We present the results of studies of the *in vitro* susceptibility of 52 isolates of *Trichophyton rubrum* and 40 of *Trichophyton mentagrophytes* to griseofulvin, terbinafine, itraconazole, ketoconazole, fluconazole and cyclopixolamine. All test strains were recovered from patients with toe nail onychomycosis and the minimum inhibitory concentration (MIC) of each antifungal against both species was individually assessed. In addition, we investigated the MIC of the combination of cyclopixolamine and itraconazole and cyclopixolamine and ketoconazole. The NCCLS approved procedure M38-A as modified by Santos and Hamdan was employed. The studies of the two drug combinations were conducted with a checkerboard design. Analysis of the data revealed that terbinafine was the most effective *in vitro* against all isolates, followed in order by itraconazole, cyclopixolamine, ketoconazole and fluconazole. We observed no significant difference in the *in vitro* susceptibility profiles between either species to any of the antifungals (*P* < 0.05). Our *in vitro* results confirm that terbinafine is the most effective of the antifungals included in this study. Furthermore, synergistic interactions were found in the two drug combinations with all of the dermatophyte test isolates. The latter results are in agreement with clinical data that show synergism between oral and topical antifungals in the treatment of onychomycosis.

Keywords dermatophytes, susceptibility tests, drug combination, synergism

Introduction

Dermatophytes are a unique group of fungi that infect keratinous tissue and the most common sites of infection are skin, hair and nails [1]. *Tinea unguium* (onychomycosis) is defined as a fungal infection of the nail with dermatophytes, and it has been recalcitrant to therapy for many years [2]. Over 90% of onychomycosis are caused by two dermatophytes: *Trichophyton rubrum* and *Trichophyton mentagrophytes* [3]. In clinical practice, there are a number of onychomycosis presentations, which are classified according to the route of infection [4]. Risk factors for this infection include aging, diabetes, poorly fitting shoes, and the presence of *Tinea pedis*.

The management of onychomycosis continues to be problematic despite the undeniable advances achieved in the development of new antifungal agents. The therapeutic objective defined by patients suffering from fungal infections of the nail is full restoration of a healthy nail plate, if possible for all 20 nails [5]. But up to 20–25% of patients with onychomycosis fall into the categories of poor responders or non-responders to treatment, which is a particular challenge to clinicians [6].

The systemic drugs used to treat onychomycosis include Griseofulvin and newer antifungal drugs such as itraconazole, ketoconazole, fluconazole and terbinafine, which are currently used in oral therapy. In addition topical agents include cyclopixolamine and amorolfine [7]. The two treatment strategies that are
available, topical and oral, have different benefits depending on the setting in which they are used [8]. The use of oral and topical drugs in combination therapy has great potential for treating fungal nail infection and could not only improve cure rates but also reduce the duration of oral therapy and reduce relapse rates. In order to characterize the likely non-responders to treatment, the first step is to ensure correct diagnosis of the condition. Missing diagnosis of onychomycosis will inevitably jeopardize the perception of the therapeutic effectiveness of any treatment modality [6]. In addition, the therapeutic also depends on the ability of the drug to attack the pathogen and the determination of in vitro susceptibility of dermatophytes to antifungal drugs may prove helpful to predict the ability of a given antimycotic agent to eradicate these fungi [2]. Dermatophytes were not included in the in vitro methods proposed by National Committee on Clinical Laboratory Standards (NCCLS) for testing molds [9]. We have evaluated, in previous work, several testing conditions for determining Minimal Inhibitory Concentration (MICs) for T. rubrum, which were used in this work [10]. The study reported here was undertaken to examine the in vitro activity of terbinafine, itraconazole, ketoconazole, fluconazole, griseofulvin, cyclopiroxolamine and drug combinations between cyclopiroxolamine with itraconazole and with ketoconazole for 52 clinical isolates of T. rubrum and 40 of T. mentagrophytes. In vitro analysis of antifungal activity is useful because it enables a comparison between different antymycotics, which in turn may clarify the reasons for lack of clinical response and assist clinicians to choose the most effective therapy for their patients.

