



In vitro Activity of Amorolfine, Ciclopirox, Itraconazole and Terbinafine Against *Aspergillus versicolor* as Agent of Onychomycosis

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Abstract

Cases of onychomycosis caused by non-dermatophyte filamentous fungi have increased over the years. It is worth noting that this group of fungi is resistant to fluconazole, thus the importance of determining the *in vitro* susceptibility patterns to the antifungals available. We determined the minimal inhibitory concentration of amorolfine, ciclopirox, itraconazole and terbinafine using the microdilution method M38-A, as described by the Clinical Laboratory and Standards Institute, of 13 isolates of *Aspergillus versicolor* obtained from onychomycosis. The final concentrations were: 0.13-64 µg/mL for amorolfine and terbinafine, 0.06 - 32 µg/mL for ciclopirox and 0.03-16 µg/mL for itraconazole. Also, we determined the interaction of these drugs by the checkerboard method. The MIC₅₀ and MIC₉₀ were ≥ 64.00 and ≥ 64.00 µg/mL for amorolfine, 4.00 and 8.00 µg/mL for ciclopirox, 1.00 and 1.80 µg/mL for itraconazole and 0.50 and 1.36 µg/mL for terbinafine, respectively. Regarding the combination of antifungals, 15.38% of the combinations of ciclopirox-itraconazole presented synergism, while the rest of the combinations showed no interaction. Thus, *in vitro* susceptibility testing indicates that terbinafine exhibited the highest antifungal activity and amorolfine the lowest against *A. versicolor*. Also, combining treatments enhances the activity of the drugs, proving a possible alternative for successful treatment of onychomycosis caused by this fungus.

Keywords: *Aspergillus versicolor*; Amorolfine; Ciclopirox; Itraconazole; Terbinafine

Abbreviations

CLSI: Clinical and Laboratory Standard Institute; FIC: Fractional Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; MIC: Minimal Inhibitory Concentration; NDFF: Non-Dermatophyte Filamentous Fungi; PDA: Potato Dextrose Agar; RPMI: Roswell Park Memorial Institute; MOPS: Morpholine Propanesulphonic Acid.

Introduction

Onychomycosis is an infection of the nail apparatus caused by fungi. Currently, it is considered a public health problem since it has been reported worldwide and cases are on the rise [1]. This disease is mainly caused by dermatophytes, followed by *Candida* spp. and finally by non-dermatophyte filamentous fungi (NDFF) [1]. The latter group is made up of fast-growing microorganisms that are widely distributed in nature. Traditionally, they have been regarded as laboratory contaminants. Therefore, in order to consider them as etiological agents of onychomycosis, their isolation

must be accompanied by the presence of septate, hyaline or dematiaceous mycelium in the clinical sample [2]. Among the genera reported are: *Acremonium*, *Aspergillus*, *Chrysosporium*, *Fusarium*, *Neoscytalidium*, *Onychocola*, *Penicillium*, *Scopulariopsis* and *Trichosporon* [3-5]. In Costa Rica, *Fusarium* spp. is the most frequently isolated NDFF from toenails and *Trichosporon* spp. from fingernails, but also *Scopulariopsis* spp. and *Aspergillus versicolor* have been isolated [2,5]. *A. versicolor*, is a ubiquitous fungus that produces velvety colonies, which at first are white and then turn yellow, orange, tan, green or pinkish. The reverse of the colony can be white, yellow, orange or red. Microscopically, it is characterized by the production of conidiophores that are enlarged at the tip, forming a swollen vesicle. This vesicle is mostly covered by two rows of phialides (biseriated) [6].

