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Potential of Ergosterol Synthesis Inhibitors To Cause Resistance or Cross-Resistance in *Trichophyton rubrum*

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Superficial mycoses caused by *Trichophyton rubrum* are among the most common infections worldwide. *T. rubrum* infections are difficult to treat and are often associated with recurrences after interruption of the antifungal therapy. Nevertheless, reports on *T. rubrum* resistance to commonly used antifungal drugs are rare. In this study, we compared the *in vitro* resistance frequencies and development of resistance to terbinafine, itraconazole, amorolfine, and ciclopirox in *T. rubrum*. Results demonstrated that naturally occurring mutants were isolated at a frequency of 10^{-7} for itraconazole and 10^{-9} for terbinafine and amorolfine. To mimic conditions of body sites in which low drug levels are reached during therapy, *T. rubrum* was propagated for 10 transfers in media containing subinhibitory drug concentrations. Resistance to itraconazole, terbinafine, and amorolfine emerged at a higher frequency than was seen with spontaneous mutation. Itraconazole-resistant mutants also showed decreased susceptibility to amorolfine as well as to terbinafine, and amorolfine-resistant mutants were also less susceptible to terbinafine. No mutant resistant to ciclopirox was isolated, suggesting no propensity of *T. rubrum* to develop resistance to this drug. How different drug mechanisms of action can influence the onset of resistance is discussed.

Dermatophytoses, the infections of keratinized tissues such as skin, hair, and nails by the highly specialized dermatophyte fungi, represent the most common type of human infection worldwide, particularly in aging, diabetic, or immunocompromised individuals (1–4). The main causative agent of dermatophytosis in developed countries is *Trichophyton rubrum*, an anthropophilic filamentous fungus that most commonly infects nails (onychomycosis) and skin (*tinea pedis*; *tinea corporis*) (1, 4–6). *T. rubrum* is known to account for almost 70% of all dermatophyte infections, and the incidence of infections due to this species has not changed in recent decades, although many efficient antifungal drugs have been introduced into the market during this period (4, 6, 7).

In clinical practice, several antimycotic agents are most frequently prescribed for the treatment of *T. rubrum* infections. Terbinafine (TRB) is a synthetic allylamine derivative that is widely used, both orally and topically, for treatment of dermatophyte onychomycosis and tinea (7). This drug is a potent inhibitor of the fungal squalene epoxidase, an enzyme involved in the biosynthesis of ergosterol (8). Azole drugs such as itraconazole (ITC) are fungistatic triazoles that block the ergosterol synthesis pathway by inhibiting the enzyme 14α-demetilase (9) and are popular for systemic and topical treatment of infections due to yeasts and dermatophytes (7). Amorolfine (AMF) is a member of the family of morpholine antimycotic agents that also block ergosterol synthesis (inhibition of sterol-Δ^{14}-reductase and sterol-Δ^{7}-Δ^{8}-isomerase) and displays activity against a wide range of pathogenic fungi (8). Topical use of AMF in the form of nail lacquer is a common treatment for onychomycosis (10), and AMF-containing creams are used to treat skin dermatophytoses (11). Ciclopirox (CPX) is a hydroxypyridone which exhibits activity against nearly all the clinically relevant dermatophytes, yeasts, and molds. The main mechanism of action of CPX is its high affinity for trivalent cations (such as Fe^{3+}), resulting in the inhibition of many metal-dependent enzymes that are responsible for the degradation of peroxides within the fungal cell (12). CPX and its olamine salt are available in multiple topical formulations, suitable for administration to the skin, nails, and vaginal mucosa (12).

T. rubrum infections and, in particular, onychomychosis are often intractable, and relapses occur frequently after cessation of the antifungal therapy (13, 14). One reason for unsuccessful antifungal management is poor adherence to long-term treatment regimens using topical antifungal drugs (7, 15). However, relapses following cessation of the antifungal therapy may also be due to *T. rubrum* acquisition of resistance, especially in the case of infections involving prolonged treatment with relatively low drug concentrations (14). Although rare, *T. rubrum* clinical isolates and *in vitro*-selected mutants resistant to ITC and TRB have already been described (14, 16–18).

