British Journal of Dermatology 2003; **149:** 296–305.

Clinical and Laboratory Investigations

In vitro susceptibility testing of ciclopirox, terbinafine, ketoconazole and itraconazole against dermatophytes and nondermatophytes, and *in vitro* evaluation of combination antifungal activity

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Accepted for publication 9 December 2002

Summary

Background With the development of newer antifungal agents with activity against both yeasts and filamentous fungi, there is an increased need to develop and standardize *in vitro* assays that will evaluate the activity of antimycotics against filamentous fungi. *In vitro* analysis of antifungal activity of these agents would also allow for the comparison between different antimycotics, which in turn may clarify the reasons for lack of clinical response or serve as an effective therapy for patients with chronic infection.

Objectives To determine the *in vitro* susceptibility of fungal organisms to ciclopirox, terbinafine, ketoconazole and itraconazole and to evaluate the *in vitro* activity and mode of interaction of ciclopirox in combination with either terbinafine or itraconazole.

Materials and methods In the minimum inhibitory concentration (MIC) study 133 strains were evaluated, including dermatophytes (110 strains; 98 from *Trichophyton* spp.), *Candida* spp. (14 strains) and nondermatophyte moulds (nine strains). *In vitro* susceptibility testing was conducted in microbroth dilutions based on the National Committee for Clinical Laboratory Standards (NCCLS) M27-A proposed standard. The testing MIC ranges were 0·003–2 μg mL^{-1} for ciclopirox and terbinafine, and 0·06–32 μg mL^{-1} for itraconazole and ketoconazole. For inoculum preparation, dermatophytes were grown on Heinz oatmeal cereal agar slants. Inoculum suspensions of dermatophytes were diluted in RPMI 1640 (Sigma-Aldrich) with the desired final concentration being 2–5 × 10^3 c.f.u. mL^{-1}. Once inoculated, the microdilution plates were set up according to the NCCLS M27-A method, incubated at 35 °C, and read visually following 7 days of incubation. For azole agents, the MIC was the lowest concentration showing 80% growth inhibition; for terbinafine and ciclopirox, the MIC was the lowest concentration showing 100% growth inhibition. In the synergy studies, 29 strains from nondermatophyte species were evaluated using a checkerboard microdilution method. The concentrations tested were: 0 and 0·06–32 μg mL^{-1} for itraconazole, and 0 and 0·003–4 μg mL^{-1} for both terbinafine and ciclopirox. Modes of interaction between drugs were classified as synergism, additivism, antagonism or indifference based on fractional inhibitory concentration index values (FIC index). Synergism was defined as an FIC index of ≤ 0·50, additivity as an FIC index of ≤ 1·0, and antagonism as an FIC index of ≥ 2·0. The drug combination was interpreted as indifferent if neither of the drugs had any visible effect on the presence of the other drug.

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Results In the MIC study, the dermatophyte MIC values (μg mL^{-1}) (mean ± SEM) were: ciclopirox (0·04 ± 0·02), terbinafine (0·04 ± 0·23), itraconazole (2·28 ± 7·42) and ketoconazole (0·83 ± 1·99). The yeast MIC values (μg mL^{-1}) (mean ± SEM) were: ciclopirox (0·05 ± 0·02), terbinafine (1·77 ± 0·58), itraconazole (0·18 ± 0·27) and ketoconazole (0·56 ± 0·60). The nondermatophyte fungi MIC values (μg mL^{-1}) (mean ± SEM) were: ciclopirox (1·04 ± 2·62), terbinafine (1·04 ± 0·95), itraconazole (17·87 ± 16·75) and ketoconazole (10·69 ± 13·09). In the synergy study, with ciclopirox in combination with terbinafine, mainly a synergistic or additive reaction was observed; there were no cases of antagonism. For ciclopirox in combination with itraconazole, there were some instances of additivism or synergism, with indifference in the majority of instances; there were no cases of antagonism.

Conclusions *In vitro* susceptibility testing indicates that ciclopirox may have a broad antimicrobial profile including dermatophytes, yeasts and other nondermatophytes. Terbinafine is extremely potent against dermatophytes. *In vitro* evaluation of activity of ciclopirox and terbinafine suggests many instances of synergy or additivism; for ciclopirox and itraconazole there may be indifference, synergy or additivism.