These infections are difficult to treat, as the nail has several intrinsic factors such as: slow growth and little vascularity which make it difficult for the antifungal agent to reach its target, the area and percentage of the affected nail, the etiological agent involved,

coexistence of various fungi and non-compliance with treatment by patients [7-9]. Also, NDFF are usually resistant to the available treatments [3]. For example, *Scopulariopsis* sp. and *A. versicolor* are resistant, *in vitro*, to fluconazole and itraconazole [10,11] and *Fusarium* to itraconazole and terbinafine [12]. As a result, studies involving the determination of the antifungal activity of the different compounds against NDFF have increased, highlighting the importance of identifying the etiological agent of the condition in order to provide the best therapeutic option for the patients [13]. Thus, the present study aims to determine the *in vitro* susceptibility patterns of two systemic antifungal agents (itraconazole and terbinafine) and two topical treatments (amorolfine and ciclopirox) against isolates of *A. versicolor* obtained from onychomycosis.

Materials and Methods

Strains

A total of thirteen clinical isolates of *A. versicolor* were included in this study. The isolates were preserved at the Fungal Collection of the Section of Medical Mycology, School of Microbiology, University of Costa Rica. They were obtained from patients with onychomycosis from 2006 to 2016; it is worth noting that each isolate originated from a unique clinical specimen. All isolates were maintained in Czapek Dox Agar and Potato Dextrose Agar (PDA) (OXOIDTM, Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 20 - 25 °C.

Preparation of the fungal inoculum

The isolates were grown for 7 days at 20 - 25 °C in test tubes containing PDA. The conidia were collected by adding 5 mL of 85% sterile saline solution to the test tubes and then probing the colonies with the tip of a sterile Pasteur pipette. Conidia concentration was determined with a Neubauer hemocytometer (Hausser Scientific Horsham, PA, USA) and standardized to $1-5 \times 10^6$ spores/mL. Suspensions were later diluted 1:50 in RPMI 1640 Medium (Roswell Park Memorial Institute) (Thermo Fisher Scientific, USA) ($2 \times 10^4 - 1 \times 10^5$ spores/mL). One hundred microliters of this suspension were added to the wells with the antifungal compounds. Hence, the final spore concentrations were $1-5 \times 10^4$ spores/mL.

Antifungal agents

The antifungal agents used in this study were: amorolfine, ciclopirox, itraconazole and terbinafine (Royal Pharm, Hangzhou, China). They were obtained as standard powder and the stock solution was prepared in RPMI 1640 with glutamine and without bicarbonate, buffered to pH 7 with morpholine propanesulphonic acid (MOPS) to yield twice the final concentration required for the test.

Microdilution method

The minimal inhibitory concentration (MIC) was determined by the microdilution method, according to the Clinical and Laboratory

Standard Institute (CLSI) document M38-A [14]. The final concentrations were: amorolfine (0.13 - 64) µg/mL, ciclopirox (0.06 - 32) µg/mL, itraconazole (0.03 - 16) µg/mL and terbinafine (0.13 - 64) µg/mL. The quality control strains used were: *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019. Spectrophotometric readings were made at 72 hours of incubation at 450 nm with a Synergy HT (BioTek Instruments, Inc., Winooski, VT, USA). Geometric means, MIC ranges, MIC₅₀ and MIC₉₀ were calculated for each antifungal agent. The significant differences in mean values were determined by a one-way ANOVA test with Tukey post-test, using the program SPSS for Windows, version 20 (SPSS Inc., Chicago, Ill, USA). P-values < 0.05 were considered statistically significant.

Drug synergy assay

The *in vitro* drug interaction study was determined using the checkerboard titration method, as described previously [15]. Briefly, synergistic/additive/antagonist interactions of ciclopirox, itraconazole and terbinafine against *A. versicolor* were evaluated by determining MICs of the drugs alone and in combination in 96-well plates. Each combination was prepared so that the initial concentration of each drug was 4-fold the MIC. Serial dilutions were made in subsequent wells. 100 µL of the fungal inoculum (final conidia concentration of $1 - 5 \times 10^4$ spores/mL) were added to each well. The plates were incubated at (20 - 25) °C for 72 hours and then read by spectrophotometry with a Synergy HT (Bio Tek Instruments, Inc., Winooski, VT, USA). The combination reductions in MICs were used to calculate the fractional inhibitory concentration (FIC). FIC indices (FICI) were interpreted as follows: ≤ 0.5 , synergism; > 0.5 to < 4.0 , no interaction/indifference; and ≥ 4.0 , antagonism [16]. All tests were performed in duplicate. FICI ranges were calculated for each antifungal combination. Finally, the significant differences in mean values between the MICs of alone and combined treatments and the differences between the FICI were determined by a one-way ANOVA test with Tukey post-test, using the program SPSS for Windows, version 20 (SPSS Inc., Chicago, Ill, USA). P-values < 0.05 were considered statistically significant.

