Pyrrolo[1,2-\(\alpha\)][1,4]benzodiazepines show potent in vitro antifungal activity and significant in vivo efficacy in a Microsporum canis dermatitis model in guinea pigs

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Background: Pyrrolo[1,2-\(\alpha\)][1,4]benzodiazepines (PBDs) have been described as a novel class of antifungal compounds with activity against dermatophytes and Aspergillus fumigatus. The initial structure–activity relationship showed that compounds with a chlorine substitution at position 7 have a higher activity compared with regioisomers or other substituents.

Methods: The present study evaluated more analogues with a 7-chlorine-substitution in vitro against a broad panel of clinically relevant fungal species. The Microsporum canis model in guinea pigs was used to assess the in vivo efficacy after oral and topical administration.

Results: IC\(50\) values in the low micromolar range (IC\(50\) 0.6–8.0 \(\mu\)M for dihydro-PBDs; 0.1–0.7 \(\mu\)M for oxidized PBDs) confirmed the potent and selective in vitro activity of PBDs against dermatophytes, while the activity against A. fumigatus and Candida parapsilosis was slightly lower. For dihydro-PBDs, para-substitution showed superior activity, while oxidized compounds with a meta-substitution performed best. Oxidized Compound O with meta-CF\(_2\)CH\(_3\)-substitution showed excellent IC\(50\) values of 0.6 \(\mu\)M against M. canis, 2.0 \(\mu\)M against Trichophyton mentagrophytes and 0.7 \(\mu\)M against Trichophyton rubrum, matching or outperforming the activity of itraconazole (IC\(50\) values of 2.0, 0.4 and 0.6 \(\mu\)M, respectively). In vivo, topical application of a 0.25% formulation of Compound O gave a lesion reduction of 90% compared with placebo-treated animals. Oral administration of this compound at 20 mg/kg showed superior therapeutic efficacy compared with the reference drug itraconazole.

Conclusions: In conclusion, PBDs with a chlorine atom at position 7 are very promising antifungal candidates with convincing in vitro and in vivo activity particularly against dermatophytes and should be studied in greater detail to explore their full potential in the treatment of dermatophytoses.

Keywords: antifungal profiling, lead discovery, dermatophytes

Introduction

Invasive fungal diseases, such as systemic aspergillosis, are associated with high morbidity and mortality and show a rising incidence that coincides with the increase in immunocompromised patients.\(^1\) Also, non-fatal cutaneous fungal infections occur much more frequently and affect up to a quarter of the world population.\(^2\) The growing importance of fungal diseases and the limitations of the existing drugs, i.e. the emergence of resistance, limited efficacy, drug–drug interactions and severe adverse effects, clearly highlight the need for new, safe and effective antifungals.\(^3\) Pyrrolo[1,2-\(\alpha\)][1,4]benzodiazepines (PBDs) represent a novel class of compounds that showed promising activity against dermatophytes and Aspergillus fumigatus.\(^4\) The initial structure–activity relationship (SAR) indicated that the compounds with a chlorine atom at position 7 performed better than their regioisomers with a chlorine atom at position 8, 9 or 10. Other substituents at position 7 (hydrogen, fluorine, methyl, thiomethyl and methoxy) were much less potent. Oxidation of the nitrogen atom in the diazepine ring enhanced the in vitro activity.

The present study further explored the SAR of PBDs with a chlorine atom at position 7 in a broad in vitro panel of clinically relevant dermatophytes, filamentous moulds and yeasts. The in
vivo efficacy after oral and topical administration was assessed in a *Microsporum canis* dermatophytosis model in guinea pigs.