Key words: antifungal agents, ciclopirox, *in vitro*, itraconazole, susceptibility testing, terbinafine

An increasing number of antimycotics have become available for the treatment of dermatophytoses; at the same time, however, there are reports suggesting recalcitrance to therapy or possibly even resistance of a dermatophyte against the antimicrobial agent.[1–3] *In vitro* analysis of the antifungal activity of these agents enables comparison between different antimycotics, which in turn may clarify the reasons for lack of clinical response and assist clinicians in choosing an effective therapy for their patients. However, it is important that the methodologies used for *in vitro* testing be standardized to facilitate the establishment of quality control parameters and interpretive break points. Such standard methods are being developed by the National Committee for Clinical Laboratory Standards (NCCLS). To date, however, efforts by the NCCLS have focused primarily on testing yeasts and have resulted in the establishment of a standardized reference method for antifungal susceptibility testing of this group of fungal organisms.[4] As newer antifungal agents with activity against both yeasts and filamentous fungi are being developed, the impetus is to develop and standardize *in vitro* assays that will evaluate the activity of antimycotics against filamentous fungi. Recently, the NCCLS proposed a standard, M38-P, for determining the minimum inhibitory concentrations (MICs) of several antifungal agents against conidium-forming filamentous fungi; however, dermatophytes were not included in this document.[5] Although a standard method for susceptibility testing of dermatophytes is lacking, there are several reports where antimicrobial susceptibility testing of dermatophytes has been conducted using either agar macrodilution or broth macrodilution/microdilution tests; these reports have generally been extensions of either the M27-A or M38-P methodologies.[6–11]

Antifungal agents used for the treatment of onychomycosis, such as terbinafine (an allylamine), itraconazole (an azole) and ciclopirox (a hydroxypiridone), have substantial activity against dermatophytes.[6,12,13] While some work has been published using the NCCLS standards to determine the antifungal susceptibility of various fungi and yeasts to terbinafine[7–10,14] and itraconazole,[7–11,14] only one study,[7] using older methodology, has looked at ciclopirox olamine. The present study evaluates ciclopirox olamine with methodology similar to that reported in other recent work, where other antifungal agents have been used for dermatophyte susceptibility testing. We also performed tests of the activity of combinations of ciclopirox and terbinafine, and ciclopirox and itraconazole. Terbinafine and itraconazole are both used as systemic agents in the treatment of onychomycosis. Recently, there has also been interest in the treatment potential of combination therapy with an oral agent and topical ciclopirox lacquer.[15,16] However, to our knowledge, there are no reports evaluating the activity of combination therapy *in vitro*, when ciclopirox is used in conjunction with terbinafine or itraconazole.

This study builds on recent work towards establishing a reference susceptibility testing method for dermatophytes, yeasts and nondermatophyte moulds. Recently, in an attempt at standardizing the antifungal susceptibility testing of dermatophytes Norris *et al.*[14]

evaluated variables such as inoculum size, temperature and duration of incubation, media and endpoint determination that can greatly affect the MICs. This study was further extended by Jessup *et al.*[9] in developing an appropriate medium for conidial formation of dermatophytes, especially *Trichophyton rubrum* and by determining the antifungal susceptibilities of a large number of dermatophytes to four antifungal agents. The parameters proposed by these investigators form the foundation for a standardized antifungal susceptibility testing method for dermatophytes.

In the present study we have used Heinz oatmeal cereal (H.J.Heinz Company of Canada Ltd, North York, ON, Canada) agar as the optimal medium to support the conidial growth of all dermatophytes and determined the antifungal activity of ciclopirox against dermatophytes, yeasts and other filamentous fungi; three commonly used antifungal agents have been used for comparison. We also evaluated the mode of interaction of ciclopirox when used *in vitro* in combination with terbinafine and itraconazole against some nondermatophyte moulds.

Materials and methods

Strains

In vitro *susceptibility testing.* One hundred and thirty-three strains from various dermatophytes, yeasts and other nondermatophyte species were evaluated (Table 1; dermatophytes are categorized based on the molecular taxonomic classification by Gräser *et al.*[17] and Summerbell *et al.*[18]). Among 110 isolates of dermatophytes, 98 were from nine *Trichophyton* spp.,

Table 1. Minimum inhibitory concentration (MIC) ranges of ciclopirox and terbinafine against 133 strains of dermatophytes, yeasts and nondermatophyte moulds

