Examination of the in vitro activity of posaconazole against clinically relevant Candida species with different methods, including time-kill curves

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Introduction

The number of the invasive and non-invasive infections caused by fungi is increasing. These infections can be serious and life-threatening especially in immunocompromised patients. The mortality rate can reach 40-80% (depending on the species) in these patient population.

From the 1990's a radical change was observed in the distribution of yeasts isolated from invasive infections. Although *Candida albicans* is still mentioned at the first place in the literature, the number of diseases caused by other non-*C. albicans* Candida species has been increasing.

We can enumerate several reasons for this increase; the number of susceptible patients has increased substantially, certain patients receive life-long treatment with broad-spectrum antibiotics, antifungal agents are extensively used for prevention in the prophylaxis, resistance can occur during the treatment with these antifungal agents. Resistance can be primary, as in case of *Candida krusei* which is intrinsically resistant to fluconazole (FLU), or secondary, like *Candida glabrata* which frequently acquires resistance to FLU. Patients undergone serious surgical interventions, or those with malignancies receiving chemotherapy are highly susceptible to the invasive infections caused by yeasts and moulds. Other well-known risk factors are low or high age. We can also mention the appearance of „new” patient groups (HIV-infected patients, etc.).

Although the numbers of the available antifungal agents increased, the mortality rate hardly changed in the past years.

From the 1990’s a new research wave has begun. This event has brought the newer triazoles (voriconazole (VOR), and posaconazole (POS)), and the introduction of the echinocandins.

These antifungal agents have broad-spectrum efficacy against pathogenic yeasts, as well as against moulds. Although triazoles are considered fungistatic, several studies reported the *in vitro* and *in vivo* fungicidal effect of VOR and POS.

I accomplished my experiments in the Medical Microbiological Institute of the University of Debrecen Medical and Health Science Centre (DE OEC). POS as a newer antifungal agent has not been studied in every aspect according to the international literature, and this fact played a role to choose my Ph.D. theme.

With this end in view, I executed POS as a new antifungal agent *in vitro* susceptibility examinations in the cases of various *Candida* species. My aim was to find a connection
between the *in vitro* susceptibility tests broth microdilution method (BMD) and Etest, as well as to investigate the *in vitro* killing activity of POS by means of determination of the minimum fungicidal concentration (MFC) and time kill curves, as described Klepser et al..
Aims

1. We would like to compare in vitro activities of the new antifungal agent POS to FLU against clinically significant Candida species by means of standard BMD.

2. We wanted to find an answer to the question whether the different solvents (dimethyl sulfoxide, recommended by CLSI in the standard BMD method versus polyethylene glycol, PEG) have an impact on the POS minimum inhibitory concentration (MIC) values.

3. Our aim was to compare the alternative susceptibility method Etest MIC values, (most widely applied method in the routine diagnostics) with the standard BMD MIC values.

4. We wished to investigate whether POS has an in vitro fungicidal effect against the clinically most significant Candida species by determining the MFC values.

5. We examined the correlation between the MFC values and time kill curves, which are the most suitable method to determine whether an antifungal agent has fungistatic or fungicidal effect.
Materials and methods

Origin of the yeasts

In our experiments we examined 209 Candida strains (32 C. albicans, 30 C. glabrata, 21 Candida tropicalis, 29 C. krusei, 28 Candida parapsilosis, 50 Candida inconspicua, 13 Candida kefyr, 5 Candida famata) isolated in the Department of Medical Microbiology between 2002 and 2005. We used three Candida lusitaniae and three Candida guilliermondii instead of Candida famata in our time kill experiments. The majority of the strains were isolated from blood samples, other strains were from respiratory, wounds, peritoneal and pleural cavity, and vaginal samples.

Identification of the yeasts

The cultures grown on Sabouraud dextrose agar (SDA) were incubated in fetal calf serum for 2 hours to detect the germ tube production.

For presumptive species identification of yeasts CHROMagar Candida (Becton Dickinson) medium was used. For further identification we used API ID32C panel.