The present *in vitro* study investigated the spontaneous occurrence of *T. rubrum* resistance to TRB, AMF, CPX, and ITC as well as the evolution of resistance to these drugs.

MATERIALS AND METHODS

Test organisms. Three strains were used for this study: a reference *T. rubrum* strain (ATCC 28188) and two *T. rubrum* clinical isolates (CI-1 and CI-2). Strains CI-1 and CI-2 were isolated from clinical samples obtained at the Microbiology Unit of the Pisa University Hospital at the end of 2012 from different patients affected by onychomycosis. Patients declared that they were not undergoing topical or systemic antifungal ther-

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apy. This study was approved by the institutional review board. Strains were seeded on Sabouraud dextrose agar (SDA) and Dermasel agar plates and identified according to their microscopic and macroscopic features. *T. rubrum* ATCC MYA-4438 was included as a quality control isolate for broth dilution antifungal susceptibility testing as recommended by the CLSI (19). Strains were kept frozen at −70°C in Sabouraud dextrose broth (SDB; Becton, Dickinson) containing 30% glycerol. For each experiment, *T. rubrum* strains were taken from frozen stocks. Test organisms were routinely grown on SDA at 30°C.

Medium. Assays were performed using RPMI 1640 medium with L-glutamine but without bicarbonate, buffered to pH 7.0 with 0.165 M 3-(*N*-morpholino)ethanesulfonic acid (Trek Diagnostic Systems Inc., Cleveland, OH).

Antifungal agents. Stock solutions of TRB (Aminochemicals, Malta), ITC (Sigma-Aldrich, Switzerland), AMF (Sigma-Aldrich, Switzerland), and CPX (Erregierre, Italy) were obtained dissolving the drug powder (1 mg/ml) in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Stock solutions were serially diluted using RPMI 1640 to yield the concentrations required by the experiments.

Inoculum. *T. rubrum* was subcultured on SDA at 30°C for 7 to 15 days. All of the tested strains sporulated well after this period. Stock inoculum suspensions were obtained from each strain by covering the fungal colonies with sterile saline solution and gently rubbing the colonies with the tip of a transfer pipette. The resulting conidial suspensions were transferred to sterile tubes. Collected conidia were allowed to settle for 10 to 15 min, counted using a hemocytometer, and then adjusted to the required density by dilution in RPMI 1640. Plate counts were performed to verify the conidium concentrations by plating aliquots of the adjusted conidial suspensions on SDA.

MIC determination by the agar dilution assay. To set up the experimental conditions to test both the natural and the induced resistance to drugs, minimum inhibitory concentration (MIC) determinations were performed at inocula of 10^8 CFU/plate and 10^5 CFU/plate by the agar dilution method described by Yamaguchi and coworkers (20), with some modifications. Serially diluted drug solutions (0.1 ml) were transferred into sterile petri dishes. Liquefied SDA medium (9.9 ml) was added at 45°C and immediately mixed. Control plates were prepared without addition of drugs. A 100-μl volume of the suspensions of dermatophytes was seeded by spot inoculation onto the control plates and on the media containing the antifungal agents. Plates were incubated at 35°C for 4 days. The antifungal concentration inhibiting growth was defined as the lowest concentration preventing growth of macroscopically visible colonies on drug-containing plates when visible growth was present on the control plates.

Natural resistance of *T. rubrum* to antifungals. The natural resistance to antifungals was evaluated essentially as described by Osborne and colleagues (16). Briefly, stock inoculum suspensions of the dermatophytes were prepared to contain a total of about 10^9 CFU/ml. An aliquot of 0.1 ml of these suspensions was seeded on each SDA plate containing the antifungal agents at the concentrations inhibiting the growth of *T. rubrum* strains seeded at 10^8 CFU/plate. Colonies were counted after incubation at 30°C for 3 weeks. The resistance frequency to antifungals was calculated by dividing the number of CFU grown on the plates containing each drug by the total number of CFU spread on these plates. The colonies grown on the plates containing the antifungal agents were transferred to SDA plates containing 1- and 2-fold levels of the drug concentrations used for selection. Growth was checked after incubation for 1 week at 30°C.