Results and Discussion

Determination of the minimal inhibitory concentration

Resistance to treatment by NDFF, that produce onychomycosis, has led to numerous studies and different therapeutic schemes. In order to improve the treatment for this fungal group, we determined the *in vitro* susceptibility patterns of two systemic antifungal agents (itraconazole and terbinafine) and two topical treatments (amorolfine and ciclopirox) against isolates of *A. versicolor* obtained from onychomycosis. Table 1 shows the distribution of MICs for the 13 isolates. Statistically significant differences were found between the MIC averages of the drugs tested ($F = 8753.73$; $df = 3$; $p < 0.0001$). As a result of these significant differences, a Tukey post-test was performed. This analysis grouped the treatments into

Antifungal agent	MIC* (µg/mL)			
	Geometric mean (SD)	Range	MIC ₅₀	MIC ₉₀
Amorolfine	64.00 (± 0.00)	-	≥64.00	≥64.00
Ciclopirox	4.89 (± 2.30)	1.60-8.00	4.00	8.00
Itraconazole	0.86 (± 0.50)	0.13-2.00	1.00	1.80
Terbinafine	0.55 (± 0.43)	0.13-1.60	0.50	1.36

Table 1: *In vitro* susceptibility patterns of Costa Rican isolates of *A. versicolor* (n = 13) isolated from onychomycosis.

*MIC: minimal inhibitory concentration.

three groups. In the first one, itraconazole and terbinafine were included, since they had the highest antifungal activity (lower MIC). On the other hand, the topical antifungals were grouped separately, in the other two groups.

In regard to the systemic treatments, a MIC₅₀ of 0.50 µg/mL and a MIC₉₀ of 1.36 µg/mL were obtained for terbinafine. These results are consistent with Torres., *et al.* (1998), since they reported a MIC₅₀ of 0.25 µg/mL and a MIC₉₀ of 0.75 µg/mL by the broth microdilution method for 12 isolates of *A. versicolor* obtained from onychomycosis in Spain [10]. This treatment is also effective against dermatophytes as shown by Díaz., *et al.* (2015) who studied 62 dermatophyte isolates from Chile obtaining MICs ranging from 0.03 to 0.06 µg/mL [17]. On the other hand, in the present study, seven of the isolates were resistant to itraconazole, with a MIC₅₀ of 1.00 µg/mL. These results are in concordance with Torres., *et al.* (1998) that reported a 91.7% resistance to this compound. However, the MICs found in the present study are higher than those published by Chavez., *et al.* (2010) in Colombia, where the authors reported a MIC₅₀ of 0.38 µg/mL for an isolate of *A. versicolor*, by using the E-Test® method [18] or by García-Martos., *et al.* (2005) from Spain, who reported a MIC₅₀ of 0.5 µg/mL for five isolates of *A. versicolor*, by the microdilution method with Sensititre Yeast One® [19]. As previously stated, *Aspergillus* are environmental fungi, so it is considered that their development of resistance could be due to the use of pesticides in agriculture; in addition, cross-resistance has been observed with other azoles such as voriconazole and posaconazole. The most common resistance mechanism is a replacement of a leucine by a histidine at position 98, in the *cyp51A* gene [20-22]. Mechanisms of azole resistance have been reported in strains of *Aspergillus fumigatus*, including point mutations in two genes; *cyp51A* and *cyp51B* encoding for 14- α lanosterol demethylase. This mutation causes the loss of drug affinity at the catalytic site [23]. In addition, an increase in the number and activity of efflux pumps (ABC: ATP Binding Cassette and MFS: Mayor Facilitators Superfamily) have been described [24,25].