In the case of C. inconspicua and C. parapsilosis strains molecular biological methods were used to confirm the ID32C results. These methods included PCR-ribotyping, and the detection of the SADH gene by restriction fragment length polymorphism (RFLP) method.

Broth microdilution method for susceptibility determination

The BMD method was executed as recommended by the CLSI M27-A2 reference document. Each investigation was executed at least two times.

POS (Schering-Plough Research Institute) was dissolved in 100% DMSO (standard CLSI method) or PEG (modified method). In case of FLU (Pfizer) sterile distilled water was used as solvent. The final concentration ranges for POS and FLU were 0.015-8 mg/L and 0.25-64 mg/L, respectively. The same POS plates were used for MFC determination. The uninoculated plates were stored at -20°C till usage.

For preparing the fungal suspensions 24 h SDA cultures were used. The density of the suspensions were 0.5 McFarland, and they were diluted in 0.85 % saline. RPMI 1640 was used for the dilution of the inoculum (10³, 10⁴ cfu/mL). Each plate contained a drug-free control and a medium control.
After 48 h incubation of the microdilution plates the contents of each well were homogenized by pipetting, and read visually based on the prominent inhibition criteria, i.e. the endpoint was the lowest concentration of drug that produced prominent (50%) decrease in turbidity compared to the drug-free control.

*C. krusei* isolates were not tested against FLU, because it is innately resistant to this antifungal agent. In the case of *C. inconspicua* the FLU susceptibility test was executed only with the 2 newest strains, and in the case of the other 48 isolates our preliminary MIC results were used.

**Etest for susceptibility determination**

Etest was carried out using RPMI-1640 agar supplemented with 2% glucose (following the manufacturer’s recommendation). The 0.5 McFarland suspensions grown on SDA for 24 h were diluted by 0.85% saline. Plates were incubated at 35 °C for 48 h, and read after 24 and 48 h.

**Determination of minimum fungicidal concentration (MFC) of posaconazole**

For the determination of MFC of POS the method described by Cantón et al. was used. All tests were run in duplicates and repeated twice.

The inoculum was $10^4$ cfu/mL which was confirmed by serial dilutions on SDA.

The trays were incubated at 35 °C for 48 h, and after determination the 48 h MIC values, the entire contents (200 µl) of the visually totally clear wells were homogenized by pipetting. Aliquots were placed as a single spot on SDA plates (100 µl/plate) and after drying, the cells were dispersed by streaking. Plates were incubated at 35 °C for 48 h.

The MFC was defined as the lowest drug concentration that resulted in a 99.9% ($\leq 3$ colonies) reduction in the starting inoculum.

**Time-kill studies**

In time-kill experiments the method described by Klepser et al. was used. All tests were repeated at least twice.

The starting inoculum was $10^5$ cfu/mL, and the final concentration range for POS was 0.5-16×MIC (0.015-16 mg/L). *C. parapsilosis* strains were also tested at concentrations 32 and 64 times the MIC.

Test tubes were incubated with agitation in the dark at 35 °C. At predetermined time points, samples (100 µl) were removed and serially diluted 10-fold in sterile saline. Four 30 µl
aliquots were subsequently plated onto SDA (if colony counts suspected to be <1000 cfu/mL, undiluted samples were plated). On the basis of our preliminary results with *C. albicans* ATCC 14053 and *C. krusei* ATCC 6258 strains, the following sampling points were chosen: 0, 6, 18, 24, 36 and 48 h. The plates were let to dry at room temperature. Colony counts were determined after incubation of the plates at 35 ºC for 48 h. Time-kill curves were prepared using the computer curve-fitting software GraphPad Prism 4.03 for Windows.

POS was defined fungicidal if there was a 99.9 % reduction in viable cfu/mL of the starting inoculum. If the reduction in the starting inoculum was lower than this, the efficacy of POS was defined as fungistatic.

**Interpretation of the results**

Read of the MIC values was executed by 2 experts and the author.

The CLSI approved quality control strains (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) were used in each test.

The following breakpoints for FLU were used: susceptible ≤8 mg/L, dose-dependent susceptible 16-32 mg/L, resistant >32 mg/L.