Evolution of the antifungal drug resistance. Strains ATCC 28188 and CI-1 were selected for evaluating the *in vitro* evolution of resistance to TRB, ITC, AMF, and CPX in *T. rubrum*. Conidia of each strain were serially propagated for 10 transfers (7 days of incubation for each transfer) in SDA containing a 0.5-fold level of the MIC of each drug. Experiments were performed essentially as previously described for *Aspergillus fumigatus* (21). In each transfer, 100 μl of a suspension containing about 10^6 conidia/ml was inoculated onto SDA plates containing the drug concentrations described above and the plates were incubated at 30°C. After the 5th and the 10th transfers, all conidia were collected and seeded on SDA plates containing 2-fold the MIC of each drug.

Antifungal susceptibility testing. MICs were determined by the microtiter broth dilution method, as described for the CLSI M38-A2 standard methodology for the susceptibility testing of dermatophytes (19). Drug stocks were serially diluted in RPMI 1640 to yield twice the final strength required for the test and added (100 μl) to each well of 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany). Inoculum *T. rubrum* suspensions were adjusted to 1×10^3 to 3×10^3 conidia/ml in RPMI 1640. Inocula (in 100-μl aliquots) were combined 1:1 with the test drug solutions in the microtiter plates to bring drug and inocula to the final desired test concentrations. Growth (RPMI 1640–1% DMSO) and sterility (RPMI 1640) controls were included for each tested isolate. Each organism was tested in duplicate, and the experiments were repeated three times. The inoculated plates were incubated at 30°C for 4 to 7 days. MICs were determined visually, and the MIC endpoint was defined as the lowest concentration that caused a reduction of ≥80% in fungal growth compared to the growth in the control well (drug-free medium). MIC differences of ±1 2-fold dilution were not considered significant.

Stability of drug-resistant mutants. To ensure that resistant phenotypes were genetically stable in the absence of the drugs, selected mutants were serially propagated for three transfers (7 days incubation for each transfer) on nonselective SDA plates. After the third transfer, conidia were collected to determine the MIC by the microtiter broth dilution method, as described above.

RESULTS

Isolation of spontaneous antifungal-resistant *T. rubrum* mutants. To isolate spontaneous mutants resistant to TRB, ITC, AMF, or CPX, a total of about 10^9 CFU of the ATCC 28188, CI-1, and CI-2 *T. rubrum* strains was plated on solid growth media containing the minimum inhibitory drug concentration determined by the agar diffusion assay for each antifungal agent. The highest incidence of resistant mutants was obtained with ITC, which selected mutants in the three strains with resistance frequencies ranging from 2.97×10^{-7} to 7.51×10^{-7} (Table 1). The frequencies of naturally occurring AMF-resistant derivatives for the three strains ranged from 1.14×10^{-9} to 5.79×10^{-9}. Mutants resistant to TRB were obtained with strain ATCC 28188 (resistance frequency, 1.38×10^{-9}) and strain CI-2 (resistance frequency, 2.89×10^{-9}). No mutant resistant to CPX was obtained from any of the three *T. rubrum* strains. All colonies grown on the plates containing TRB or AMF and 10 randomly selected ITC-resistant colonies were able to grow when transferred to fresh plates containing 1- or 2-fold the amount of drug used for selection.

***In vitro* evolution of TRB, ITC, AMF, or CPX resistance in *T. rubrum*.** To analyze any *in vitro* evolution of resistance to TRB, ITC, AMF, or CPX, one clinical *T. rubrum* isolate (CI-1) and the reference ATCC 28188 strain were serially subcultured (10 transfers) on plates containing 0.5-fold the minimum drug concentration inhibiting growth on solid medium. Abundant fungal growth was obtained with both strains using these subinhibitory drug concentrations. After the 5th and the 10th transfer, conidia were collected and seeded on SDA plates containing 4-fold the drug concentrations used for transfers. After the 5th transfer, mutants with increased resistance to ITC were isolated from ATCC 28188 (resistance frequency, 9.6×10^{-5}) and CI-1 (resistance frequency, 9.0×10^{-6}) (Table 2). From ATCC 28188, mutants with increased resistance to TRB were also selected (resistance frequency, 1.3×10^{-7}). After the 10th transfer, ATCC 28188 and CI-1 derivatives resistant to TRB, ITC, and AMF concentrations were isolated (see