When it comes to topical treatments, the postulated antimycotic mechanism of action of ciclopirox is the chelation of polyvalent metal cations, especially iron (Fe³⁺), which leads to the inhibition

of metal-dependent enzymes (cytochromes, catalases and peroxidases) [26]. For this drug, we obtained a MIC₅₀ of 4.00 µg/mL and a MIC₉₀ of 8.00 µg/mL. To our knowledge, this is the first study which aims to determine the *in vitro* susceptibility of *A. versicolor* to ciclopirox. However, in 2017, Liu., *et al.* published a case report of a patient with primary cutaneous aspergillosis caused by *A. fumigatus* that was successfully treated with oral itraconazole and ciclopirox olamine ointment [27]. Finally, 100% of the isolates had a MIC ≥ 64.00 µg/mL for amorolfine. This data is consistent with Li., *et al.* (2004) who reported an isolate of *Aspergillus flavus* obtained from a patient with dermatomycosis with a MIC ≥ 64.00 µg/mL for this drug. In the other hand, this antifungal agent has proven to be a possible therapeutic option for infections caused by dermatophytes such as *Trichophyton rubrum* (MIC₅₀ 0.01 µg/mL), *Trichophyton mentagrophytes* (MIC₅₀ 0.04 µg/mL) and *Epidermophyton floccosum* (MIC₅₀ 0.02 µg/mL) [28] and other NDFF like *Fusarium solani* (MIC₅₀ 1.25 µg/mL) [12] and *Scopulariopsis* spp. (MIC₅₀ 0.13 µg/mL) [11].

Drug synergy assay

Tables 2 - 4 show the MICs of the antifungal drugs obtained after their combination with other treatments and the FICIs for the combinations studied. 15.38% of the combinations of ciclopirox-itraconazole presented synergism while the rest of the combinations showed no interaction. However, it is worth noting that, statistical differences were found between the geometric means of the MICs obtained for ciclopirox (F = 12,311; df = 2; p < 0,001) and terbinafine (F = 5,127; df = 2; p < 0,05) when compared to the MICs obtained after the combination. In both cases, Tukey test grouped the MIC of the antifungal when tested alone in one group and the MICs in combination in another. In contrast, there were no statistical differences found between the MICs for itraconazole alone or in combination with terbinafine or ciclopirox (F = 3,007; df = 2; p = 0,062). Nevertheless, 53.85% (n = 7) of the isolates were resistant to itraconazole (MIC ≥ 1,00 µg/mL) when tested alone but only 15.39% (n = 2) were resistant when combined with terbinafine or ciclopirox. Finally, there were no statistical differences found between the FICI of the three combinations (F = 1,731; gl = 2; p = 0,191).

When analyzing the drug-drug interaction, through the FICI, within the results obtained in our research, a possible enhancing effect was found between the combinations itraconazole - terbinafine and ciclopirox - terbinafine and ciclopirox - itraconazole. In fact, synergism was found within the latter combination. Dorsthorst., *et al.* (2002) and Gupta (2003) obtained similar results to ours, the first obtained synergism for all strains of *A. fumigatus* subjected to the combination itraconazole - terbinafine [15] and Gupta synergism and/or additive (0.5 < FICI ≤ 1.00) reactions for the combinations ciclopirox - terbinafine and ciclopirox - itraconazole [29]. Like us, none of them obtained cases of antagonism [15,29]. The usage of antifungal combinations is beneficial for the patient since

