In vitro profiling of pramiconazole and in vivo evaluation in Microsporum canis dermatitis and Candida albicans vaginitis laboratory models

In vitro and in vivo evaluation of pramiconazole

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The triazole antifungal pramiconazole (Stiefel, a GSK company) was compared with itraconazole, miconazole and terbinafine in vitro and in vivo. A potent in vitro activity against Candida spp. (IC$_{50}$ 0.04-1.83 µM) and Microsporum and Trichophyton spp. (IC$_{50}$ 0.15-1.34 µM) was obtained, however, not against other filamentous moulds and zygomycetes. In the M. canis guinea pig model and C. albicans vulvovaginitis rat model, pramiconazole was superior to the reference compounds after oral and topical administration.
Although considerable research is invested in finding novel strategies for the treatment of fatal invasive mycoses (6), non-fatal superficial mycoses believed to infect about 25% of the world population should not be overlooked (1). The most widespread dermatomycoses are caused by Trichophyton, Epidermophyton and Microsporum species. Treatment is oral or topical with the allylamine terbinafine or any of the azoles (4, 13). Yeasts also cause superficial infections of skin and mucous membranes, whereby vulvovaginal candidiasis (VVC) affects at least 75% of all women at least once in their life (15, 21). Standard therapy involves intravaginal application of clotrimazole or miconazole or oral treatment with fluconazole or itraconazole (15). Although current treatment options may suffice, new antifungals would still be acceptable to improve treatment compliance or reduce adverse effects and drug-interactions. The triazole pramiconazole shows good in vitro and clinical activity against dermatophytes and yeasts (12, 16, 17) and Malassezia infections (10, 11, 19). Laboratory data always refer to oral treatment of mice and guinea pigs (16, 17), however, no data on topical application are available. No data have yet been published on pramiconazole in VVC in comparison with reference drugs, although it is in clinical development for these indications (8, 9). The specific aims of this laboratory study were: 1/ to perform an in vitro profiling of pramiconazole and 2/ to evaluate oral and topical treatment schemes against M. canis in guinea pigs and C. albicans VVC in rats.

Miconazole (MC), itraconazole (ITC) and terbinafine (TRB) were purchased from Sigma, while pramiconazole (PRC) was provided by Stiefel-GSK. The fungal isolates were
obtained from the Scientific Institute of Public Health (IHEM, Brussels) and cultivated on Sabouraud Dextrose Agar (SDA, Oxoid). For all species, a stock of $5 \times 10^6$ colony forming units (cfu)/ml was prepared in RPMI-MOPS medium with 10% glycerol and stored in liquid nitrogen for later use in all *in vitro* tests. Fresh inocula were used for animal infections. The *in vitro* susceptibility screens were performed as previously described (7). Briefly, 10 µl of pre-diluted compound solution was spotted onto 96-well plates (U-bottom, Greiner Bio-One) with 64 µM as highest concentration; $10^3$ cfu in 200 µl RPMI-MOPS were added to each well. After incubation, growth inhibition was measured after adding 10 µl/well of 0.005% (w/v) resazurin (Sigma) allowing fluorimetric reading ($\lambda_{ex}$ 550nm - $\lambda_{em}$ 590nm) (23). Activity is expressed as IC$_{50}$, *i.e.* the concentration that inhibits growth for 50% compared to non-treated controls. Cytotoxicity was simultaneously tested on human lung fibroblasts (MRC-5$_{SV40}$) (Invitrogen). Five independent replicates were performed for each observation.

The *in vitro* IC$_{50}$ values for reference drugs were comparable to the ranges in literature (2, 5, 20) and available data on pramiconazole were also confirmed (16, 17) (Table 1). TRB performs marginally better against dermatophytes. Except for *T. quinckeanum*, PRC activity remained below 0.5 µM. Against *Candida spp.*, activities remained below 1 µM, except for *C. albicans* B2630. PRC failed to show activity (IC$_{50}$ >64 µM) against the other filamentous moulds and zygomycetes (data not shown).

All animal experiments were approved by the Ethical Commission of the University of Antwerp (2008/015). Compounds were formulated in polyethylene glycol 200 (PEG$_{200}$)
for oral (PO) dosing and in PEG<sub>400-1500</sub> (3:2 w/w) for topical (TP) administration. Each treatment was evaluated in 6 animals divided over 2 independent experiments. Group averages of lesion scores (LS) or intravaginal burdens were used to plot graphs and the area under the infection curve (AUC) was calculated for each animal as a measure for infection burden. An unpaired t-test (two-tailed, p ≤ 0.05) was used to determine levels of significance between the different experimental groups.

The dorsum of female guinea-pigs was shaved and scarified with a steel brush. An inoculum (\textit{M. canis} B68128) of $10^6$ cfu in 100 µl was applied to the scarified skin. Oral dosing at 10 mg/kg started about 2 hours before infection and was continued for 5 days. Topical treatment using a 1% formulation was applied twice daily for 4 days starting on the morning after infection. Skin lesions were evaluated every 3-4 days (Fig. 1). Lesion scoring systems as found in literature (18, 24) were slightly modified to include both lesion size and severity. Upon oral administration at 10 mg/kg (Fig 1A), PRC performed much better than ITC and TRB with complete suppression of lesion development, which contrasts with the \textit{in vitro} data where TRB was better than PRC (p = 0.004) and ITC (p = 0.005). The latter can be explained by the better pharmacokinetic properties of PRC (Table 2), the lower protein-binding (17) and higher metabolic stability (3). After TP application, PRC was better than TRB (p = 0.041), but no difference was observed between PRC and ITC (Fig 1B).