TABLE 1 Spontaneous drug-resistant *T. rubrum* mutants obtained following direct selection on plates containing inhibitory drug concentrations

Strain	Parameter[a]	Value			
		Terbinafine	Itraconazole	Amorolfine	Ciclopirox
ATCC 28188	Total no. of CFU plated	7.2×10^8	7.2×10^8	7.2×10^8	7.2×10^8
	No. of resistant colonies	1	490	3	0
	Resistance frequency	1.4×10^{-9}	6.8×10^{-7}	4.1×10^{-9}	$<1.4 \times 10^{-9}$
CI-1	Total no. of CFU plated	8.7×10^8	8.7×10^8	8.7×10^8	8.7×10^8
	No. of resistant colonies	0	654	1	0
	Resistance frequency	$<1.1 \times 10^{-9}$	7.5×10^{-7}	1.1×10^{-9}	$<1.1 \times 10^{-9}$
CI-2	Total no. of CFU plated	6.9×10^8	6.9×10^8	6.9×10^8	6.9×10^8
	No. of resistant colonies	2	205	4	0
	Resistance frequency	2.9×10^{-9}	3.0×10^{-7}	5.8×10^{-9}	$<1.4 \times 10^{-9}$

[a] Resistance frequency data were calculated by dividing the number of CFU grown on the plates containing each drug by the total number of CFU spread on plates.

Table 2 for resistance frequencies). In the presence of subinhibitory drug concentrations, resistance to AMF, TRB, and ITC emerged rapidly in *T. rubrum*, with a frequency almost 100-fold higher than that of spontaneous drug resistance (Table 2). No CPX-resistant mutant emerged following exposure to subinhibitory CPX concentrations after 5 and 10 transfers.

Antifungal resistance of selected mutants. To evaluate the spectrum of resistance of the isolated mutants, MIC values of TRB, ITC, AMF, and CPX were determined by the broth microdilution assay. We analyzed all TRB- and AMF-resistant mutants and 5 randomly selected ITC-resistant mutants obtained from each parental strain following direct selection (naturally occurring mutants [S mutants]) or exposure to subinhibitory drug concentrations before selection (I mutants). The parental *T. rubrum* strains ATCC 28188, C-1, and CI-2 were assayed in parallel. For all these strains, the MICs of TRB, ITC, AMF, and CPX were 0.01 μg/ml, 0.08 μg/ml, 0.02 μg/ml, and 0.5 μg/ml, respectively. The results obtained with the quality control strain *T. rubrum* MYA-4438, which served as an internal control, were within the acceptable ranges recommended by the CLSI (i.e., MIC of CPX = 0.5 μg/ml).

Table 3 reports the fold increase in the MIC values of TRB, ITC, AMF, and CPX for the mutants compared to their parental strains. Mutants are clustered together on the basis of the drug used for selection, the parental strain from which they derived, and the method used for isolation (S or I mutants).

A considerable increase in the MIC values of the antifungals that were used for selection was observed for the mutants compared to the parental strains. TRB-resistant mutants showed a 500- or 1,000-fold increase in the MIC values of TRB, ITC-resistant mutants a 4- or 8-fold increase in the MIC values of ITC, and AMF-resistant mutants 16- to 64-fold increases in the MIC values of AMF. Interestingly, the ITC-resistant mutants also showed increased MIC values of AMF (8- or 32-fold) and TRB (4- or 8-fold) and AMF-resistant mutants increased resistance to TRB (from 4- to 16-fold). No variation in the MICs of ITC and AMF was observed for TRB-resistant mutants. No substantial differences were observed in the MICs of resistant S and I mutants.

None of the mutants with increased resistance to ITC, AMF, or TRB showed altered MICs of CPX compared to the parental strains.

Analysis of the stability of the drug-resistant mutants following three transfers on nonselective medium revealed that all AMF-resistant mutants were genetically stable, while 5 of the 11 TRB-resistant mutants and 7 of the 25 ITC-resistant mutants exhibited restored susceptibility to TRB and ITC, respectively.

DISCUSSION

Routine antifungal susceptibility testing is not carried out in the case of isolation of dermatophytes from clinical samples. Therefore, it is difficult to define whether common relapses of dermatophytoses after the interruption of an antifungal therapy are due to poor compliance with the treatment or to infection with antifungal-resistant strains.