Established interpretive criteria for POS are not available.

MIC values by the different methods were compared to the standard MIC values. Discrepancies of no more than ±1 dilution between MICs were defined as agreement.

In the case of Etest, MIC values that fell between the two-fold dilutions used in BMD were increased to the next two-fold level of the BMD for comparison.
Results

Quality control strains
POS MICs of the CLSI approved quality control strains were always within the control limits (C. parapsilosis ATCC 22019: 0.06-0.25 mg/L and C. krusei ATCC 6258: 0.12-0.5 mg/L).

Posaconazole and fluconazole susceptibility using BMD

C. inconspicua FLU MIC values varied between 16-128 mg/L, that is all tested isolates fell into the dose-dependent susceptible and resistant categories. C. albicans, C. tropicalis, C. parapsilosis, C. kefyr, C. famata isolates were generally susceptible to FLU (MIC₉₀: 0.12-1 mg/L). Two out of the 32 C. albicans, and three out of the 30 C. glabrata isolates exhibited FLU MIC values that fell into the resistant category (MIC >32 mg/L). A single C. glabrata isolate MIC value was 16 mg/L (dose-dependent susceptible). All the other C. albicans and C. glabrata isolates were susceptible.

The geometric mean of POS MICs of the eight tested Candida strains varied between 0.04-0.71 mg/L in our experiments (Table1.). C. albicans, C. tropicalis, C. parapsilosis, C. kefyr, C. famata isolates showed the lowest MIC values. Only one of the isolates out of the two C. albicans isolates resistant to FLU had high POS MIC value (MIC>8 mg/L), the other isolate showed 0.12 mg/L POS MIC. Similarly, out of the three C. glabrata isolates resistant to FLU two isolates showed elevated POS MIC values (MIC > 8mg/L), the third isolate had POS MIC value of 1 mg/L.

In case of C. krusei and C. inconspicua isolates the geometric mean of POS MIC values was 0.22 mg/L and 0.21 mg/L, respectively.

Results of Etest

All Candida isolates grew well after 24 h, all plates could be easily evaluated.

Etest results read after 24 h incubation, with the exception C. tropicalis, C. kefyr, C. famata isolates, showed good correlation with the standard BMD method (≥ 86 %).

The single C. albicans strain that had a BMD MIC of > 8 mg/l exhibited an Etest MIC of 0.03 mg/L at 24 h and 8 mg/L at 48 h.

The two C. glabrata strains with elevated POS MICs by BMD showed elevated MICs (> 8 mg/L) by Etest at 24 h.
After 48 h incubation, the geometric mean of MIC values were elevated. This was especially observable in case of *C. glabrata* isolates; they showed almost four times higher geometric means. The correlation with the standard method was poor within ± 1 dilution (16.7 %).

**Effect of solvents**

The effect of using PEG instead of DMSO as a solvent for POS had only a minor impact on MIC values, with the exception of *C. parapsilosis* (85.7 %), the overall agreement within ± 1 dilution was 97-100 %. The MIC values obtained using PEG for *C. glabrata* and *C. kefyr* were generally higher.

Other species showed similar MIC values using the two solvents.

**Results of the minimum fungicidal concentration**

MFC determinations were performed using 10-fold higher inoculum, $10^4$ cfu/mL. The MICs obtained using the higher inoculum were the same as, or one dilution higher than, those obtained using the standard CLSI inoculum ($10^3$ cfu/mL).

POS was fungicidal at low concentrations ($\leq 2$ mg/l) against all *C. inconspicua* and *C. lusitaniae* isolates and against the majority of *C. krusei* (24/29, 83 %), *C. parapsilosis* (20/28, 71 %) and *C. kefyr* (9/13, 69 %) isolates.

In contrast, the drug was uniformly fungistatic against all *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. guilliermondii* isolates tested.

**Time-kill study results**

The MIC and MFC values of the tested isolates and ATCC quality strains are shown in Table 1.