Organism	Isolates (*n*)	MIC range [MIC_{50}] (μg mL^{-1})	
		Ciclopirox	Terbinafine
Dermatophytes[a]			
Trichophyton rubrum clade			
T. rubrum	68	0·015–0·125 [0·03]	0·003–1 [0·003]
T. raubitschekii	3	0·015–0·03 [0·03]	0·003 [0·003]
T. soudanense	2	0·03 [0·03]	0·003–0·007
T. violaceum	1	0·03	0·003
Arthroderma vanbreuseghemii clade			
T. mentagrophytes	14	0·03–0·125 [0·03]	0·003–0·5 [0·003]
T. krajdenii	2	0·03	0·003–0·015
Arthroderma benhamiae clade			
A. benhamiae	2	0·03	0·003
T. verrucosum	1	0·06	0·007
T. tonsurans	5	0·03–0·06 [0·06]	0·003–2 [0·003]
T. terrestre	2	0·03	0·003–0·5
Microsporum gypseum	3	0·03–0·06 [0·03]	0·003–0·015 [0·003]
M. cookei	2	0·03 [0·03]	0·003 [0·003]
M. canis	1	0·03	0·015
M. persicolor	1	0·03	0·003
Epidermophyton floccosum	3	0·015–0·03 [0·03]	0·003
Yeasts			
Candida parapsilosis	6	0·06 [0·06]	0·5 ≥ 2 [2]
C. albicans	3	0·03 [0·03]	> 2 [> 2]
C. glabrata	3	0·03–0·06 [0·06]	> 2 [> 2]
C. tropicalis	1	0·03	> 2
C. krusei	1	0·06	> 2
Nondermatophytes			
Scytalidium dimidiatum	3	0·125–0·25 [0·25]	0·125–2 [2]
Aphanoascus fulvescens	1	0·03	2
Aspergillus sydowii	1	0·5	0·015
Chaetomium globosum	1	0·03	2
Fusarium proliferatum	1	8	0·125
Onychocola canadensis	1	0·03	0·125
Scopulariopsis brevicaulis	1	0·125	1

[a]Dermatophytes are categorized on the basis of molecular taxonomic classification of these species by Gräser *et al.*[17] and Summerbell *et al.*[18] MIC_{50}, MIC for 50% of the isolates.

seven were from four *Microsporum* spp. and three were from *Epidermophyton* spp.; additionally two strains of *Arthroderma benhamiae* (a teleomorph of *Trichophyton*) were also included (Tables 1 and 2). All strains were clinical isolates obtained from nail, skin or hair specimens received by Mediprobe Laboratories Inc. (London, ON, Canada), or the Public Health Laboratories of Ontario (PHLO) for routine mycological and culture analysis. In addition, there were 14 yeast isolates from five *Candida* species and nine isolates from seven other nondermatophyte species. Two American Type Culture Collection (ATCC) *Candida* strains were also included as reference controls. The technologists at Mediprobe Laboratories Inc. or PHLO performed primary isolation and identification of all strains to the species level. The pure cultures were maintained on plain Sabouraud (SAB) or Sabouraud–cycloheximide, chloramphenicol, gentamicin (SAB–CCG) slants. The isolates were stored in water at room temperature until the time of use. Freshly growing cultures of dermatophytes on SAB–CCG slants were transferred to oatmeal agar slants 2 weeks prior to set-up of the experiment to enhance conidial production, as recommended by Jessup *et al.*[9]

Table 2. Comparison of minimum inhibitory concentration (MIC) values of ciclopirox, terbinafine, itraconazole and ketoconazole against dermatophytes

		MIC statistics (μg mL^{-1})			
Species (*n*)	Antifungal agent	MIC range	G-MIC	MIC_{50}	MIC_{90}
T. rubrum (68)	Ciclopirox	0·015–0·125	0·031	0·03	0·06
	Terbinafine	0·003–1	0·003	0·003	0·003
	Itraconazole	0·06–32	0·143	0·06	1
	Ketoconazole	0·06–8	0·165	0·06	2
T. raubitschekii (3)	Ciclopirox	0·015–0·03	0·024	0·03	0·03
	Terbinafine	0·003	0·003	0·003	0·003
	Itraconazole	0·06–0·125	0·077	0·06	0·125
	Ketoconazole	0·03–1	0·122	0·06	1
T. soudanense (2)	Ciclopirox	0·03	0·03	–[a]	–
	Terbinafine	0·003–0·007	0·005	–	–
	Itraconazole	0·06	0·06	–	–
	Ketoconazole	0·06–0·5	0·173	–	–
T. violaceum (1)	Ciclopirox	0·03	–	–	–
	Terbinafine	0·003	–	–	–
	Itraconazole	0·06	–	–	–
	Ketoconazole	0·06	–	–	–
T. mentagrophytes (14)	Ciclopirox	0·03–0·125	0·039	0·03	0·06
	Terbinafine	0·003–0·5	0·005	0·003	0·015
	Itraconazole	0·06–32	0·129	0·06	0·25
	Ketoconazole	0·125–2	0·344	0·25	1
T. krajdenii (2)	Ciclopirox	0·03	0·03	–	–
	Terbinafine	0·003–0·015	0·007	–	–
	Itraconazole	0·06–0·5	0·173	–	–
	Ketoconazole	0·25–0·5	0·354	–	–
Arthroderma benhamiae (2)	Ciclopirox	0·03	0·03	–	–
	Terbinafine	0·003	0·003	–	–
	Itraconazole	0·25–32	2·828	–	–
	Ketoconazole	0·06–8	0·693	–	–
T. verrucosum (1)	Ciclopirox	0·06	–	–	–
	Terbinafine	0·007	–	–	–
	Itraconazole	2	–	–	–
	Ketoconazole	4	–	–	–
T. tonsurans (5)	Ciclopirox	0·03–0·06	0·05	0·06	0·06
	Terbinafine	0·003–2	0·015	0·003	2
	Itraconazole	0·06–32	0·288	0·06	32
	Ketoconazole	0·06–1	0·173	0·25	1