### Materials and methods

The compounds were synthesized and kindly supplied by Janssen, Beerse, Belgium. The *in vitro* susceptibility was profiled against a broad range of fungal species in accordance with the CLSI standard guidelines M27-A3 and M38-A2.5.6 To assess selectivity of inhibition, cytotoxicity was evaluated on human lung fibroblasts (MRC-5 SV 40).1 Activity is expressed as the IC₅₀ value in µM, i.e. the concentration that results in a 50% growth inhibition compared with the untreated controls on the same test plate. For each compound–fungus combination, three independent replicates were analysed. The stock concentration of all compounds was 20 mM in DMSO. The final in-test concentration of DMSO was 0.5%. The oxidized PBDs were analysed. The stock concentration of all compounds was 20 mM in DMSO. The final in-test concentration of DMSO was 0.5%. The in *in vivo* efficacy of the oxidized PBDs was evaluated in an *M. canis* dermatitis model in guinea pigs.7 Oral treatment started 2 h before infection and was continued for 5 consecutive days at 40 or 20 mg/kg. Topical treatment (1%, 0.25% or 0.06% formulations) started the day after infection and was applied twice daily for 4 days. Compounds were formulated in polyethylene glycol (PEG200) for oral dosing and in a mixture of PEG400 and PEG1500 (3:2 w: w) for topical treatment. Within each experiment, a vehicle-treated infected control group was included and itraconazole (oral administration of 20, 10, 5 and 2.5 mg/kg; topical administration of 1%, 0.25% and 0.06%) and terbinafine (oral administration of 10 mg/kg; topical administration of 1%) were included as reference antifungal controls. Each treatment was evaluated in three animals. Skin lesions were scored every 3 days for 3 weeks. The lesion scores were plotted over time and the maximal lesion score (MLS) and area under the curve (AUC) were determined as measures for lesion burden using GraphPad Prism version 4.01 (San Diego, CA, USA). The AUC and MLS of each treatment were expressed as percentages of the AUC or MLS obtained in the vehicle-treated infected control group. One-way ANOVA was used to identify significant activity (P≤0.05). All experiments with animals were approved by the Animal Trials Ethics Committee of the University of Antwerp (Id. 2008–14) and conducted in agreement with ethical standards.

### Results and discussion

Table S1 (available as Supplementary data at JAC Online) shows the chemical structure of the PBDs and their *in vitro* activity profile against dermatophytes, filamentous moulds and yeasts. Several reference antifungals were included and showed IC₅₀ values in agreement with those found in the literature.8 The dihydro-PBDs are designated A–L, whereas M–P represent the oxidized PBDs. Overall, PBDs showed good *in vitro* activity (IC₅₀ <10 µM) against at least one of the fungal groups. All compounds were particularly active against dermatophytes, with IC₅₀ values in the low micromolar range. For example, Compound O showed IC₅₀ values of 0.6 µM against *M. canis*, 2.0 µM against *Trichophyton mentagrophytes* and 0.7 µM against *Trichophyton rubrum*, approaching or outperforming the activity of reference drug itraconazole against these dermatophytes (IC₅₀ values of 2.0, 0.4 and 0.6 µM, respectively). The potency against filamentous moulds was mainly seen for oxidized PBDs, which is in accordance with the initial SAR.6 In particular, compounds with a CF₂CH₃-substitution showed activity against the *Scedosporium* species. The metra-substitutions of the oxidized PBDs appeared to be the most potent forms, while the para-substitutions were more active for the dihydro-PBDs. *Candida parapsilosis* proved to be the sole susceptible yeast species. No activity could be detected against other *Candida* spp., *Fusarium* spp., *Rhizomucor* spp., *Rhizopus* spp. and *Sporothrix schenkii*. Our results endorse and expand the previously reported PBD activity against the dermatophytes *M. canis* and *Trichophyton* spp., *A. fumigatus* and *C. parapsilosis*. None of the compounds showed significant cytotoxicity against MRC-5 cells and hence can be considered as highly selective.