**Table 1.: Candida species examined in time-kill experiments**

<table>
<thead>
<tr>
<th>Tested isolates (number)</th>
<th>MIC range of the tested isolates (mg/L)$^1$</th>
<th>MFC range of the tested isolates (mg/L)$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em> (4)</td>
<td>0.03-&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td><em>C. glabrata</em> (3)</td>
<td>0.5-&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td><em>C. tropicalis</em> (2)</td>
<td>0.06-0.25</td>
<td>&gt;8</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> (12)</td>
<td>0.06-0.12</td>
<td>0.5-&gt;8</td>
</tr>
<tr>
<td><em>C. krusei</em> (9)</td>
<td>0.25</td>
<td>0.5-8</td>
</tr>
<tr>
<td><em>C. inconspicua</em> (4)</td>
<td>0.12-0.25</td>
<td>0.5-1</td>
</tr>
<tr>
<td><em>C. lusitaniae</em> (3)</td>
<td>0.06</td>
<td>2</td>
</tr>
<tr>
<td><em>C. guilliermondii</em> (3)</td>
<td>0.25</td>
<td>&gt;8</td>
</tr>
<tr>
<td><em>C. kefyr</em> (3)</td>
<td>0.06</td>
<td>0.5-8</td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC 14053</td>
<td>0.12</td>
<td>&gt;8</td>
</tr>
<tr>
<td><em>C. tropicalis</em> ATCC 750</td>
<td>0.5</td>
<td>&gt;8</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> ATCC 22019</td>
<td>0.12</td>
<td>&gt;8</td>
</tr>
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</table>
Out of the clinically significant *Candida* species POS showed fungicidal effect against *C. krusei*, *C. lusitaniae*, *C. kefyr*, and *C. inconspicua* clinical isolates.

The single *C. krusei* strain exhibiting a fungicidal effect within 24 h was the ATCC 6258 strain, at the drug concentration 4 mg/L. Though in case of a single clinical isolate POS showed to be fungicidal at 1 mg/L after 36 h, the best fungicidal effect was observed after 48 h. POS exhibited fungicidal activity against seven out of nine clinical isolates at 1 mg/L. The remaining 2 isolates exhibited 99.9 % reduction in cfus at POS concentration of 2 mg/L.

In case of *C. lusitaniae* POS exhibited fungicidal effect at the concentration 0.25 mg/L after 48 h. Two isolates showed 99.9 % reduction in cfus at concentration 1 mg/L after 24 h, and this fungicidal effect could be observed after 36 h in case of all three isolates.

POS proved to be fungicidal at the concentration of 1 mg/L after 24 h in case of two *C. kefyr* isolates. These strains showed at least 99.9 % reduction in the starting inoculum at concentration 0.12 mg/L after 36 h. POS showed fungistatic effect in case of the third *C. kefyr* isolate, in correlation with the MFC value.

Three *C. inconspicua* clinical isolates, similarly to the ATCC quality control strain, showed 99.9 % reduction in the starting inoculum at POS concentration 1 mg/L after 24 h (fungicidal effect). After 36 h POS exhibited fungicidal effect at concentration 0.06 mg/L against these isolates. POS showed fungicidal effect at concentrations 0.25-2 mg/L after 48 h against a single isolate.

Against the *C. norvegensis* ATCC 22977 strain, POS displayed good fungicidal activity after 36 h at concentrations 1-16-fold higher than the MIC.

Confirming the obtained MFC values, POS exhibited fungistatic effect in case of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. guilliermondii*. *C. albicans*, *C. tropicalis*, *C. guilliermondii* isolates were strongly inhibited at drug concentrations ranging from 2-16-fold higher than the respective MICs. The time-kill curve of the *C. albicans* isolate exhibiting high (> 8 mg/L) POS MIC was similar to that of the control at concentrations 16-32 mg/L, that is no inhibition was observed.

