Accumulation and activity of cethromycin (ABT-773) within human polymorphonuclear leucocytes

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Received 19 December 2002; returned 19 February 2003; revised 25 March 2003; accepted 15 April 2003

Objectives: To evaluate the penetration of ketolide cethromycin (ABT-773) into human polymorphonuclear leucocytes (PMNs) and its intracellular activity.

Methods: The uptake of radiolabelled cethromycin by PMNs was determined by a velocity gradient centrifugation technique. The activity of cethromycin against intracellular Staphylococcus aureus ATCC 25923 in PMNs was also evaluated.

Results: The cellular to extracellular concentration (C/E) ratio for cethromycin was >200 at an extracellular concentration of 2 mg/L. The uptake of cethromycin into PMNs was rapid and saturable. Cethromycin was slowly released from the loaded PMNs (cell associated drug >50% after 2 h of incubation). Intracellular penetration was significantly affected by the environmental temperature (C/E ratio at 4°C and 37°C: 13 ± 6 and 226 ± 31, respectively; P < 0.05), by cell viability (C/E ratio for dead and viable cells: 100 ± 38 and 226 ± 31, respectively; P < 0.05), by pH (C/E ratio was significantly increased at basic pH) and by the metabolic inhibitors 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone. The intracellular accumulation of cethromycin also decreased significantly when cells were activated with phorbol myristate acetate or opsonized zymosan. These data indicate that a potentially active mechanism could be involved in the uptake of cethromycin by PMNs. At high extracellular concentrations of 5 and 10 mg/L, cethromycin showed significant intracellular activity against S. aureus.

Conclusions: Cethromycin achieves high intracellular concentrations within human PMNs, remaining active intracellularly.

Keywords: phagocytes, intracellular concentration, intracellular activity, ketolides

Introduction

The penetration and intracellular activity of an antimicrobial agent into phagocytic cells is essential for activity against microorganisms which are known to survive and multiply within them. Erythromycin A-derivatives (macrolides and ketolides) are able to penetrate and concentrate intracellularly, reaching high intracellular concentrations.1-6 Macrolide uptake by human polymorphonuclear leucocytes (PMNs) shows two patterns of intracellular pharmacokinetics, related to their chemical structure. Monobasic macrolides display saturable accumulation kinetics, characteristic of a carrier-mediated transport system and a rapid efflux, whereas the uptake of dibasic macrolides, such as azithromycin, is characterized by a progressive accumulation, without saturation and a slow efflux.6 However, the kinetic profiles of other ketolides evaluated cannot be included in any group, showing intermediate kinetic characteristics.1,4 Although these compounds have been shown to be strongly incorporated and accumulated by human PMNs, achieving high intracellular concentrations, they display a lower intraphagocytic activity than expected from the intracellular concentrations obtained.3,5,7

Cethromycin is a new ketolide, a semisynthetic derivative of macrolide erythromycin A. This antimicrobial agent displays good activity against a broad bacterial spectrum (Gram-positive organisms, some Gram-negative organisms and intracellular bacteria).8-12 Initially, ketolides are developed for respiratory tract infections, since they show good activity against common respiratory pathogens, including those that have acquired resistance to macrolides. Cethromycin shows greater activity against macrolide-resistant streptococci (Streptococcus pneumoniae and Streptococcus pyogenes) compared with telithromycin.13,14

The purpose of this study was to evaluate the uptake of cethromycin by human PMNs. The mechanism involved in the penetration...
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of this agent into human PMNs and its intracellular activity was also evaluated.

Materials and methods

Isolation of PMNs

PMNs were recovered from the heparinized venous blood of healthy donors and isolated by methods which have been previously described. \(^{15}\) PMN preparations were 97% pure. Final cell suspensions were adjusted to 5 x 10^6 PMNs per mL in Hanks balanced salt solution (HBSS) containing 1% gelatin. The PMNs were 95% viable by Trypan Blue exclusion.

Cethromycin uptake by human PMNs

The uptake of radiolabelled cethromycin by human PMNs was determined by means of a velocity gradient centrifugation technique, described by Klempner & Styrt. \(^{16}\) [14C]Cethromycin (51.51 mcg/g) and cethromycin were kindly supplied by Abbott Laboratories (North Chicago, IL, USA). In these experiments, human PMNs were incubated in HBSS containing different concentrations of cethromycin (2–200 mg/L). After different incubation times at 37°C, cells were separated from the extracellular solution by centrifugation through a water-impermeable silicone-oil barrier (density 1.029 g/cm^3) in a microcentrifuge tube. A 10 µL aliquot of the extracellular medium and the entire cell pellet, obtained by cutting off the portion of the microfuge tube containing the pellet, were placed in 3 mL of scintillation fluid (Ready Micro, Beckman Instruments, Inc., Fullerton, CA, USA) and counted in a liquid scintillation counter (model LS 1801; Beckman).

