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Comparison of In Vitro Activities of 17 Antifungal Drugs against a Panel of 20 Dermatophytes by Using a Microdilution Assay

Bertrand Favre,† Bettina Hofbauer, Kwang-Soo Hildering,‡ and Neil S. Ryder§*

Novartis Research Institute, Vienna, Austria

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The in vitro activities of 17 antifungal drugs against a panel of 20 dermatophytes comprising 6 different species were determined using a microdilution assay according to the NCCLS M38-P method with some modifications. Terbinafine was the most potent systemic drug while tolnaftate and amorolfine were the most active topical agents.

Most superficial infections caused by dermatophytes can be rapidly eradicated with topical antifungals. However, two common dermatophytoses, tinea capitis and tinea unguium, do not respond well to such treatment and require the use of systemic antimycotics to be cured (2, 8, 23). Numerous topical agents and several systemic ones are available, but comparison of their in vitro activity against dermatophytes has been hampered by the lack of a well accepted MIC assay for these fungi (1, 5, 9, 10, 13, 14, 18–20, 25). Recently, several groups have adapted the proposed reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi (17) for developing a more specific assay for dermatophytes (6). Since the preparation of conidia inoculum is sometimes a challenge with dermatophytes, a microdilution assay appears to be the ideal format (5, 6, 13, 20). However, assay parameters, such as the temperature, duration, or growth inhibition endpoint, are still the subject of debate (11, 12, 21).

The NCCLS guidelines are primarily aimed toward susceptibility testing of clinical isolates. The aim of the present study was to establish an NCCLS-compatible assay, which was optimized for our primary purpose of evaluating investigative antifungal agents.

Twenty strains of dermatophytes, *Trichophyton rubrum* (n = 5), *Trichophyton tonsurans* (n = 5), *Trichophyton mentagrophytes* (n = 4), *Microsporum canis* (n = 4), *Microsporum gypseum* (n = 1), and *Epidermophyton floccosum* (n = 1), were employed. Five strains were obtained from either the fungal biodiversity center (Centralbureau voor Schimmelcultures, Utrecht, The Netherlands), *T. mentagrophytes* strain 560.66 (Novartis Fungal Index [NFI] 5606), *T. tonsurans* strains 171.65 (NFI 5177) and 729.88 (NFI 5178), or American Type Culture Collection (Manassas, Va.), *T. rubrum* strain 18759 (NFI 5182) and *T. tonsurans* strain 10217 (NFI 5176). The others were clinical isolates.

RPMI 1640 medium (Invitrogen) with L-glutamine and without bicarbonate was buffered at pH 7.0 with 0.165 M morpholinepropanesulfonic acid (Sigma). Terbinafine, naftifine, butenafine, voriconazole, and itraconazole were synthesized at Novartis. Fluconazole was extracted and purified from Diflucan tablets (Pfizer). Miconazole, amorolfine, and tolciclate were obtained from Janssen, Roche, and Montedison, respectively. Clotrimazole, econazole, ketoconazole, ciclopiroxolamine, tolnaftate, griseofulvine, and undecylenic acid were purchased from Sigma, while tioconazole was bought from U.S. Pharmacopeia. All drugs were dissolved and twofold serially diluted in dimethyl sulfoxide (DMSO).

All standard media were purchased from Merck. *T. mentagrophytes*, *T. tonsurans*, and *E. floccosum* were grown on Kimmig agar, *T. rubrum* was grown on potato dextrose agar, and *M. canis* and *M. gypseum* were grown on malt extract agar at 26°C for 2 to 3 weeks. Mycelium and spores were scraped from the plates and dispersed in a small volume of Sabouraud 2% dextrose broth (usually 20 ml for 25 plates) using a sterile glass homogenizer. After addition of 5% DMSO as a cryoprotectant, the fungal suspension was stored at −80°C (7). The viable count was determined by serially diluting the stock in 0.86% NaCl and spreading 50 μl/plate on the same agar medium as the one used for the inoculum preparation.