Strain	Antifungal agent	MIC ^a of ITZ (µg/mL)		Interaction	
		Alone	Combination	FICI ^b	Type
AS VE 01	ITZ ^c + CIC ^d	0.50	0.50	1.25	No interaction
	ITZ + TB ^e	0.50	0.25	1.00	No interaction
AS VE 02	ITZ + CIC	0.13	0.03	0.48	Synergy
	ITZ + TB	0.13	0.02	0.65	No interaction
AS VE 03	ITZ + CIC	1.00	1.00	2.00	No interaction
	ITZ + TB	1.00	0.50	0.63	No interaction
AS VE 04	ITZ + CIC	1.00	0.50	0.63	No interaction
	ITZ + TB	1.00	0.06	0.56	No interaction
AS VE 05	ITZ + CIC	2.00	1.00	0.75	No interaction
	ITZ + TB	2.00	2.00	2.00	No interaction
AS VE 06	ITZ + CIC	0.50	0.25	0.75	No interaction
	ITZ + TB	0.50	1.00	3.00	No interaction
AS VE 07	ITZ + CIC	0.50	0.13	0.29	Synergy
	ITZ + TB	0.50	0.50	1.50	No interaction
AS VE 08	ITZ + CIC	1.50	0.75	0.75	No interaction
	ITZ + TB	1.50	0.75	0.62	No interaction
AS VE 09	ITZ + CIC	1.00	0.50	1.00	No interaction
	ITZ + TB	1.00	0.50	1.01	No interaction
AS VE 10	ITZ + CIC	0.50	0.50	0.63	No interaction
	ITZ + TB	0.50	0.13	0.76	No interaction
AS VE 11	ITZ + CIC	0.50	0.13	0.76	No interaction
	ITZ + TB	0.50	0.25	0.75	No interaction
AS VE 12	ITZ + CIC	1.00	0.13	0.63	No interaction
	ITZ + TB	1.00	0.50	1.00	No interaction
AS VE 13	ITZ + CIC	1.00	0.50	0.63	No interaction
	ITZ + TB	1.00	0.50	1.04	No interaction

Table 2: Minimal inhibitory concentration of itraconazole alone and in combination with ciclopirox or terbinafine of Costa Rican isolates of *A. versicolor* (n = 13) isolated from onychomycosis

a MIC: minimal inhibitory concentration.

bFICI: fractional inhibitory concentration index. Drug interactions were classified as synergistic (FICI ≤ 0.5), no interaction (0.5 < FICI < 4) or antagonistic (FICI ≥ 4).

cITZ: Itraconazole.

dCIC: Ciclopirox.

eTB: Terbinafine.

Strain	Antifungal agent	MIC ^a of ITZ (µg/mL)		Interaction	
		Alone	Combination	FICI ^b	Type
AS VE 01	CIC ^c + ITZ ^d	8.00	2.00	1.25	No interaction
	CIC + TB ^e	8.00	4.00	1.00	No interaction
AS VE 02	CIC + ITZ	2.00	0.50	0.48	Synergy
	CIC + TB	2.00	1.00	0.65	No interaction
AS VE 03	CIC + ITZ	6.00	6.00	2.00	No interaction
	CIC + TB	6.00	3.00	0.75	No interaction
AS VE 04	CIC + ITZ	8.00	1.00	0.63	No interaction
	CIC + TB	8.00	4.00	1.00	No interaction
AS VE 05	CIC + ITZ	2.00	0.50	0.75	No interaction
	CIC + TB	2.00	1.00	1.04	No interaction
AS VE 06	CIC + ITZ	4.00	1.00	0.75	No interaction
	CIC + TB	4.00	2.00	0.76	No interaction
AS VE 07	CIC + ITZ	8.00	0.25	0.29	Synergy
	CIC + TB	8.00	4.00	1.02	No interaction
AS VE 08	CIC + ITZ	4.00	1.00	0.75	No interaction
	CIC + TB	4.00	2.00	1.02	No interaction
AS VE 09	CIC + ITZ	6.00	3.00	1.00	No interaction
	CIC + TB	6.00	3.00	0.85	No interaction
AS VE 10	CIC + ITZ	4.00	0.50	0.63	No interaction
	CIC + TB	4.00	2.00	0.63	No interaction
AS VE 11	CIC + ITZ	4.00	2.00	0.76	No interaction
	CIC + TB	4.00	2.00	0.76	No interaction
AS VE 12	CIC + ITZ	6.00	3.00	0.63	No interaction
	CIC + TB	6.00	0.19	0.53	No interaction
AS VE 13	CIC + ITZ	1.60	0.20	0.63	No interaction
	CIC + TB	1.60	0.80	0.58	No interaction