Materials and methods

Strains

We tested 92 strains of dermatophytes (52 strains of T. rubrum and 40 of T. mentagrophytes), all of them were clinical isolates obtained from patients with onychomycosis. They were recovered from nails samples and were identified by routine mycological and culture analysis and were maintained in sterile saline (0.9%) at 4°C and on Sabouraud Dextrose Agar. The quality control strains Candida parapsilosis (ATCC 22019), Candida krusei (ATCC 6258) and the strains T. rubrum (ATCC 40051) and T. mentagrophytes (ATCC 40004) were included each time isolates were tested.

Inocula preparation

Inocula suspensions were prepared from 7-day-old cultures of dermatophytes grown on Potato Dextrose Agar at 28°C. We performed the inoculum preparation according to the method developed by Santos and Hamdan (10). Briefly, colonies were covered with 5 ml of sterile saline, and then rubbed carefully with the tip of a Pasteur pipette. The suspensions were passed through Whatman filter model 40 (pores 8 μm), to produce a filtrate consisting of only microconidia of dermatophytes. The final inoculum size was adjusted with a spectrophotometer at a wavelength of 520 nm to a transmittance of 70–72%. These suspensions were diluted in RPMI 1640 test medium (1:50) (buffered with 0.165 M morpholinepropanesulfonic acid (MOPS), 34.54 g per liter at pH of 7.0) to obtain a cell number ranging from 1 × 10^4 to 4 × 10^4 CFU/ml.

Antifungal agents

Antifungal agents used were terbinafine (Novartis), itraconazole (Janssen-Cilag), ketoconazole (Janssen-Cilag), fluconazole (Pfizer), Griseofulvin (Shering Plough) and cyclopiroxolamine (Pratti, Donaduzzi). They were obtained as standard powders and stock solutions of all tested drugs were prepared in 100% dimethylsulfoxide prior to dilution in RPMI-1640 test medium to yield twice (when drug was tested individually) or four times (when drug was tested in combination with another drug) the final strength required for the test.

Broth microdilution method

All tests were performed in flat-bottomed 96-well microplates. When drugs were tested individually, aliquots of 100 μl of the twice drug dilutions were inoculated into the wells with a multichannel pipette followed by 100 μl of the diluted inocula suspensions to bring the drug dilutions to the final test concentrations. Tested concentrations for fluconazole and griseofulvin ranged from 0.125–64.0 μg/ml, terbinafine from 0.031 to 16.0 μg/ml, cyclopiroxolamine from 0.015–1.0 μg/ml, itraconazole from 0.003–2.0 μg/ml and ketoconazole from 0.007–4.0 μg/ml (these last three drugs were tested in combination as described below). Terbinafine was not tested in combination with other drugs because of its low MIC against dermatophytes. In addition, growth and sterility control wells were included for each isolate tested. We also tested two drug pair combinations as follows: cyclopiroxolamine with ketoconazole and cyclopiroxolamine with itraconazole. Drug interaction was evaluated in a checkerboard microdilution design as described previously [11] that easily provides a matrix of all possible drug combinations in the required concentration ranges mentioned above.
Reading and interpretation of results

When drugs were tested individually, MIC endpoints were determined for terbinafine and cyclopiroxolamine as the lowest concentrations that showed complete growth inhibition. For azoles and griseofulvin MIC endpoints were the lowest concentrations that showed 80% growth inhibition. When tested in combination, interaction between drugs was quantitatively evaluated by means of the fractional inhibitory concentration index (FIC), which was calculated by the following formula: (MIC A in combination/MIC A) + (MIC B in combination/MIC B). The interaction was defined as synergistic if the FIC index was ≤0.5, indifferent if FIC was >0.5 but ≤4.0, and antagonistic if FIC was >4.0. The interaction was also represented as profiles of the curves of drug combination. Comparison MIC data for different species of dermatophytes were performed by Wilcoxon (Mann-Whitney) and Kruskal-Wallis tests. A P value of <0.05 was considered to be significant.

Results

We observed that all analyzed isolates produced clearly detectable growth after seven days of incubation. Table 1 summarizes individual MIC data for all tested drugs. Terbinafine was the most active drug against all tested isolates, since all FIC indexes were ≤0.5, indifferent if FIC was >0.5 but ≤4.0, and antagonistic if FIC was >4.0. The interaction was also represented as profiles of the curves of drug combination. Comparison MIC data for different species of dermatophytes were performed by Wilcoxon (Mann-Whitney) and Kruskal-Wallis tests. A P value of <0.05 was considered to be significant.