Because of the promising *in vitro* activity against *M. canis* (Table S1), the four oxidized PBDs (Compounds M, N, O and P) were dose-titrated in the guinea pig *M. canis* dermatitis model (Table 1). In untreated controls, skin lesions develop by day 4, reach maximal severity by day 11 and then spontaneous healing starts. Almost full recovery is obtained by day 25 (Figure 1 and Tables S2 and S3 available as Supplementary data at JAC Online). None of the compounds showed adverse effects in the guinea pigs within the tested dose range. After oral administration of 40 mg/kg, all compounds significantly reduced the lesion burden and Compound O led to nearly complete lesion suppression (P≤0.05). Even at 20 mg/kg, Compound O demonstrated superior oral activity compared with reference drugs itraconazole (at 20 mg/kg) and terbinafine (at 10 mg/kg). Topical application of Compound O in a 0.25% formulation showed excellent lesion reduction of >90% compared with the vehicle-treated infected

**Table 1. In vivo efficacy of oxidized PBDs after oral treatment (5 days) or topical treatment (4 days), compared with itraconazole (ITC) and terbinafine (TRB)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose</th>
<th>AUC (%)</th>
<th>±SEM</th>
<th>MLS (%)</th>
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<tbody>
<tr>
<td>ITC</td>
<td>20 mg/kg</td>
<td>31</td>
<td>±3</td>
<td>52</td>
</tr>
<tr>
<td>ITC</td>
<td>10 mg/kg</td>
<td>37</td>
<td>±2</td>
<td>59</td>
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<td>ITC</td>
<td>2.5 mg/kg</td>
<td>83</td>
<td>±5</td>
<td>100</td>
</tr>
<tr>
<td>TRB</td>
<td>10 mg/kg</td>
<td>22</td>
<td>±3</td>
<td>73</td>
</tr>
<tr>
<td>M</td>
<td>40 mg/kg</td>
<td>43</td>
<td>±15</td>
<td>85</td>
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<tr>
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<td>85</td>
</tr>
<tr>
<td>N</td>
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<td>67</td>
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<td>20 mg/kg</td>
<td>70</td>
<td>±5</td>
<td>73</td>
</tr>
<tr>
<td>O</td>
<td>40 mg/kg</td>
<td>8</td>
<td>±0</td>
<td>6</td>
</tr>
<tr>
<td>O</td>
<td>20 mg/kg</td>
<td>17</td>
<td>±7</td>
<td>30</td>
</tr>
<tr>
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<td>20 mg/kg</td>
<td>77</td>
<td>±18</td>
<td>73</td>
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</tbody>
</table>

### Parameters for efficacy include (i) the area under the infection curve (AUC) that plots individual lesion scores over time; and (ii) the maximal obtained lesion score per group (MLS). Both parameters are shown as a percentage of the values reached in the vehicle-treated infected control animals within the same experiment (low percentages indicate high efficacy (n=3)).
control group, largely outperforming the reference drugs itraconazole and terbinafine (Table 1 and Figure 1).

In conclusion, PBDs with a chlorine atom at position 7 are very promising antifungal candidates with convincing in vitro and in vivo activity against dermatophytes. Compound O deserves further study in greater detail to explore its full potential in the treatment of dermatophytosis. In addition, PBDs with alternative substitution patterns should be investigated for their in vitro activity spectrum and in vivo potential in order to identify candidate compounds that comply with the target product profiles and address the need for new antifungal therapies.

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We thank An Matheeussen for her help in performing the in vitro assays and Pim-Bart Feijens for his contribution in running the guinea pig model.

Figure 1. In vivo efficacy of oral treatment with itraconazole (ITC) at 20 mg/kg and Compound O at 20 mg/kg and topical treatment with ITC at 0.25% and Compound O at 0.25% in the M. canis model in guinea pigs. To evaluate the effect on lesion development, mean lesion scores (n=9) are plotted against the day of scoring. VIC, vehicle-treated infected control group.

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Transparency declarations
Lieven Meerpoel is Head of Medicinal Chemistry at Janssen Research and Development. The remaining authors have none to declare.

Supplementary data
Tables S1, S2 and S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References