For VVC, female rats were ovariectomized 3 weeks before infection and estrus was induced with 1 mg oestradiol-benzoate + 200 µg progesterone on days -3, 2 and 7. Rats were infected intravaginally with $10^7$ cfu \textit{C. albicans} B2630. Treatment schedules were
identical to the guinea pig model. At days 4, 9 and 14 after the infection, vaginal swabs were taken to estimate *Candida* burdens. Oral PRC and ITC at 10 mg/kg were both highly effective, but not significantly different. At 5 mg/kg, PRC outperformed ITC (*p* = 0.021) (Fig. 2A). After intravaginal application, superiority of PRC over ITC and MC was significant (Fig. 2B).

In conclusion, pramiconazole has potent *in vitro* anti-dermatophyte and anti-yeast activity comparable to current reference drugs. In dermatomycosis and VVC animal models, oral pramiconazole performs better than itraconazole and terbinafine and shows a higher intrinsic *in vivo* efficacy, as also demonstrated after topical application. Our findings support the potential of pramiconazole as a promising candidate for treatment of topical mycoses.

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References


**Table 1** Cytotoxicity (CC$_{50}$) and activity (IC$_{50}$) against 4 dermatophyte and 4 *Candida* species. The IC$_{50}$ is the concentration in µM at which growth is inhibited for 50% compared to untreated controls, CC$_{50}$ is the concentration at which 50% of the MRC-5 cells are killed. Averages of 5 independent repeats are expressed together with the standard deviation (SD).

<table>
<thead>
<tr>
<th>Fungal isolates</th>
<th>TRB CC$_{50}$ ± SD</th>
<th>MC IC$_{50}$ ± SD</th>
<th>ITC</th>
<th>PRC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. canis</em></td>
<td>63.00 ± 1.73</td>
<td>29.67 ± 13.32</td>
<td>49.33 ± 14.50</td>
<td>53.33 ± 18.48</td>
</tr>
<tr>
<td><em>T. mentagrophytes</em></td>
<td>B70554</td>
<td>0.06 ± 0.04</td>
<td>0.40 ± 0.28</td>
<td>0.37 ± 0.39</td>
</tr>
<tr>
<td><em>T. rubrum</em></td>
<td>B68183</td>
<td>0.07 ± 0.05</td>
<td>0.33 ± 0.26</td>
<td>0.56 ± 0.48</td>
</tr>
<tr>
<td><em>T. rubrum</em></td>
<td>J941704</td>
<td>0.03 ± 0.02</td>
<td>0.14 ± 0.09</td>
<td>0.98 ± 1.31</td>
</tr>
<tr>
<td><em>T. quinckeanaum</em></td>
<td>B68683</td>
<td>0.01 ± 0.01</td>
<td>0.79 ± 0.49</td>
<td>2.93 ± 2.91</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>B59163</td>
<td>3.57 ± 1.59</td>
<td>0.30 ± 0.22</td>
<td>1.41 ± 1.20</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>B2630</td>
<td>64.00 ± 0.00</td>
<td>2.50 ± 2.08</td>
<td>1.39 ± 1.86</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>B63155</td>
<td>30.66 ± 23.68</td>
<td>0.12 ± 0.06</td>
<td>3.74 ± 4.63</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>B46120</td>
<td>6.33 ± 4.80</td>
<td>0.03 ± 0.03</td>
<td>0.40 ± 0.35</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>B68404</td>
<td>64.00 ± 0.00</td>
<td>1.40 ± 0.57</td>
<td>4.21 ± 4.90</td>
</tr>
</tbody>
</table>

*Conversion factor to be used for the IC$_{50}$ values in µg/ml: TRB x 0.33; MC x 0.42; ITC x 0.70; PRC x 0.66*
Table 2 Pharmacokinetics (PK) after oral administration of pramiconazole, itraconazole and terbinafine to guinea pigs (literature and unpublished data)

<table>
<thead>
<tr>
<th>PK parameter</th>
<th>PRC *</th>
<th>ITC b</th>
<th>TRB c</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
<td>0.18</td>
<td>0.35</td>
<td>0.06</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>240</td>
<td>120</td>
<td>42</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>23</td>
<td>13.9</td>
<td>6.6</td>
</tr>
</tbody>
</table>

* plasma concentrations were normalized to a dose of 10 mg/kg

* unpublished data (Janssen Pharmaceutica) 40 mg/kg

* Sobue et al. (2004) 20 mg/kg (ref. 22)

* unpublished data (Janssen Pharmaceutica) 10 mg/kg
Figure 1 Comparative efficacy of pramiconazole, terbinafine and itraconazole after oral dosing (s.i.d.) at 10 mg/kg (1A) and after topical treatment (b.i.d.) with 1% (w/w) cream (1B) against *M. canis* in guinea pigs. The scores assigned to the animals are shown in the Y-axis, the X-axis represents the days after infection.

Legend: VIC = vehicle treated control group; DPI = days post infection; s.i.d. = once daily; b.i.d. = twice daily
Figure 2 Comparative efficacy of pramiconazole (PRC) and itraconazole (ITC) after oral treatment (s.i.d.) at 10 and 5 mg/kg (2A) and comparison with itraconazole (ITC) and miconazole (MC) after topical treatment (b.i.d.) with 1% (w/w) cream (2B) in the C. albicans vaginitis model in rats. The AUC representing the entire infection burden over the 3 days of sampling is shown in the Y-axis. The different groups are shown on the X-axis.

Legend: VIC = vehicle treated control group; PRC = pramiconazole, ITC = itraconazole, MC = miconazole

* p = 0.01-0.05 (two-tailed t-test), *** p < 0.001 (two-tailed t-test)

s.i.d. = once daily; b.i.d. = twice daily