Table 1 Antifungal drugs in vitro susceptibility data for 92 dermatophyte isolates. Statistical analysis revealed no significant difference between MIC values for Trichophyton rubrum and Trichophyton mentagrophytes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC range (µg/ml)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;* (µg/ml)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;** (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Griseofulvin</td>
<td>0.125–4.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>0.125–&gt;64.0</td>
<td>64.0</td>
<td>&gt;64.0</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.031–0.5</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.0625–4.0</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Cyclopiroxolamine</td>
<td>0.031–1.0</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>&lt;0.031</td>
<td>&lt;0.031</td>
<td>&lt;0.031</td>
</tr>
</tbody>
</table>

*MIC<sub>50</sub>: MIC at which 50% of the isolates were inhibited. **MIC<sub>90</sub>: MIC at which 90% of the isolates were inhibited.

Our MIC data for quality control strains C. parapsilosis (ATCC 22019) and C. krusei (ATCC 6258) were within the expected range [12] and MIC values found here for reference dermatophytes T. rubrum (ATCC 40051) and T. mentagrophytes (ATCC 40004) were similar to that found in a previous study [10].

Evaluation of the drug combination between itraconazole and cyclopiroxolamine and ketoconazole and cyclopiroxolamine revealed a synergistic effect for all tested isolates, since all FIC indexes were ≤0.5 (data not shown). The MIC values for drugs tested alone (Table 1) were higher than when tests were performed in combination of itraconazole with cyclopiroxolamine and of ketoconazole with cyclopiroxolamine. Our in vitro data confirm theoretical synergism between azoles and cyclopiroxolamine and it is shown on Fig. 1 for reference dermatophytes. This figure illustrates what was obtained for all tested isolates.

Discussion

The NCCLS approved document M38-A does not describe a test guideline for dermatophytes, which makes a comparison of data from different authors difficult. With respect to antifungal drugs tested in this work, Cyclopiroxolamine is a topical antismycotic agent that adheres well to keratin and exhibits good penetration through the nail plate [2]. Its mechanism of action depends in part on the chelation of heavy metal ions, such as the ferrous ion [11]. Clinical data demonstrates that cyclopiroxolamine is more effective than previous topical approaches to treatment [4]. Although it has widely been used as topical treatment for onychomycosis, there is a shortage data of in vitro activity of cyclopiroxolamine against dermatophytes. The concentrations in the stratum corneum range from 2.3–4.5 µg/g, and those in the deeper epidermal layers range from 20–30 µg/g [2]. Considering the MICs of cyclopiroxolamine obtained in the present work, it is plausible that its use in topical application as nail lacquer would be effective in cases of onychomycosis, since results found here were within the expected range for nails. Our MIC values were higher (one to two dilutions) than data from previous studies [1,11] and lower than results from Korting et al. [2].

We observed that MICs of itraconazole and terbinafine were all well within the range that can be expected for treating the nail with conventional doses, similar data were found by Korting et al. [2], Nimura et al. [13] and Fernández-Torres et al. [14]. Itraconazole concentrations of more than 0.5 µg/g were found up to 6 months post treatment in distal nail clippings and the use of terbinafine (250 mg per day) led to
average concentrations of 0.25–0.55 µg/g that could be detected as early as 4 weeks after initiating therapy [2]. The low MIC values found here for itraconazole and particularly for terbinafine can help to explain the superior efficacy of these drugs in treating onychomycosis and any dermatophytic infection. Because dermatophytes generally exhibit a high susceptibility profile to terbinafine, the low MIC values obtained here for this drug makes it difficult to test it in combination with another drug. This is also demonstrated by Gupta and Kohli [11].

Griseofulvin was the first systemic antifungal drug but today it is not used extensively. The new-generation oral antifungal agents, such as terbinafine and itraconazole (both mentioned above), produce much better cure rates and less toxicity than their predecessor, griseofulvin [4]. When griseofulvin is administrated at the doses of 500 mg every 12 hours, concentrations of 6–12 µg/g are reached in stratum corneum after 30 h. If those doses are continued for several weeks, concentrations of 12–25 µg/g are achieved, but it is highly probable that its persistence in the nails is short-term [15], explaining its low cure rates. Our MIC values for this drug were directly comparable to those of our previous study [10]. Develoux [15] and Artis et al. [16] considered isolates resistant to griseofulvin when MIC values were equal or greater than 3.0 µg/ml. According to this standard, our findings suggest that all isolates were in vitro susceptible to griseofulvin; however, to reflect success or failure to therapy, further tests are still needed to correlate MIC data with clinical outcome.