Table 2. (Continued)

Species (*n*)	Antifungal agent	MIC statistics (μg mL^{-1})			
		MIC range	G-MIC	MIC_{50}	MIC_{90}
T. terrestre (2)	Ciclopirox	0·03	0·03	–	–
	Terbinafine	0·003–0·5	0·039	–	–
	Itraconazole	0·06–0·25	0·122	–	–
	Ketoconazole	0·06–0·125	0·087	–	–
Microsporum gypseum (3)	Ciclopirox	0·03–0·06	0·038	0·03	0·06
	Terbinafine	0·003–0·015	0·005	0·003	0·015
	Itraconazole	0·06–1	0·247	0·25	1
	Ketoconazole	0·25–8	1·0	0·5	8
Microsporum cookei (2)	Ciclopirox	0·03	0·03	–	–
	Terbinafine	0·003	0·003	–	–
	Itraconazole	1–2	1·414	–	–
	Ketoconazole	1–2	1·414	–	–
Microsporum canis (1)	Ciclopirox	0·03	–	–	–
	Terbinafine	0·015	–	–	–
	Itraconazole	1	–	–	–
	Ketoconazole	16	–	–	–
Microsporum persicolor (1)	Ciclopirox	0·03	–	–	–
	Terbinafine	0·003	–	–	–
	Itraconazole	0·25	–	–	–
	Ketoconazole	1	–	–	–
Epidermophyton floccosum (3)	Ciclopirox	0·015–0·03	0·024	0·03	0·03
	Terbinafine	0·003	0·003	0·003	0·003
	Itraconazole	0·06–0·25	0·123	0·125	0·5
	Ketoconazole	0·125–0·25	0·157	0·125	0·25

[a]Calculations could not be performed because of small sample size. G-MIC, geometric mean MIC; MIC_{50}, MIC for 50% of the isolates; MIC_{90}, MIC for 90% of the isolates.

Synergy testing. Twenty-nine strains from six non-dermatophyte species were tested (Table 3). These strains were obtained from the reference laboratory at the University of Alberta Mycological Herbarium (UAMH) as lyophilized powder, and cultures were revived and maintained as per the recommended protocol by UAMH. Cultures were maintained at ambient temperature on potato dextrose agar (PDA) slants and were stored in water at room temperature until the time of use.

Antifungal agents

Susceptibility testing. For *in vitro* testing we used four antifungal agents: ciclopirox (Aventis, Dermik Laboratories, Berwyn, PA, U.S.A.), itraconazole and ketoconazole (Janssen Pharmaceutica, Beerse, Belgium) and terbinafine (Novartis-Pharma AG, Basel, Switzerland). Ciclopirox, itraconazole and ketoconazole were dissolved, as recommended by the drug manufacturer, in 100% dimethyl sulphoxide (Sigma-Aldrich, Mississauga, ON, Canada) and terbinafine was dissolved in dimethyl sulphoxide with 5% Tween 80 (Sigma-Aldrich), following the protocol of Jessup *et al.*[9] All drugs were prepared as stock solutions of 5120 μg mL^{-1} and were stored at −86 °C.

Synergy testing. Three antifungal agents (ciclopirox, terbinafine and itraconazole) were tested in double combinations utilizing a checkerboard microdilution method.[19] Ciclopirox was tested in combination with either terbinafine or itraconazole; itraconazole and terbinafine were not tested against each other. The same stock solutions as prepared for susceptibility testing were used for these tests.

In vitro *susceptibility testing*

Medium. The medium used for broth microdilution susceptibility testing was RPMI 1640 (Sigma-Aldrich) with L-glutamine but without sodium bicarbonate and buffered at pH 7·0 with 3-(*N*-morpholino) propanesulphonic acid, monosodium salt (MOPS).