POS had only a minor inhibitory impact on *C. glabrata* isolates; growth could be observed at all tested POS concentrations. The best inhibitory effect could be observed at 4-8-

<table>
<thead>
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<th>MIC (mg/L)</th>
<th>FMC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. krusei</em> ATCC 6258</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td><em>C. inconspicua</em> ATCC 16783</td>
<td>0.12</td>
<td>0.5</td>
</tr>
<tr>
<td><em>C. guilliermondii</em> ATCC 6260</td>
<td>0.25</td>
<td>&gt;8</td>
</tr>
<tr>
<td><em>C. norvegensis</em> ATCC 22977</td>
<td>0.12</td>
<td>0.25</td>
</tr>
</tbody>
</table>

1 Determination of MIC/MFC values based on CLSI M27-A document with the starting inoculum $10^4$ cfu/mL.
fold MIC values. The time-kill curve of the *C. glabrata* isolate exhibiting high (> 8 mg/L) POS MIC was similar to that of the control at concentrations 16-32 mg/L.

Although, on the basis of the MFC values (0.5-8 mg/L), POS was fungicidal against the majority of the *C. parapsilosis* isolates, the time-kill curves did not confirm it in any cases. POS exhibited fungistatic effect at 0.5×MIC against all isolates tested, and after 48 h the drug reduced the viable cfu in the starting inoculum at 1-16×MIC.
Discussion, conclusions

In my Ph.D. theses I examined the efficacy of POS, the newest marketed triazole antifungal agent against *Candida* clinical isolates, including the most frequent *Candida* species. Among the tested isolates, *C. krusei* and *C. inconspicua* showed reduced susceptibility against FLU, the most widely used antifungal agent.

Correlating with international data, POS exhibited excellent *in vitro* activity against *Candida* species isolated from clinical samples. The highest MIC\textsubscript{90} value was obtained in the case of *C. glabrata*, but that these data are notably lower than those obtained by Pfaller et al. (MIC\textsubscript{90} 4 mg/L).

We demonstrated that FLU resistant *C. albicans* and *C. glabrata* isolates do not show reduced susceptibility to POS in all cases. This phenomenon can be explained by the fact that the mechanisms of resistance to these two antifungal agents are different. Accordingly, the efficacy of POS is less influenced by the mutations of the lanosterole-demethylase gene, and, in contrast to FLU, POS does not seem to be a good substrate of the main efflux pumps.

On the basis of our results, POS can inhibit the growth of *C. krusei* and *C. inconspicua* even at low concentrations. These results also differ from those obtained by Pfaller et al., but they examined only three *C. inconspicua* isolates in their experiments, and the POS MIC value was > 8 mg/L in one single case out of the three. The authors assumed that probably there is a cross resistance between POS and FLU. Analysing our results suggest that this cross resistance is rather an exception, and not a common phenomenon.

Our results also showed that the solvent (DMSO vs. PEG) used for dissolving of the antifungal agent generally had only a minor impact on MIC values; with the exception of *C. parapsilosis*, the overall agreement was 97-100 % in case of the tested isolates. These data are similar to those obtained by Ostrosky-Zeichner. For better comparison of the POS MIC values obtained by the different authors, we recommend using DMSO (as described in the standard method) instead of PEG in the macro-, and microdilution experiments.

In our study we extended the Etest database with numerous *C. inconspicua* isolates. POS MIC values with Etest read after 24 h showed excellent correlation with the standard method, confirming that Etest is an excellent method for MIC determination. Consequently, POS Etest can safely be used in the routine laboratory work.

Correlating with the preliminary data concerning triazoles, POS showed to be fungistatic against clinically relevant *Candida* species, such as *C. albicans*, *C. glabrata*, *C.
tropicalis, C. parapsilosis, C. guilliermondii. The fungistatic efficacy was observed at low POS concentrations, with the exception of C. glabrata. In case of C. glabrata the fungistatic activity was poor, no reduction was observed in the starting inoculum in case of the examined isolates at any tested concentrations. Only the 48 h Etest MIC results refer to the low fungistatic activity from the different methods. Although there is only a minor agreement between the 48 h Etest MIC results and the BMD MIC results, we cannot exclude the clinical relevance of the 48 h Etest MIC values in case of C. glabrata.