Table 3: Minimal inhibitory concentration of ciclopirox alone and in combination with itraconazole or terbinafine of Costa Rican isolates of *A. versicolor* (n = 13) isolated from onychomycosis

a MIC: minimal inhibitory concentration

bFICI: fractional inhibitory concentration index. Drug interactions were classified as synergistic (FICI ≤ 0.5), no interaction (0.5 < FICI < 4) or antagonistic (FICI ≥ 4).

cCIC: Ciclopirox

dITZ: Itraconazole

eTB: Terbinafine.

Strain	Antifungal agent	MIC ^a of ITZ (µg/mL)		Interaction	
		Alone	Combination	FICI ^b	Type
AS VE 01	TB ^c + CIC ^d	0.50	0.25	1.00	No interaction
	TB + ITZ ^e	0.50	0.25	1.00	No interaction
AS VE 02	TB + CIC	0.13	0.02	0.65	No interaction
	TB + ITZ	0.13	0.07	0.65	No interaction
AS VE 03	TB + CIC	1.00	0.25	0.75	No interaction
	TB + ITZ	1.00	0.13	0.63	No interaction
AS VE 04	TB + CIC	0.50	0.25	1.00	No interaction
	TB + ITZ	0.50	0.25	0.56	No interaction
AS VE 05	TB + CIC	0.13	0.07	1.04	No interaction
	TB + ITZ	0.13	0.13	2.00	No interaction
AS VE 06	TB + CIC	0.50	0.13	0.76	No interaction
	TB + ITZ	0.50	0.50	3.00	No interaction
AS VE 07	TB + CIC	0.50	0.26	1.02	No interaction
	TB + ITZ	0.50	0.25	1.50	No interaction
AS VE 08	TB + CIC	0.25	0.13	1.02	No interaction
	TB + ITZ	0.25	0.03	0.62	No interaction
AS VE 09	TB + CIC	0.38	0.13	0.85	No interaction
	TB + ITZ	0.38	0.19	1.01	No interaction
AS VE 10	TB + CIC	1.00	0.13	0.63	No interaction
	TB + ITZ	1.00	0.50	0.76	No interaction
AS VE 11	TB + CIC	0.50	0.13	0.76	No interaction
	TB + ITZ	0.50	0.13	0.75	No interaction
AS VE 12	TB + CIC	1.60	0.80	0.53	No interaction
	TB + ITZ	1.60	0.80	1.00	No interaction
AS VE 13	TB + CIC	0.13	0.01	0.58	No interaction
	TB + ITZ	0.13	0.07	1.04	No interaction

Table 4: Minimal inhibitory concentration of terbinafine alone and in combination with ciclopirox or itraconazole of Costa Rican isolates of *A. versicolor* (n = 13) isolated from onychomycosis

a MIC: minimal inhibitory concentration

bFICI: fractional inhibitory concentration index. Drug interactions were classified as synergistic (FICI ≤ 0.5), no interaction (0.5 < FICI < 4) or antagonistic (FICI ≥ 4).

c TB: Terbinafine

d CIC: Ciclopirox

e ITZ: Itraconazole.

this therapeutical scheme may reduce toxicity and the development of resistance [30].

Conclusion

In vitro susceptibility testing indicates that terbinafine exhibits the highest antifungal activity and amorolfine the lowest against *A. versicolor*. Also, combining treatments enhances the activity of the drugs, proving a possible alternative for successful treatment of onychomycosis caused by this fungus.

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Conflict of Interest

The authors declare that there is no conflict of interest involved in this article.

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