Uptake and intracellular activity of voriconazole in human polymorphonuclear leucocytes

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Objectives: The intracellular penetration of voriconazole into human polymorphonuclear leucocytes (PMNs) and its intracellular activity against Candida spp. were evaluated.

Methods: The intracellular penetration of voriconazole into PMNs was evaluated by a radiometric assay. The effect of cell viability, environmental conditions, metabolic inhibitors and membrane stimulation was also studied. The intracellular activity was determined by incubation of PMNs containing intracellular blastospores in the presence of voriconazole for 3 h.

Results: The uptake of voriconazole by PMNs was rapid and not saturable. The cellular to extracellular concentration (C/E) ratio for voriconazole was $8.5 \pm 1.3$. Voriconazole was rapidly released from loaded PMNs. The uptake of voriconazole was not affected by environmental temperature and cell viability. Neither the external pH nor the metabolic inhibitors affected the uptake of voriconazole. The ingestion of opsonized zymosan, but not of opsonized Candida spp., significantly decreased the levels of PMN-associated voriconazole. At the extracellular concentrations evaluated, voriconazole did not affect the intracellular survival of Candida.

Conclusions: Voriconazole reached high intracellular concentrations within human PMNs. The uptake was rapid and not saturable but it did not affect the intracellular killing of Candida spp.

Keywords: Candida, azoles, accumulation

Introduction

Polymorphonuclear leucocytes (PMNs) are an important component of the host’s defence mechanisms against infection by Candida spp. The effect of antifungal agents on PMN activity could be an important factor in the treatment of candidiasis, particularly in immunosuppressed patients.1

Fluconazole has been a convenient and effective treatment for candidiasis. However, with extensive use of fluconazole, strains of Candida albicans have emerged that are resistant to fluconazole.2 In addition, some Candida spp., such as Candida krusei, are intrinsically resistant to fluconazole.3

Voriconazole, a new triazole agent, is commonly used for treatment of invasive aspergillosis; moreover this drug is also active against many fluconazole-resistant Candida spp.4 There is little information on the ability of this antifungal agent to penetrate and concentrate within PMN cells.

The purpose of this study was to evaluate the intracellular penetration of voriconazole into human PMNs. Additionally, the possible mechanism involved in the membrane transportation of this antifungal agent was also evaluated. Finally, the correlation between intracellular penetration and activity against Candida spp. within human PMNs was also studied.

Materials and methods

The uptake of radiolabelled voriconazole (50.5 µCi/mg; Pfizer Laboratories) by human PMNs was determined by a radiometric assay described by Klempner & Styrt.5 In these experiments, human PMNs were incubated in HBSS containing different concentrations of voriconazole (1–40 mg/L). After various incubation times at 37°C, cells were separated from extracellular solution by centrifugation through a water-impermeable silicone–oil barrier. A 10 µL aliquot of the extracellular medium and the entire cell pellet were counted in a liquid scintillation counter (model LS 1801; Beckman). After determination of the cell volume with radiolabelled polyethylene glycol and water, the accumulation ratio of the antifungal agent in PMNs was calculated and expressed as the cellular to extracellular concentration (C/E) ratio.

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Further studies to elucidate the mechanism of voriconazole uptake by PMNs were performed, as described previously. The influence of cell viability, environmental temperature, pH and metabolic inhibitors was evaluated.

In a series of experiments, voriconazole uptake by human PMNs was measured after stimulation of cells with phorbol myristate acetate (PMA) and after phagocytosis of either opsonized zymosan or opsonized C. albicans (strain CNMCL1017, fluconazole-susceptible; and strain CNMCL1518, fluconazole-resistant) and C. krusei ATCC 6258.

The efflux of PMN-associated voriconazole was also studied. PMNs were incubated for 20 min at 37°C with voriconazole (extracellular concentration of 2 mg/L), collected by centrifugation and rapidly resuspended in antifungal agent-free medium. Cell-associated voriconazole was quantitated at various time intervals after the removal of the extracellular antifungal agent.

The partition coefficients of voriconazole were determined by the modified method of Niaikado. To evaluate the intracellular activities of antifungal agents, a previously described method was used. C. albicans (a fluconazole-susceptible strain and a fluconazole-resistant strain) and C. krusei were used for killing assays. C. albicans strains were kindly provided by Dr M. Cuenca-Estrella, Centro Nacional de Microbiología, Madrid. Susceptibility studies were performed using the colorimetric microdilution test (Yeastone®; Trek Diagnostic Systems Ltd, UK). The MICs of voriconazole for C. albicans (fluconazole-susceptible and fluconazole-resistant) and C. krusei were 0.06, 0.12 and 1 mg/L, respectively.

Yeast suspension and PMNs were incubated for 60 min. After this, extracellular blastospores were removed by differential centrifugation. Cells were then suspended in RPMI medium. At this time, different concentrations of antifungal agents (1, 2 and 5 mg/L) were added, and the cells reincubated for 3 h at 37°C. Cells were lysed in distilled water, and samples were diluted and pour plated onto Sabouraud agar. Colonies were counted after 48 h of incubation at 37°C. The data were expressed as percentages of Candida surviving compared with levels in controls (without antifungal agent).