In this study, we aimed to evaluate the *in vitro* emergence of naturally occurring *T. rubrum* mutants resistant to TRB, ITC, AMF, and CPX and the evolution of resistance to these antifungals following exposure to subinhibitory drug concentrations.

The analysis of how frequently spontaneous drug-resistant *T. rubrum* mutants occur *in vitro* showed that the highest incidence of mutants is obtained with ITC (frequency of about 10^{-7}). This result can be explained by the fact that fungistatic drugs, such as

TABLE 2 *In vitro* evolution of drug resistance in *T. rubrum* following exposure to subinhibitory drug concentrations for 5 or 10 transfers before selection

Strain	Transfer	Resistance frequency[a]			
		Terbinafine	Itraconazole	Amorolfine	Ciclopirox
ATCC 28188	5th	1.3×10^{-7}	9.6×10^{-5}	$<1.5 \times 10^{-8}$	$<6.0 \times 10^{-9}$
	10th	9.1×10^{-6}	2.4×10^{-4}	2.2×10^{-8}	$<1.3 \times 10^{-8}$
CI-1	5th	$<5.2 \times 10^{-8}$	9.0×10^{-6}	$<3.4 \times 10^{-8}$	$<1.7 \times 10^{-8}$
	10th	4.4×10^{-7}	2.6×10^{-5}	9.1×10^{-8}	$<1.2 \times 10^{-8}$

[a] Resistance frequency data were calculated by dividing the number of CFU grown on the plates containing each drug by the total number of CFU spread on plates.

TABLE 3 Fold increase in the MICs of terbinafine, itraconazole, amorolfine, and ciclopirox for drug-resistant *T. rubrum* mutants compared to their parental strains[a]

Mutant and drug susceptibility	Fold MIC increase			
	Terbinafine	Itraconazole	Amorolfine	Ciclopirox
ATCC 28188				
TRB-S ($n = 1$)	50	ns	ns	ns
ITC-S ($n = 5$)	4–8	4–8	4–8	ns
AMF-S ($n = 3$)	4–8	ns	16–32	ns
TRB-I ($n = 3$)	500–1,000	ns	ns	ns
ITC-I ($n = 5$)	8	4–8	32	ns
AMF-I ($n = 3$)	8–16	ns	64	ns
CI-1				
ITC-S ($n = 5$)	4–8	4–8	4–8	ns
AMF-S ($n = 1$)	4	ns	16	ns
TRB-I ($n = 5$)	500	ns	ns	ns
ITC-I ($n = 5$)	8	4–8	32	ns
AMF-I ($n = 5$)	8–16	ns	32–64	ns
CI-2				
TRB-S ($n = 2$)	500–1,000	ns	ns	ns
ITC-S ($n = 5$)	4–8	4–8	4–8	ns
AMF-S ($n = 4$)	4–8	ns	8–16	ns

[a] Mutants were obtained following direct selection on plates containing inhibitory drug concentrations on the basis of selection (TBR, terbinafine; ITC, itraconazole; AMF, amorolfine) and the parental strain from which they derived. *n*, number of strains; ns, not significant (MIC differences of ±1 2-fold dilution were considered not significant).

the triazoles, have the potential to leave more fungal survivors than fungicidal drugs (such as TRB, AMF, and CPX), and this larger effective population can contribute to a higher probability of resistance in the pathogen (21). A variety of biochemical and molecular mechanisms have been shown to contribute to drug resistance in eukaryotes (22). The resistance of dermatophytes to inhibiting agents involves the participation of target-enzyme modifiers and overexpression of ATP-binding cassette (ABC) transporters and stress-related proteins (23). In *T. rubrum*, two ABC transporters, TruMDR1 and TruMDR2, were identified as responsible for resistance to various antifungal drugs, such as ITC and TRB (24, 25). *Candida albicans* clinical isolates resistant to azole compounds and overexpressing the ABC transporter gene products CDR1 and CDR2 are less susceptible to the morpholine derivative AMF (26). Our finding that ITC-resistant mutants display increased MIC values of TRB and AMF (Table 3) suggests that ABC transporters play a role in the cross-resistance to these antifungals in *T. rubrum*. However, the observation that 28% of the ITC-resistant mutants exhibit restored susceptibility following three passages on nonselective medium suggests that other mechanisms can be involved in the development of ITC resistance in *T. rubrum*.