Table 3. Mode of interaction of antifungal agents in combination against nondermatophyte species

Species	Isolates[a] (*n*)	Mode of interaction (no. of isolates)	
		Ciclopirox/terbinafine	Ciclopirox/itraconazole
Scopulariopsis brevicaulis	6	S (5)	I (6)
		A (1)	
Aspergillus sydowii	6	A (1)	
		NG (5)	A (6)
Onychocola canadensis	5	S (1)	S (2)
		A (2)	A (2)
		NG (2)	I (1)
Scytalidium dimidiatum	5	S (2)	S (2)
		A (2)	I (3)
		I (1)	
Fusarium solani	4	S (1)	S (1)
		A (1)	I (3)
		I (2)	
Fusarium proliferatum	3	A (3)	I (3)

[a]No. of isolates of each nondermatophyte species tested for the combinations of antifungal agents. S, synergistic reaction observed; A, additive reaction; I, indifference; NG, no growth was observed in the combination of the two drugs. Minimum inhibitory concentration for one of the two drugs was very low, which did not allow for the calculations of fractional inhibitory concentration index values.

Drug dilutions. Serial twofold dilutions were prepared according to the NCCLS M27-A proposed standard.[4] Ciclopirox and terbinafine had MIC ranges of 0·003–2 μg mL^{-1}. Itraconazole and ketoconazole had MIC ranges of 0·06–32 μg mL^{-1}. Drug dilution plates were prepared in batches of 10 plates at a time and were stored at − 86 °C for up to a maximum of 3 months.

Inoculum preparation. Stock inoculum suspensions of dermatophytes were prepared from 2-week-old cultures of dermatophytes grown on Heinz oatmeal cereal agar slants or plates, as recommended by Jessup *et al.*[9] Inoculum preparation was performed according to the methods developed by Norris *et al.*[14] For every actively growing culture, conidial production was accessed microscopically by making tape slides. Any culture producing abundant conidia [i.e. between 50 and 100 conidia per field under the microscope (× 40), for at least five different fields of view] was chosen for setting up the tests. Sterile normal saline (0·85%) was added to the slant, and culture was gently swabbed with a cotton tip applicator to dislodge conidia from the hyphal mat. The resulting mixture (conidia and hyphal particles) was withdrawn and transferred to a sterile tube. Heavy particles of the suspension were allowed to settle for 15–20 min at room temperature and the upper part of the homogeneous suspension (≈ 2 mL) was adjusted to 0·5 MacFarland standard for 80–85% transmission using a Vitek Colorimeter (Hach Company, Loveland, CO, U.S.A.). At this transmission, the MacFarland standard corresponds to $1–5 \times 10^6$ c.f.u. mL^{-1}. The volume of the inoculum was adjusted to 5 mL and was further diluted in RPMI 1640 to the desired concentration of $2–5 \times 10^3$ c.f.u. mL^{-1}.

Test procedure. Microdilution plates were set up in accordance with the NCCLS M27-A reference method.[4] Each microdilution well containing 100 μL of the twofold drug concentration was inoculated with 100 μL of the diluted inoculum suspension. For each test plate, two drug-free growth controls were included, one with the media alone (growth control) and the other with media containing an equivalent amount of solvent used to dissolve the drug (solvent control).

Incubation time and temperature. The microdilution plates were incubated at 35 °C and were read visually after 7 days of incubation. Growth in the control wells and other wells for each plate was checked every day, 48 h following the initial inoculation.

Reading and interpretation of MICs. Endpoint determination readings were performed visually based on comparison of the growth in wells containing the drug with that of the growth in a drug-free solvent control well. For azole agents, the MIC was defined as the lowest concentration showing prominent growth inhibition (a drop in growth corresponding to approximately 80% of the growth in the control well with the appropriate solvent used to dissolve the drug). A standard of 80% growth inhibition is generally used for testing azole drugs because of the trailing endpoint characteristic of this class of agents. For terbinafine and ciclopirox, the MIC was defined as the lowest concentration showing 100% growth inhibition. MIC ranges were obtained for each species–drug combination tested. Geometric mean MICs were determined to facilitate comparisons of the activities of the drugs, as well as readings of the MIC at which 50% of the isolates were inhibited (MIC_{50}); similarly, MIC_{90} is the MIC at which 90% of the isolates were inhibited.

Synergy testing

The medium used, drug dilutions and preparation of inoculum were the same as performed for the susceptibility testing. However, a checkerboard microdilution

method[19,20] was utilized to test the susceptibility of drugs in double combinations.