The intracellular water space was measured by using tritiated water and the extracellular marker \(^{14}C\)(polyethylene glycol (1.4 mg/mL; New England Nuclear Corp., Boston, MA, USA)). \(^{16}\) Cells were incubated with these radiolabelled compounds for 2 min at 37°C and processed as described above. From the values obtained by this procedure, cell-associated antimicrobial agent concentrations were calculated and expressed as cellular to extracellular concentration (C/E) ratios. \(^{16}\)

Characterization of cethromycin uptake

Further studies were carried out to elucidate the mechanism of cethromycin uptake by the PMNs, as previously described. \(^{17}\) The importance of cell viability was studied by using PMNs killed by exposure to 10% formalin for 30 min. These cells were then washed and suspended in fresh medium. In addition, the influence of environmental temperature, pH and metabolic inhibitors was evaluated. The influence of temperature was examined by comparing the antimicrobial uptake at 4 and 37°C. The pH profiles of cethromycin uptake were measured in media pre-adjusted to different external pHs (pH 6, 8 and 9; control 7.2) by the addition of 10 N HCl or 10 N NaOH. An inhibitor of glycolysis (sodium fluoride 1.5 x 10^-5 M) (Sigma), an inhibitor of mitochondrial oxidative metabolism (sodium cyanide, 1.5 x 10^-5 M) (Sigma), a blocker of the proton gradient (carbonyl cyanide m-chlorophenylhydrazone, 1.5 x 10^-5 M) (CCCP) (Sigma) and an uncoupler of oxidative phosphorylation (2,4-dinitrophenol, 1 x 10^-4 M) (2,4-DNP) (Sigma) were used as metabolic inhibitors. PMNs in Hanks balanced salt solution, with and without metabolic inhibitors, were incubated for 30 min at 37°C. Cethromycin (final concentration, 2 mg/L) was then added and the uptake measured, as described above.

In a series of experiments, cethromycin uptake (extracellular concentration: 2 mg/L) by human PMNs was measured after stimulating the cells with 200 nM phorbol myristate acetate (PMA; Sigma) and after phagocytosis of either opsonized zymosan (0.9 mg/L; Sigma) or S. aureus ATCC 25923, opsonized in 5% pooled human serum (15 min; 37°C) at a 10:1 ratio of bacteria to PMNs. PMA or opsonized particles were added to PMN suspensions at the same time as the antimicrobial agent, and the uptake was measured as described above.

The efflux or reversibility of binding of cell-associated cethromycin was also studied. PMNs were incubated for 20 min at 37°C with cethromycin (extracellular concentration: 2 mg/L) collected by centrifugation and rapidly suspended in ketolide-free medium. Cell-associated cethromycin was quantified at various time intervals (1, 5, 10 and 20 min and 1 and 2 h) after removal of the extracellular antimicrobial agent.

Organism and susceptibility testing

Staphylococcus aureus ATCC 25923 was used for killing assays. Susceptibilities were determined by dilution assay. The MIC and MBC of cethromycin against this strain were 0.004 and ≥16 mg/L respectively.

Intracellular activity of antimicrobial agents

To evaluate the intracellular activity of cethromycin, a previously described method was used. \(^{18}\) Briefly, 0.1 mL of bacterial suspension pre-opsonized with 5% pooled human serum (5 x 10^5 cfu/mL) and 0.1 mL of PMNs (5 x 10^6 PMNs per mL) were combined in a series of polypropylene biowells (Beckman) and incubated in a shaker (50 rpm) for 60 min at 37°C. After incubation, the mixtures were washed three times with 2.5 mL of ice-cold phosphate-buffered saline (pH 7.2) using differential centrifugation (160g; 5 min at 4°C) to remove the extracellular bacteria. Cells were then suspended in 0.2 mL of RPMI medium (Sigma). At this time (designated time 0), different concentrations of ketolide were added, and the vials were incubated in a shaker (50 rpm) at 37°C. Controls (samples without antimicrobial agent) were also included. Controls were removed at time zero and controls and samples with antimicrobial agent after 3 h of incubation. Cells were lysed in distilled water and samples diluted and poured plated in agar. Colonies were counted after 24 h of incubation at 37°C. Data were expressed as percentages of surviving staphylococci compared with control levels at 3 h. In addition to determining bacterial survival, morphological studies were also routinely carried out at time zero and after 3 h of incubation to evaluate the disposition of bacteria (cell associated or extracellular). Samples (50 µL) were removed from biowells and deposited on glass slides. After being stained with Wright stain, samples were examined by light microscopy. Morphologic studies showed that most bacteria were cell-associated. All assays were carried out in duplicate with PMNs from five different donors.