Spontaneous resistance to TRB occurred at a low frequency (about 10^{-9}), in agreement with previous data reported by Osborne and coworkers (16). As already suggested, this low frequency of isolation appears to be compatible with resistance based on a single nonsilent nucleotide substitution in the gene encoding squalene epoxidase (27). Three results of the present study further support this hypothesis: (i) the high level of resistance to TRB displayed by TRB-resistant mutants (Table 3), (ii) the observation that TRB-resistant mutants do not display increased resistance to ITC and AMF (Table 3), which inhibit the activity of different enzymes in the ergosterol biosynthesis pathway (8, 9), and (iii) the restored susceptibility to TRB observed in 45% of the TRB-resistant mutants when drug pressure was removed.

To our knowledge, this is the first report describing the acquisition of resistance to AMF by *T. rubrum*. Spontaneous mutants resistant to AMF were isolated at a low frequency (about 10^{-9}). In some yeasts, resistance to AMF has been associated with overexpression of ABC transporter genes and with cross-resistance to TRB (26, 28). Although the mechanisms that underlie resistance of dermatophytes to AMF are unknown, our finding that AMF-resistant *T. rubrum* mutants are also resistant to TRB suggests that drug efflux by ABC transporters can also contribute to drug resistance in this organism. The finding that AMF resistance is conserved after three passages in the absence of the drug further supports this hypothesis.

The emergence of drug resistance in all pathogenic microorganisms is an evolutionary process initiated by the exposure to antimicrobial agents. The emergence of resistance is a function of the rate of mutation to resistance and the size of the surviving population. *T. rubrum* acquisition of drug resistance during therapy may be responsible for relapses following cessation of the antifungal therapy, especially in the case of infections involving prolonged therapy with relatively low drug concentrations. The emergence of *T. rubrum* strains with greatly reduced susceptibility to TRB was previously reported in patients following prolonged therapy for onychomycosis (14). In order to investigate whether *T. rubrum* develops resistant mutants upon exposition to drugs *in vitro*, we grew *T. rubrum* in media containing subinhibitory concentrations of TRB, ITC, AMF, or CPX. In the presence of ITC, mutants with elevated resistance to ITC were isolated at a higher frequency than was seen with spontaneous mutation. A decreased susceptibility to ITC has already been described for *T. rubrum* subjected to sequential passages in the presence of the azole compound (17). Our results determined with TRB and AMF indicate that prolonged exposure to subinhibitory concentrations of these drugs also leads to significant loss of susceptibility (resistance frequency, about 10^{-7} to 10^{-8}). It can be speculated, therefore, that *T. rubrum* can more easily develop resistance to ITC, TRB, and AMF at infection sites in which drug concentrations are not appropriately reached during therapy.

In this study, no difference was observed in the MICs of mutants obtained following direct selection (S mutants) or following exposure to subinhibitory drug concentrations before selection (I mutants). This result suggests that growth in the presence of subinhibitory drug concentrations, despite increasing the frequency of isolation of resistant strains, does not cause variations in the level of resistance compared to naturally occurring mutants.

CPX is a hydroxypyridone that is chemically and mechanically different from other antifungal agents, such as azole derivates, allylamines, and morpholines. Unlike those antifungal agents, CPX does not affect sterol biosynthesis and is not metabolized via cytochrome P450 (12). Interestingly, in the present study, no mutant resistant to CPX was obtained, thus suggesting that, under our experimental conditions, *T. rubrum* has no biochemical or molecular capacity to develop resistance to this drug, even after prolonged exposure to subinhibitory drug concentrations for several growth generations. Lack of tolerance of or resistance to ciclopirox in tests of this drug against *C. albicans* has previously been published (29). The multiplicity of the mechanisms of action

of CPX may explain why there is no development of resistance to this drug, unlike the results seen with other antifungal drugs, and why this is the case not only in yeasts but also in dermatophytes.