Test procedure. Two 96-well flat-bottomed microtitre plates (P1 and P2) were used for each strain being tested for each of the combination of two drugs. The concentrations tested for each of the three antifungals were: 0 and 0·06–32 μg mL^{-1} for itraconazole, 0 and 0·003–4 μg mL^{-1} for both terbinafine and ciclopirox. Row (R)1/P1 and R8/P2 were filled with 200 μL of RPMI 1640 to serve as a sterility control. R2/P1 and R7/P2 were filled with 100 μL of RPMI 1640 (testing media) to serve as the drug-free growth control. Plates were set up so that the 12 columns (C1–C12 from left to right) on every plate corresponded to an individual final testing concentration of either itraconazole (0 and 0·06–32 μg mL^{-1}) or terbinafine (0 and 0·003–4 μg mL^{-1}); 50 μL of the appropriate drug (six times the final testing concentration) were dispensed into C1 (with 0 μg mL^{-1} of the appropriate drug) through C12 (with 32 or 4 μg mL^{-1} of the appropriate drug). Similarly, the rows corresponded to individual final testing concentration of ciclopirox; 50 μL of ciclopirox (six times the final testing concentration) were dispensed into R3–R8 of P1 and into R1–R6 of P2 in descending order. The order of loading was such that the final testing concentration in R3/P1was 4 μg mL^{-1} and in R5/P2 was 0·003 μg mL^{-1}. The first plate of each pair contained the six highest and the second plate of the pair contained the six lowest concentrations (including 0) of ciclopirox. For the final testing, each well, from R2–R8 in P1 and R1–R7 in P2, for the two test plates per strain was inoculated with 100 μL of the appropriate inoculum for each strain.

Reading and interpretation of results. Modes of interaction between drugs were classified as synergism, additivism, antagonism or indifference based on the calculations of fractional inhibitory concentration index values (FIC index).[19,20] In this method, the FIC for each drug is derived by dividing the concentration of that drug necessary to inhibit (for terbinafine and ciclopirox) or reduce growth (80% reduction for itraconazole) in a given row or column by the MIC of the test organism for that drug alone. The FIC index is then calculated by summing the separate FICs for each of the two drugs present in that well. With this method, synergism is defined as an FIC index of ≤ 0·5, additivity as an FIC index of ≤ 1·0, and antagonism as an FIC index of ≥ 2·0. More recent criteria suggest that an FIC index of more than 4 should be applied to definitions of antagonism to account for inherent variability in the technique and because an FIC index of 2·0 is probably indicative of an indifferent, rather than a true antagonistic, effect. The drug combination was interpreted as indifferent if neither of the drugs had any visible effect on the presence of the other drug in terms of the growth of a strain in different wells. Other instances were (i) a precise MIC could not be determined, because the marked susceptibility to a drug resulted in no growth even at the lowest testing concentration, and (ii) where no growth was observed in any of the wells where the antifungals were used in combination.

Results

Susceptibility testing

The MIC ranges of ciclopirox and terbinafine against dermatophytes ($n = 110$), yeasts ($n = 14$) and other filamentous fungi ($n = 9$) are given in Table 1. Overall, the dermatophyte MIC values (mean ± SEM) for the four antifungals were: 0·04 ± 0·02 μg mL^{-1} for ciclopirox, 0·04 ± 0·23 μg mL^{-1} for terbinafine, 2·28 ± 7·42 μg mL^{-1} for itraconazole and 0·83 ± 1·99 μg mL^{-1} for ketoconazole. The yeast MIC values (mean ± SEM) for the four antifungals were: 0·05 ± 0·02 μg mL^{-1} for ciclopirox, 1·77 ± 0·58 μg mL^{-1} for terbinafine, 0·18 ± 0·27 μg mL^{-1} for itraconazole and 0·56 ± 0·60 μg mL^{-1} for ketoconazole. The nondermatophyte fungi MIC values (mean ± SEM) for the four antifungals were: 1·04 ± 2·62 μg mL^{-1} for ciclopirox, 1·04 ± 0·95 μg mL^{-1} for terbinafine, 17·87 ± 16·75 μg mL^{-1} for itraconazole and 10·69 ± 13·09 μg mL^{-1} for ketoconazole. Collectively, the activity of the agents against dermatophytes, yeasts and other nondermatophytes was 0·10 ± 0·69 μg mL^{-1} for ciclopirox, 0·29 ± 0·67 μg mL^{-1} for terbinafine, 3·16 ± 9·31 μg mL^{-1} for itraconazole and 1·48 ± 4·54 μg mL^{-1} for ketoconazole.

MIC statistics, including geometric mean MIC, MIC_{50} and MIC_{90} values for the three dermatophyte species are summarized in Table 2. Among the dermatophytes, the observed MICs of all the drugs tested showed a broad range of variability against the different species of *Trichophyton*, *Microsporum* and *Epidermophyton* as shown in Table 2.

Terbinafine was the most potent drug against dermatophytes. Ciclopirox, while less potent than terbinafine, was more active than itraconazole and ketoconazole against all species. Among the azoles, itraconazole was more effective than ketoconazole, except against *A. benhamiae*.

Considering the yeasts, the mean and geometric mean MIC values for ciclopirox were lower than that of itraconazole and ketoconazole. The geometric mean MIC values with the four antifungal agents for the yeasts were 0·05 μg mL^{-1} for ciclopirox, 0·18 μg mL^{-1} for itraconazole, 0·56 μg mL^{-1} for ketoconazole and 1·77 μg mL^{-1} for terbinafine. Lower MIC values for ciclopirox could be an artefact of a lower testing range of 0·003–8 μg mL^{-1} for ciclopirox as compared with 0·06–32 μg mL^{-1} for itraconazole.

