought to be responsible for this effect. It was shown that when roxithromycin was added to the culture medium of the Int-407 cells, adherence was markedly reduced and the effect was maintained at the lowest concentration tested (MIC/32).

Overall, these results suggest that the inhibitory effect of roxithromycin is related to a modification of the Int-407 cells or to a disturbance in the interaction between bacteria and eukaryotic cells since both cells and bacteria were in contact with roxithromycin.

Two further experiments suggest that extracellular roxithromycin has the major inhibitory effect on adhesion. Firstly, using bicarbonates which promote the intracellular penetration of roxithromycin, we observed that its inhibitory effect on adhesion was reduced. Secondly, when Int-407 cells were incubated with roxithromycin before adding bacteria, a weak inhibitory effect was observed. If we assume that during pre-incubation roxithromycin has penetrated the intestinal cells, intracellular roxithromycin did not appear to act on bacterial adhesion. Thus, when the intracellular concentration of roxithromycin was augmented there was a reduction in the inhibitory effect observed when roxithromycin was added to the cell culture medium.

The addition of roxithromycin to adherent bacteria also produced interesting results. After a period of 2 h, bacteria are bound to the monolayer of intestinal cells. Roxithromycin was able to release bacteria from the cells, suggesting that it could lead to the breakdown of the binding between bacteria and intestinal cells.

The inhibitory effect of roxithromycin is clear when the adhesion factor or the cellular receptor contain a polysaccharide fraction—for example, the cellular receptor for \textit{K. pneumoniae} 3051 type 1 fimbriae\textsuperscript{10} or the cellular receptor of \textit{K. pneumoniae} CF504 CF29K adhesin. Di Martino \textit{et al.}\textsuperscript{15} showed that the eukaryotic receptor of adhesin CF29K contains a sialic acid (N-acetylneuraminic acid) fraction.
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Mannose and other N-acetyl-D-glucosamine. Thus, competition could occur between roxithromycin and either the receptor sites on the intestinal cells, or the polysaccharide fraction of the adhesion factor, via steric hindrance.

Analysis of surface proteins by SDS–PAGE revealed that the adhesin CF29K was not altered by exposure to roxithromycin. Likewise, the quantity of capsular polysaccharide synthesized by *K. pneumoniae* LM21 was not modified in the presence of subinhibitory concentrations of roxithromycin. Similar results have been reported for erythromycin and *Pseudomonas aeruginosa.*

In conclusion, we have shown an inhibitory effect of roxithromycin on the adhesion of three *K. pneumoniae* strains to intestinal cells; this effect is seen whatever adhesion factor is involved. Roxithromycin seems to modify the interaction between the adhesion factors and the eukaryotic receptor. Further work is now required to elucidate the target of roxithromycin and to investigate whether the components of the cladinose and desosamine residues of roxithromycin could inhibit adhesion.

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**References**