POS showed concentration-, and mainly time-dependent fungicidal activity against all tested C. krusei, C. inconspicua, C. lusitaniae isolates as well as against the two C. kefyr isolates exhibiting low MFC values. Fungicidal effect of POS could be observed after 24 h at 1 mg/L concentration in case of two C. lusitaniae and two C. kefyr, and four C. inconspicua strains (including the quality control strains). It is noteworthy that 1 mg/L POS concentration can be easily reached in blood, so these results can have therapeutical significance.

The fungicidal activity of POS at 1 mg/L could be observed after 48 h in case of the majority of C. krusei isolates and, in case of one single C. inconspicua isolate. The fungicidal effect could be observed only at 2 mg/L POS concentration in case of two C. krusei clinical isolates. Since C. krusei and C. inconspicua show reduced susceptibility to FLU and, VOR exhibits only poor fungicidal effect against these two species, POS can be a real therapeutic alternative in the treatment of infections caused by C. krusei and C. inconspicua.

Our MFC results showed excellent correlation with the time-kill study results (with the exception of C. parapsilosis isolates). Since MFC determination is much easier to perform, MFC can be used to determine whether POS has fungistatic or fungicidal effect. Although out of 28 tested C. parapsilosis isolates 22 isolates had MFC values ≤ 2 mg/L, the time-kill curves did not confirm fungicidal activity in any cases, despite the fact that we executed the experiments even at 32-64×MIC. The explanation for this observation is unknown and requires further studies. POS probably has long-life post-antifungal effect against C. parapsilosis strains, therefore it is possible that changing the incubation time from 48 h to 72 h could help to detect the inhibited, but viable C. parapsilosis cells.

On the basis of our results, POS has excellent in vitro activity against the clinically significant yeasts, considering the MIC values and the time-kill results. POS proved to be fungicidal against numerous clinically significant yeasts, but the investigation of its possible in vivo therapeutical effect is a task for the future. Although POS showed as good therapeutical efficacy as FLU in the treatment of oral-, and oesophageal candidiasis, there are no data available in the literature about the treatment of patients with candidemia, or
neutropenia. These investigations can be limited by the fact that POS has no parenteral formulation, and the rapid absorption can be reached only by consuming fatty food. POS balanced concentration in blood is 689-817 ng/mL (range: 0-3710 ng/mL) with 800 mg daily POS treatment. These data are based on a survey executed with 194 patients with invasive candidiasis. Moreover, POS binding to proteins in the serum is higher than 90 %, reducing the free, biologically active POS concentration. These facts strongly set a limit to the applicability of POS in the treatment of severe invasive infections, while POS seems to be eligible in prophylaxis in the case of bone-marrow recipient patients.

In conclusion, the development of the intravenous formula of POS is the urgent task in the future, in order to avoid the disappearance of this excellent antifungal agent from the antifungal therapy.
Summary of the results

1. Posaconazole exhibited excellent in vitro activity (irrespectively of solvent) against the clinically significant Candida species, including species which show reduced susceptibility to FLU.

2. For the better comparison of POS MIC values we recommend dimethyl sulfoxide instead of polyethylene glycol in the macro-, and microdilution experiments.

3. The MIC values obtained by broth microdilution method showed good correlation with the Etest MIC values, thus Etest can be applied safely in the routine laboratory work.

4. On the basis of the MFC values, POS showed fungicidal effect against numerous Candida species (C. inconspicua, C. lusitaniae, C. krusei, C. parapsilosis, C. kefyr), while it exhibited fungistatic effect against C. albicans, C. glabrata, C. tropicalis, C. guilliermondii.

5. The time-kill curves fully confirmed the MFC values, with the exception of C. parapsilosis.
Publications used in thesis


Other publications

1. Varga I., G. Sóczó, G. Kardos, Á. Kemény-Beke, B. Kelentey, I. Márton, L. Majoros. Differences in killing activity of caspofungin and paradoxical growth between *C. albicans* and *C. krusei* clinical isolates in different media. Journal of Chemotherapy. 2008. (accepted for publication). **IF:** 0,922


**Total IF:** 18,753