Synergy testing

Testing for antifungal agents in combination was performed for ciclopirox with terbinafine and itraconazole in pairs. Combinational testing of terbinafine with itraconazole was not performed. Drug interaction studies could not be performed for the dermatophytes because of the low MIC values of ciclopirox and terbinafine against all species; as the MIC values of dermatophytes against ciclopirox were either mainly 0·003 or 0·03 μg mL^{-1}, this did not allow for the testing of four to five dilutions below the MIC value for each drug alone, as per the requirements of the checkerboard method. Nondermatophyte moulds and not yeasts were chosen because relatively higher MICs were obtained for these species against all three antifungals when tested alone. Among the 29 nondermatophyte species from six genera, four different modes of interactions were observed for the antifungal agents in combination (Table 3).

For ciclopirox in combination with terbinafine, mainly a synergistic or additive reaction was observed (Table 3). Additionally, for two strains of *Onychocola canadensis* and five strains of *Aspergillus sydowii*, no growth was observed in any of the wells with the drug combinations. For each one of these interactions, the MIC for terbinafine alone was below the lowest testing concentration. A typical synergy reaction between ciclopirox and terbinafine for a strain of *O. canadensis* is shown in Figure 1(a). No instance of antagonism was observed for ciclopirox in combination with terbinafine.

For ciclopirox in combination with itraconazole, indifference (that is, the presence of one drug had no effect on the other drug in the testing well) was observed for five of the six nondermatophyte genera tested (Table 3). For *A. sydowii*, an additive effect of the drugs in combination was observed for six strains. The strain of *O. canadensis* that showed a synergistic reaction between ciclopirox and terbinafine (Fig. 1a) showed an additive interaction between ciclopirox and itraconazole (Fig. 1b). No antagonism was observed for ciclopirox combined with itraconazole.

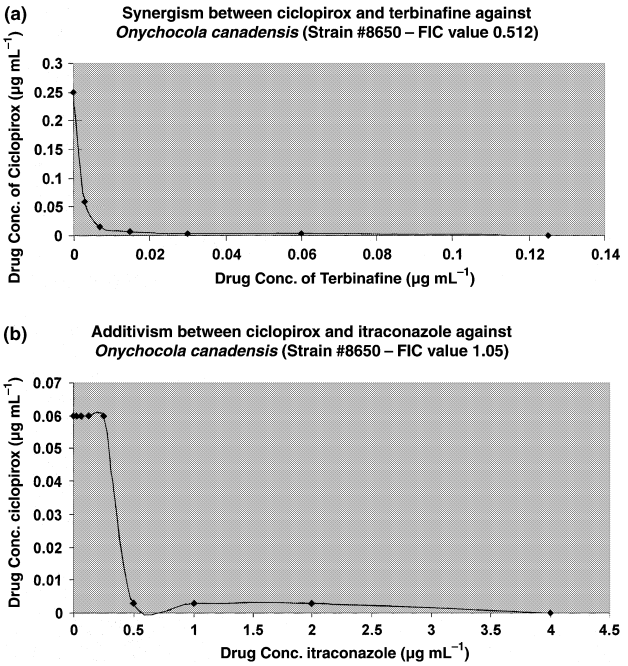

Figure 1. (a) Synergism between ciclopirox and terbinafine against *Onychocola canadensis* (strain no. 8650; FIC value 0·512); (b) additivism between ciclopirox and itraconazole against *Onychocola canadensis* (strain no. 8650; FIC value 1·05). FIC, fractional inhibitory concentration.

Discussion

In this study we employed the microdilution method using the RPMI 1640 medium along with the oatmeal agar medium to determine the MICs of antifungal agents for dermatophytes. Our results are in agreement with those reported in other studies that have utilized either the microbroth, macrobroth or agar dilution methods.[9,21–23] Our experience suggests that the use of NCCLS proposed standards for filamentous fungi (M38-P) and the utilization of oatmeal agar slants for conidial production of dermatophyte species as recommended by Jessup *et al.*[9] should be considered for developing a standardized antifungal susceptibility testing method for dermatophytes.