In conclusion, the overall results obtained in this study indicate that spontaneous *T. rubrum* mutants resistant to AMF, TRB, and ITC can be isolated, although at low frequency, and that subinhibitory drug concentrations facilitate the emergence of resistant strains. In addition, our data indicate that, among the tested drugs, CPX is the only compound able to maintain the same efficacy against a wild-type population of *T. rubrum* and after prolonged use at subinhibitory concentrations. Its use in the topical therapy of dermatophytoses could contribute to reducing the relapse rate due to selection of drug-resistant strains.

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REFERENCES

1. **Vena GA, Chieco P, Posa F, Garofalo A, Bosco A, Cassano N.** 2012. Epidemiology of dermatophytoses: retrospective analysis from 2005 to 2010 and comparison with previous data from 1975. New Microbiol. **35:** 207–213.
2. **Drakensjö IT, Chryssanthou E.** 2011. Epidemiology of dermatophyte infections in Stockholm, Sweden: a retrospective study from 2005–2009. Med. Mycol. **49:**484–488. http://dx.doi.org/10.3109/13693786.2010.540045.
3. **Welsh O, Vera-Cabrera L, Welsh E.** 2010. Onychomycosis. Clin. Dermatol. **28:**151–159. http://dx.doi.org/10.1016/j.clindermatol.2009.12.006.
4. **Havlickova B, Czaika VA, Friedrich M.** 2008. Epidemiological trends in skin mycoses worldwide. Mycoses **51**(Suppl 4)**:**2–15. http://dx.doi.org/10.1111/j.1439-0507.2008.01606.x.
5. **Simonnet C, Berger F, Gantier JC.** 2011. Epidemiology of superficial fungal diseases in French Guiana: a three-year retrospective analysis. Med. Mycol. **49:**608–611. http://dx.doi.org/10.3109/13693786.2011.558929.
6. **Seebacher C, Bouchara JP, Mignon B.** 2008. Updates on the epidemiology of dermatophyte infections. Mycopathologia **166:**335–352. http://dx.doi.org/10.1007/s11046-008-9100-9.
7. **Gupta AK, Cooper EA.** 2008. Update in antifungal therapy of dermatophytosis. Mycopathologia **166:**353–367. http://dx.doi.org/10.1007/s11046-008-9109-0.
8. **Georgopapadakou NH, Walsh TJ.** 1996. Antifungal agents: chemotherapeutic targets and immunologic strategies. Antimicrob. Agents Chemother. **40:**279–291.
9. **Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL.** 2003. A point mutation in the 14alpha-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **47:**1120–1124. http://dx.doi.org/10.1128/AAC.47.3.1120-1124.2003.
10. **Finlay AY.** 1994. Global overview of Lamisil. Br. J. Dermatol. **130:**1–3. http://dx.doi.org/10.1111/j.1365-2133.1994.tb06082.x.
11. **Banerjee M, Ghosh AK, Basak S, Das KD, Gangopadhyay DN.** 2011. Comparative evaluation of effectivity and safety of topical amorolfine and clotrimazole in the treatment of *tinea corporis*. Indian J. Dermatol. **56:**657–662. http://dx.doi.org/10.4103/0019-5154.91823.
12. **Subissi A, Monti D, Togni G, Mailland F.** 2010. Ciclopirox: recent nonclinical and clinical data relevant to its use as a topical antimycotic agent. Drugs **70:**2133–2152. http://dx.doi.org/10.2165/11538110-000000000-00000.
13. **Weitzman I, Summerbell RC.** 1995. The dermatophytes. Clin. Microbiol. Rev. **8:**240–259.
14. **Mukherjee PK, Leidich SD, Isham N, Leitner I, Ryder NS, Ghannoum MA.** 2003. Clinical *Trichophyton rubrum* strain exhibiting primary resistance to terbinafine. Antimicrob. Agents Chemother. **47:**82–86. http://dx.doi.org/10.1128/AAC.47.1.82-86.2003.
15. **Bouchara JP, Mignon B, Chatuvedi V.** 2008. Dermatophytes and dermatophytosis: a reappraisal for the twenty-first century. Mycopathologia **166:**235–237. http://dx.doi.org/10.1007/s11046-008-9156-6.