Statistical analysis of data

Data were expressed as means ± standard deviation. Analysis of variance and Student’s t-test for paired data were used to assess statistical significance at P < 0.05.

Results

Uptake of cethromycin by human PMNs

Figure 1 shows the kinetics of the uptake of cethromycin by human PMNs. Intracellular penetration was rapid and high, reaching a plateau within 20 min. With extracellular concentrations of 2 mg/L, the C/E ratios were higher than 60 and 220 after 1 min incubation time and at equilibrium, respectively. The kinetics of the efflux of cethromycin are also shown in Figure 1. Reversibility of binding of cethromycin to human PMNs was slow. At equilibrium (1–2 h after incubation in an antimicrobial-free medium), the percentage of cethromycin released was only 58%. At this time, the intracellular and extracellular concentrations reached were 189 and 0.2 mg/L, respectively. For an estimated C/E ratio of 220, the corresponding intracellular
concentration would be 44 mg/L, a value significantly lower than that obtained experimentally (189 mg/L).

The effect of extracellular concentrations (2 to 200 mg/L) of cethromycin on PMN uptake was also studied. The C/E ratios decreased progressively from 215 (at an extracellular concentration of 2 mg/L) to 25 (at an extracellular concentration of 200 mg/L). Cethromycin uptake showed saturation kinetics characteristic of that of a carrier-mediated transport system. The $K_m$ (Michaelis–Menten constant) and $V_{max}$ values (at 5 min of incubation time) calculated from Lineweaver–Burk reciprocal plots were $16.6 \text{mg/L (20.88} \mu\text{M}$ and $11 198 \text{ng/10}^6 \text{cells per 5 min}$, respectively.

The effect of environmental temperature, cell viability and pH on the intracellular penetration of cethromycin in human PMNs ($n = 5$)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>C/E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable cells, $37^\circ C$, pH 7.2</td>
<td>$226 \pm 31$</td>
</tr>
<tr>
<td>Viable cells, $4^\circ C$</td>
<td>$13 \pm 6^*$</td>
</tr>
<tr>
<td>Dead cells, $37^\circ C$</td>
<td>$100 \pm 38^*$</td>
</tr>
<tr>
<td>pH 6</td>
<td>$64 \pm 13^*$</td>
</tr>
<tr>
<td>pH 8</td>
<td>$359 \pm 40^*$</td>
</tr>
<tr>
<td>pH 9</td>
<td>$419 \pm 26^*$</td>
</tr>
</tbody>
</table>

Experiments were carried out for 20 min at an extracellular concentration of 2 mg/L. *$P < 0.05$ compared with the control.

Intracellular activity of cethromycin against S. aureus

The intracellular activity of cethromycin against S. aureus ATCC 25923 was evaluated by a 3-h assay ($n = 5$). Data are expressed as percentages of surviving staphylococci compared to controls without antimicrobial agents (100%: surviving intracellular bacteria without antimicrobial agents at 3 h: $4.8 \times 10^4$ cfu). *$P < 0.05$ compared with the controls without antimicrobial agents.

Table 2. Effect of metabolic inhibitors and stimuli on cethromycin uptake by human PMNs ($n = 5$)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>C/E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable cells, $37^\circ C$, pH 7.2 (control)</td>
<td>$220 \pm 37$</td>
</tr>
<tr>
<td>Metabolic inhibitors</td>
<td></td>
</tr>
<tr>
<td>2,4-dinitrophenol</td>
<td>$128 \pm 38^*$</td>
</tr>
<tr>
<td>sodium cyanide</td>
<td>$422 \pm 62^*$</td>
</tr>
<tr>
<td>sodium fluoride</td>
<td>$219 \pm 42$</td>
</tr>
<tr>
<td>carbonyl cyanide $m$-chlorophenylhydrazine</td>
<td>$61 \pm 17^*$</td>
</tr>
<tr>
<td>Stimulus</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>$143 \pm 31^*$</td>
</tr>
<tr>
<td>zymosan</td>
<td>$73 \pm 17^*$</td>
</tr>
<tr>
<td>S. aureus</td>
<td>$233 \pm 44$</td>
</tr>
</tbody>
</table>

Experiments were carried out for 20 min at an extracellular concentration of 2 mg/L. *$P < 0.05$ compared with the control.