The results of *in vitro* susceptibility testing of ciclopirox against dermatophytes, yeasts and other nondermatophytes presented in this study support earlier findings that the antimicrobial profile of ciclopirox may

be broader than that of some other commonly used antimycotics.[24] While ciclopirox exhibited a broad spectrum of effectiveness, terbinafine was the most potent against dermatophytes. The findings are consistent with studies on terbinafine and other antifungal agents.[7–10,21]

Our results are directly comparable with the report by Jessup *et al.*[23] as all parameters, except for a shorter incubation period of 4 days compared with 7 days in the present study, were the same. The MIC_{50} and MIC_{90} of terbinafine and itraconazole for *T. rubrum* and *T. mentagrophytes* are similar. However, for *T. tonsurans* we observed a relatively high MIC_{90} for both terbinafine and itraconazole. The difference could be attributed to the smaller sample size of *T. tonsurans* (Table 1), five isolates in our study compared with 42 in the study by Jessup *et al.*[23] Moreover, there were differences in the MIC_{50} and MIC_{90} values of terbinafine and itraconazole for *E. floccosum;* again, our values were two- to threefold higher than the MICs reported by Jessup *et al.*[23] As both studies had only three isolates of *E. floccosum*, testing of a larger sample size would help provide a more reliable MIC range for this genus.

Our results on *in vitro* activity of ciclopirox are in contrast with those from other studies on dermatophytes and other fungi. We have found MICs in the range of 0·015–0·125 μg mL^{-1} against dermatophytes, 0·03–0·06 μg mL^{-1} for yeasts and in the range of 0·03–8 μg mL^{-1} for nondermatophytes. The high MIC of 8 μg mL^{-1} was observed only for *Fusarium proliferatum*. In contrast to the low MICs for ciclopirox observed in the present study, high MICs in the range of 0·9–3·9 μg mL^{-1} against dermatophytes and yeasts, and as high as 7·8 and 15·6 μg mL^{-1} against nondermatophytes and some selected strains of *Aspergillus* have been reported previously.[24,25] These differences can be attributed to the different methodology and culture medium used for susceptibility testing. While we have used the NCCLS recommended RPMI 1640 medium for testing the antifungal activity *in vitro*, other studies reported the use of Sabouraud's dextrose that contains beef peptone, which is free of certain metals.[25] The MIC of ciclopirox may vary depending upon the susceptibility method employed, the composition and pH of the media and the utilization of nutritional supplements.[26] Also, the mechanism of action of ciclopirox depends in part on the chelation of heavy metal ions, e.g. the ferrous ion. Therefore, the composition of the medium used may have an important role upon the potential activity of this hydroxypyridone.

Few studies have examined antifungal combinations *in vitro* or *in vivo*. Synergy, demonstrated *in vitro*, might lead to heightened antifungal activity *in vivo* with subsequent cure, which may not have occurred otherwise. Furthermore, synergy might permit lower doses of the individual antifungal agents to be used more effectively and safely. There have been reports of *in vitro* drug interaction studies on *Aspergillus* species,[27,28] suggesting that the demonstration of synergy *in vitro* may translate into therapeutic benefit *in vivo* against fungi such as *Cryptococcus neoformans*.[29]

In the present study we investigated the *in vitro* interactions of ciclopirox with the two commonly used antifungal agents. Synergy was demonstrated between ciclopirox and terbinafine, and ciclopirox and itraconazole against various nondermatophyte strains. Synergy testing could not be performed for the dermatophytes because of their low MICs for both terbinafine and ciclopirox. No antagonism was observed when ciclopirox was evaluated in combination with terbinafine or itraconazole. While we were only able to test combinations of drugs for nondermatophyte moulds, our findings that there is no antagonism between ciclopirox and terbinafine or between ciclopirox and itraconazole can probably be generalized to dermatophytes as well.

It is important to be cognisant of the potential flaws of the checkerboard method employed for testing antifungal combinations. First, as we read the plates based on macroscopic (visible) evidence of growth, this methodology yields only inhibitory data, reflecting fungistatic activity. Because of sampling difficulty, microbicidal activity cannot be measured and this lack of microbicidal data may be a limitation for any *in vivo* correlation of the test results obtained *in vitro*. Secondly, although checkerboard results are often used to characterize the dose–response relationship between an antimicrobial and a microorganism, the checkerboard method as usually performed provides either growth or no growth responses. Thus, this method is not able to measure the graded response necessary to define dose–response curves. Finally, the checkerboard method provides a static rather than dynamic view of antimicrobial interactions.

Overall, our findings contribute to a growing bank of data on *in vitro* susceptibility testing for dermatophytes obtained using similar parameters; furthermore, the results provide interlaboratory agreements that may enhance the development of a reference method for dermatophyte susceptibility testing. This paper is the first to investigate the antifungal activity of ciclopirox,

alone and in combination, using these new parameters. Because of this, our results with ciclopirox can be directly compared with results obtained using other antimycotics. Overall, however, much work is still needed to correlate the MICs of ciclopirox and other antifungal agents with clinical outcome, and to develop interpretive breakpoints for the various dermatophytes.

Acknowledgment

This study was supported in part by a grant from Dermik Laboratories, Berwyn, PA, U.S.A.

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