16. **Osborne CS, Hofbauer B, Favre B, Ryder NS.** 2003. *In vitro* analysis of the ability of *Trichophyton rubrum* to become resistant to terbinafine. Antimicrob. Agents Chemother. **47:**3634–3636. http://dx.doi.org/10.1128/AAC.47.11.3634-3636.2003.
17. **Hryncewicz-Gwóźdź A, Kalinowska K, Plomer-Niezgoda E, Bielecki J, Jagielski T.** 2013. Increase in resistance to fluconazole and itraconazole in *Trichophyton rubrum* clinical isolates by sequential passages *in vitro* under drug pressure. Mycopathologia **176:**49–55. http://dx.doi.org/10.1007/s11046-013-9655-y.
18. **Khan MS, Ahmad I.** 2011. Antifungal activity of essential oils and their synergy with fluconazole against drug-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum*. Appl. Microbiol. Biotechnol. **90:**1083–1094. http://dx.doi.org/10.1007/s00253-011-3152-3.
19. **Clinical and Laboratory Standards Institute.** 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard—second ed M38-A2. CLSI, Wayne, PA.
20. **Yamaguchi H, Uchida K, Hiratani T, Hara T, Fukuyasu H, Kazuno Y, Inouye S.** 1986. In vitro activity of ME1401, a new antifungal agent. Antimicrob. Agents Chemother. **30:**705–712. http://dx.doi.org/10.1128/AAC.30.5.705.
21. **da Silva Ferreira ME, Capellaro JL, dos Reis Marques E, Malavazi I, Perlin D, Park S, Anderson JB, Colombo AL, Arthington-Skaggs BA, Goldman MH, Goldman GH.** 2004. *In vitro* evolution of itraconazole resistance in *Aspergillus fumigatus* involves multiple mechanisms of resistance. Antimicrob. Agents Chemother. **48:**4405–4413. http://dx.doi.org/10.1128/AAC.48.11.4405-4413.2004.
22. **Peres NTA, Maranhão FCA, Rossi A, Martinez-Rossi NM.** 2010. Dermatophytes: host-pathogen interaction and fungal resistance. An. Bras. Dermatol. **85:**657–667. http://dx.doi.org/10.1590/S0365-05962010000500009.
23. **Martinez-Rossi NM, Peres NT, Rossi A.** 2008. Antifungal resistance mechanisms in dermatophytes. Mycopathologia **166:**369–383. http://dx.doi.org/10.1007/s11046-008-9110-7.
24. **Maranhão FCA, Paião FG, Fachin AL, Martinez-Rossi NM.** 2009. Membrane transporter proteins are involved in *Trichophyton rubrum* pathogenesis. J. Med. Microbiol. **58:**163–168. http://dx.doi.org/10.1099/jmm.0.002907-0.
25. **Fachin AL, Ferreira-Nozawa MS, Maccheroni W, Jr, Martinez-Rossi NM.** 2006. Role of the ABC transporter TruMDR2 in terbinafine, 4-nitroquinoline N-oxide and ethidium bromide susceptibility in *Trichophyton rubrum*. J. Med. Microbiol. **55:**1093–1099. http://dx.doi.org/10.1099/jmm.0.46522-0.
26. **Vanden Bossche H, Dromer F, Improvisi I, Lozano-Chiu M, Rex JH, Sanglard D.** 1998. Antifungal drug resistance in pathogenic fungi. Med. Mycol. **36**(Suppl 1)**:**119–128.
27. **Osborne CS, Leitner I, Favre B, Ryder NS.** 2005. Amino acid substitution in *Trichophyton rubrum* squalene epoxidase associated with resistance to terbinafine. Antimicrob. Agents Chemother. **49:**2840–2844. http://dx.doi.org/10.1128/AAC.49.7.2840-2844.2005.
28. **Vanden Bossche H.** 1997. Mechanisms of antifungal resistance. Rev. Iberoam. Micol. **14:**44–49.
29. **Niewerth M, Kunze D, Seibold M, Schaller M, Korting HC, Hube B.** 2003. Ciclopirox olamine treatment affects the expression pattern of *Candida albicans* genes encoding virulence factors, iron metabolism proteins, and drug resistance factors. Antimicrob. Agents Chemother. **47:**1805–1817. http://dx.doi.org/10.1128/AAC.47.6.1805-1817.2003.