All data were expressed as means ± SD. Differences among groups were compared by analysis of variance, to assess statistical significance at $P<0.05$.

### Results and discussion

The intracellular penetration of voriconazole into PMNs was rapid, reaching intracellular concentrations 8.5 times higher than extracellular concentrations after 20 min of incubation. To evaluate whether voriconazole, which had been taken up by human PMNs, was tightly bound to cellular components, we evaluated the kinetics of the efflux. The elution of voriconazole from human PMNs was very fast. After 5 min of incubation in the voriconazole-free medium, the percentage of antifungal agent released was 85% (Figure 1). The uptake of voriconazole by PMNs was not saturable at extracellular concentrations in the range 1–40 mg/L. The C/E ratio ranged from 9.8 (extracellular concentration: 1 mg/L) to 9.7 (extracellular concentration: 40 mg/L). These C/E ratios of voriconazole were higher than those observed for fluconazole. The greater uptake of voriconazole by PMNs could be related to its high hydrophobicity (voriconazole is three times more liposoluble than fluconazole).

The uptake of voriconazole was not dependent on environmental temperature or cell viability and was not affected by external pH. None of the metabolic inhibitors used affected the intracellular penetration of voriconazole. All these data indicate that a passive mechanism is involved in the cell association of this agent. Particle phagocytosis or a soluble stimuli membrane may modify the uptake of antimicrobial agents by human PMNs. We observed a significant reduction in the cell association of voriconazole when human PMNs were stimulated with opsonized zymosan. However, neither the stimulation of the PMNs by PMA, nor the phagocytosis of opsonized Candida spp. affected its uptake (Table 1). The differences in voriconazole uptake observed with zymosan and microorganisms could be related to the fact that zymosan induces higher membrane internalization than Candida spp., which might decrease the uptake surface, as has been observed with other antimicrobials.

#### Table 1. Effects of several conditions on voriconazole-uptake by human PMNs

<table>
<thead>
<tr>
<th>Conditions</th>
<th>C/E</th>
</tr>
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<tbody>
<tr>
<td>Viable cells, 37°C, pH 7.2 (control)</td>
<td>8.5 ± 1.3</td>
</tr>
<tr>
<td>Viable cells, 4°C</td>
<td>9.5 ± 0.6</td>
</tr>
<tr>
<td>Dead cells, 37°C</td>
<td>9.9 ± 0.5</td>
</tr>
<tr>
<td>pH 4</td>
<td>9.5 ± 1.5</td>
</tr>
<tr>
<td>pH 5</td>
<td>8.8 ± 0.7</td>
</tr>
<tr>
<td>pH 6</td>
<td>8.7 ± 0.7</td>
</tr>
<tr>
<td>pH 8</td>
<td>9.3 ± 0.3</td>
</tr>
<tr>
<td>pH 9</td>
<td>8.5 ± 0.7</td>
</tr>
</tbody>
</table>

**Metabolic inhibitors**
- Sodium fluoride 1.5 mM 10.3 ± 0.8
- Sodium cyanide 1.5 mM 8.3 ± 0.7
- 2,4-dinitrophenol 0.1 mM 9.3 ± 1.5
- M-chlorophenylhydrazine 0.015 mM 8.2 ± 1.3

**Stimulus**
- PMA 200 nM 7.8 ± 1.9
- Opsonized zymosan 0.9 mg/L 4.6 ± 0.6
- Opsonized C. albicans (fluconazole-susceptible) 9.3 ± 1.6
- Opsonized C. albicans (fluconazole-resistant) 8.9 ± 0.6
- C. krusei 9.5 ± 0.5

$^a$Experiments (n = 4) were carried out for 20 min at an extracellular concentration of 2 mg/L.
$^b$P < 0.05 compared with the control.
At extracellular concentrations evaluated (1, 2 and 5 mg/L), voriconazole showed slight intracellular activity against C. krusei (the percentage of bacterial inhibition was 80, 76 and 80%, respectively). At these extracellular concentrations, voriconazole did not affect the intracellular survival of C. albicans (fluconazole-susceptible and fluconazole-resistant strains). Intracellular activity of voriconazole against Candida spp. seems to be very limited; it was lower than expected from the MIC values obtained and the intracellular concentration reached. A possible explanation for the low intracellular activity might be due to methodological limitations. According to studies of time–kill curves with voriconazole and fluconazole, a 3 h incubation period may be too short to demonstrate activity with a fungistatic antimicrobial agent, such as voriconazole. Nevertheless, longer incubation periods seriously compromise the viability of human PMNs.

In summary, voriconazole penetrates into human phagocytic cells, reaching intracellular concentrations several times higher than extracellular concentrations and greater than those described for other azoles. Using our assay system, the intracellular survival of Candida spp., however, is not affected by voriconazole.

References