Discussion

Antimicrobial intracellular penetration and activity are important considerations in treating infections due to intracellular pathogens. Ketolides, such as telithromycin, are able to penetrate and concentrate intracellularly, reaching high intracellular concentrations.1-5 In this study, we have evaluated the uptake of a new ketolide, cethromycin, by human PMNs. At therapeutic extracellular concentrations, cethromycin reached intracellular concentrations in PMNs which were 200 times higher than extracellular ones. The cethromycin accumulation kinetics were rapid and saturable over a 20–30 min incubation time. The C/E ratio was similar to that observed for
telithromycin and slightly lower than that observed for ketolides HMR3004, HMR3562 and HMR3787. Saturability over time has also been presented for all previously evaluated ketolides, except telithromycin. It is worth noting that cethromycin reached equilibrium more rapidly than other ketolides evaluated (20–30 min versus 2–3 h). These differences may be related to cethromycin structure. Previous studies report that a transmembrane transport system is involved in the uptake of ketolides by human PMNs and that molecular physico-chemical properties may regulate binding affinity to a membrane carrier involved in an active transport mechanism. In HMR ketolides, the carbamate at the C11/C12 position has an alkyl-aryl extension, whereas in cethromycin, an extension is placed on the 6-O position. These changes might confer on cethromycin a greater affinity with the binding point and/or minor steric interferences.

The intracellular penetration of cethromycin was saturable at the extracellular concentrations evaluated. Cethromycin uptake displayed saturation kinetics characteristic of a carrier-mediated transport system. The $K_m$ value obtained was lower than that obtained with telithromycin, indicating a higher affinity for the carrier. The slow efflux and the high percentage of retained agent displayed by cethromycin has also been shown for all previously evaluated ketolides. These antimicrobial agents are weak basic compounds which could be protonated and trapped within acidic compartments, accounting for the prolonged cellular retention of the agents.

Similar to dibasic macrolides and other ketolides, cethromycin uptake was sensitive to external pH, related to the weak basic character of these antibiotics. Likewise, carbonyl cyanide m-chlorophenyl-hydrazone (an inhibitor of the transmembrane pH gradient) also inhibited cethromycin accumulation. Cethromycin uptake was dependent on both temperature and cellular viability. These data point to the fact that an active mechanism may be involved in the uptake of this ketolide by human PMNs. However, PMN pre-incubation with sodium fluoride, an inhibitor of glycolysis (a major source of energy in PMNs) did not alter cethromycin uptake. These data indicate that this process is metabolically inactive or perhaps, as has been proposed by other authors, that the concentration of sodium fluoride used would be insufficient to reduce the ATP levels. Higher fluoride concentrations cannot be used because they affect cell viability. The contradictory effects observed with both evaluated oxidative respiratory inhibitors, 2,4-dinitrophenol and sodium cyanide, are noteworthy. The inhibitory action of 2,4-dinitrophenol on the cethromycin uptake cannot be related to any effect on oxidative respiration (a minor pathway in PMNs). This phenomenon, also observed with some fluoroquinolones, may be related to secondary or minor activity of this inhibitor in other metabolic functions of PMNs. As has been described for azithromycin, the increased C/E ratio observed in the presence of sodium cyanide is related to the basic pH of a medium containing cyanide.

Particle phagocytosis or soluble activators, such as PMA, membranes may modify the uptake of an antimicrobial agent by human PMNs. For cethromycin, we observed a significant reduction in its intracellular accumulation when human PMNs were stimulated with PMA or opsonized zymosan. Phagocytosis of opsonized S. aureus could be related to the fact that bacteria and zymosan activate these kinases to varying degrees. On the other hand, the internalization of the membrane might also be involved in the decrease in cethromycin uptake. The contradictory effect observed with zymosan and bacteria could be related to the fact that zymosan induces higher membrane internalization than bacteria.

The findings suggest that the mechanisms whereby cethromycin accumulates in human PMNs are not simple. Although passive diffusion mechanisms and trapping in acidic compartments are involved in cethromycin uptake, an active mechanism of membrane transport is also presented.

Intraphagocytic activity of cethromycin was lower than that expected from the intracellular concentrations obtained, although higher than that previously observed with telithromycin. Given that both ketolides present high intracellular concentrations, these differences can be related to the intrinsic activities of both compounds. The contrast between phagocytic antimicrobial agent uptake and intracellular activity has been previously described. This phenomenon is particularly noticeable with cethromycin and telithromycin, as we have shown high C/E ratios. A possible explanation for the low intracellular activity may be related either to methodological limitations or to drug inhibition by intracellular pH. Studies of time-kill curves with cethromycin and telithromycin at 24 h demonstrated that these ketolides are predominantly bacteriostatic and only slowly bactericidal at higher concentrations.

Perhaps 3 h of incubation is too short a period to demonstrate any bactericidal activity with a bacteriostatic antimicrobial agent such as cethromycin.

In summary, cethromycin penetrates into human PMNs, reaching intracellular concentrations several times higher than extracellular ones. A potentially active mechanism may be involved in the uptake of cethromycin by the PMNs. Cethromycin showed slight intracellular activity against S. aureus at high extracellular concentrations.

Acknowledgements

We gratefully acknowledge the help of Janet Dawson in the preparation of the manuscript. This study was partially supported by Abbott Laboratories (North Chicago, IL, USA).

References