MIC data obtained for fluconazole were higher than other tested drugs, even though it has been demonstrated to be effective in treating onychomycosis (150 mg weekly), with levels being achieved in the stratum corneum of 7.1 µg/ml after 7 days of an oral administration of 150 mg [17]. According to Korting et al. [2], the higher MIC values to this drug might be

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**Fig. 1** Drug combination of two pairs of drugs (ketoconazole/cyclopiroxolamine and itraconazole/cyclopiroxolamine) against *Trichophyton rubrum* (ATCC 40051) and *Trichophyton mentagrophytes* (ATCC 40004). The curves demonstrate synergistic interaction between azoles and cyclopiroxolamine.
due to technical problems, which may include interactions with particular media, or dissolution problems at higher concentrations.

Ketoconazole was the first orally active imidazole but it is also well known for its high hepatotoxicity in long-term therapy and its usual dose, 200 mg per day, results in maximum plasma concentrations of 3.24 µg/ml, and 0.91 µg/ml in cutaneous layer [2]. Our MIC data for ketoconazole were lower than levels of this drug in cutaneous layer; however these tissue levels are commonly obtained for only short periods of time [2], which explain low clinical and mycological cure rates reported when this drug is used to treat onychomycosis.

In spite of multiple advances in antifungal drug development and therapy, onychomycosis remains difficult to treat. Furthermore, synergy between drugs is not a new concept, and combination drug therapy has widely been used in various fields of medicine. The combination of two or more drugs can result in increased efficacy, speed of action, a broader spectrum of activity and better patient tolerability [4,18]. According to this, treatment strategies for onychomycosis include topical and systemic antifungal therapies in combination that has resulted in a marked improvement of mycological and clinical outcomes associated with onychomycosis [18]. The rationale for the combination of topical and oral therapies in the treatment of onychomycosis is a simple one: oral antifungal agents reach the fungal pathogen via the nail bed, and topical antifungal agents are absorbed through the nail keratin from the surface [7]. In addition, combination of more than one mode of drug administration may allow for complementary drug penetration into areas of infected tissue where each drug alone does not accumulate in sufficient concentrations. Clinical trials have been revealing a successful combination of topical nail lacquers and oral antifungals for treatment of severe onychomycosis. Amorolfine (5%) and ciclopiroxolamine (8%) are the most common topical antifungal drugs used in combination with oral drugs [4]. Lecha et al. [8] in a clinical trial demonstrated that amorolfine nail lacquer/oral itraconazole combination therapy is highly efficacious in treatment of severe toenail onychomycosis in comparison to itraconazole alone. Higher cure rates of drug combination in treating onychomycosis were obtained also by Baran et al. [19] when comparing amorolfine plus oral terbinafine with terbinafine alone. Although data from several clinical trials are currently available, there are few studies of the in vitro activity of drug combinations and especially against dermatophytes. Recently, Gupta and Kohli [11] reported the synergistic interaction of ciclopiroxolamine plus terbinafine drug combination, and an additive interaction of ciclopiroxolamine and itraconazole for Onychola canadensis using the same methodology used in this work, but this study did not include dermatophytes. Data from our study are different to those from Gupta and Kohli [11], who obtained additive interaction for combination between itraconazole and ciclopiroxolamine, but testing Onychola canadensis. Synergistic in vitro interaction has also been demonstrated for terbinafine with itraconazole or amphotericin B against Zygomycota [20]. Our results were consistent with data from the literature, confirming theoretical synergism between azoles and ciclopiroxolamine. Our data also suggests that the microdilution checkerboard methodology is useful for determining interaction between antifungal drugs against dermatophytes.

In conclusion, our in vitro data demonstrates the higher activity of terbinafine in comparison to other tested antifungal drugs, followed by ciclopiroxolamine. Among azoles, itraconazole was the most active, followed by ketoconazole and fluconazole. The in vitro results revealed a synergistic interaction between ciclopiroxolamine and itraconazole or ketoconazole, confirming clinical data that a high cure rate is achieved with drug combinations compared to topical or oral antifungal agents used individually. These data suggest a possible correlation between MIC values and clinical outcomes.

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References