Microdilution plates with flat-bottom well (Greiner) were set up in accordance with the NCCLS M38-P reference method (17). The final concentration of DMSO was 1%, and the inoculum size was 5×10^3 CFU/ml. Plates were incubated for 4 to 5 days, depending on the growth in control wells without drug, at 30°C for *E. floccosum* and *M. canis* and 35°C for the other dermatophytes. Growth inhibition was scored visually with the aid of an inverted magnifying mirror from 4 to 0 according to the NCCLS M38-P reference method, and MIC of all tested drugs corresponded to the lowest concentration giving a score of 1 (equivalent to about 75% inhibition). After MIC determination, the total volume of each well, starting from the last well in which growth was observed up to the highest drug concentration tested, was transferred into glass tubes containing 5 ml of Sabouraud 2% dextrose broth (pH 6.5). Tubes were incubated for 1 week at 30°C, and growth was inspected visually after shaking. The minimal fungicidal concentration (MFC) corresponded to the lowest drug concentration (in the assay plate) at which no viable fungus remained.

* Corresponding author. Mailing address: Infectious Diseases Biology, Room 4210, Novartis Institutes for Biomedical Research, Inc., 100 Technology Square, Cambridge, MA 02139. Phone: (617) 871-3143. Fax: (617) 871-7047. E-mail: neil.ryder@pharma.novartis.com.

† Present address: Department of Dermatology, University Hospital CHUV, Lausanne, Switzerland.

‡ Present address: Igeneon, Vienna, Austria.

§ Present address: Novartis Institutes for Biomedical Research, Inc., Cambridge, MA 02139.

TABLE 1. MIC of 17 antifungals against a panel of 20 dermatophytes[a]

Drug	MIC for species[b]																				MIC50	MIC90	Geom. mean MIC	MFC50	MFC90	Geom. mean MFC
	E. floccosum	*M. canis*				*M. gypseum*	*T. mentagrophytes*				*T. rubrum*					*T. tonsurans*										
	0167	0150	5154	5167	5168	5164	0158	5137	5165	5606	5132	5139	5140	5143	5182	0105	5175	5176	5177	5178						
TER	0.008	0.008	0.016	0.016	0.016	0.016	0.004	0.004	0.004	0.008	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.008	0.008	0.008	0.004	0.016	0.006	0.063	0.125	0.07
ITR	0.250	0.500	0.250	0.250	0.250	0.500	1.000	0.250	0.125	0.063	0.250	0.500	0.250	0.250	0.500	0.125	0.016	0.500	0.125	0.125	0.25	0.5	0.23	>4	>4	NC
KET	0.13	1.00	1.00	1.00	1.00	1.00	1.00	0.50	0.13	0.13	0.06	0.13	0.13	0.06	0.03	0.25	0.13	0.25	0.06	0.06	0.13	1	0.22	>8	>8	NC
VOR	0.031	0.031	0.063	0.063	0.063	0.125	0.063	0.063	0.016	0.031	0.016	0.016	0.063	0.016	0.016	0.031	0.016	0.063	0.008	0.031	0.031	0.063	0.033	>2	>2	NC
FLU	4	16	16	32	32	64	64	16	2	4	1	2	4	1	1	8	4	8	2	4	4	32	6.3	>128	>128	NC
GRI	0.50	0.025	0.25	0.050	0.25	0.25	1.00	0.25	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.13	0.13	0.50	0.50	0.25	0.5	0.5	0.37	8	>32	NC
BUT	0.031	0.031	0.031	0.063	0.031	0.031	0.008	0.016	0.008	0.008	0.016	0.008	0.031	0.008	0.004	0.008	0.008	0.008	0.016	0.008	0.008	0.031	0.014	0.125	0.25	0.14
NFT	0.031	0.063	0.063	0.063	0.063	0.063	0.016	0.031	0.031	0.031	0.031	0.016	0.016	0.016	0.016	0.016	0.031	0.016	0.031	0.031	0.031	0.063	0.029	1	2	0.44
TCI	0.063	0.063	0.063	0.063	0.063	0.063	0.016	0.063	0.031	0.063	0.016	0.016	0.031	0.031	0.008	0.008	0.016	0.004	0.031	0.016	0.031	0.063	0.028	0.25	>2	0.55
TLN	0.008	0.063	0.004	0.008	0.008	0.004	0.002	0.004	0.004	0.004	0.002	0.001	0.002	0.002	0.001	0.001	0.002	0.001	0.004	0.004	0.004	0.008	0.003	>0.25	>0.25	NC
CLT	0.250	0.063	0.016	0.031	0.063	0.063	0.500	0.031	0.063	0.250	0.125	0.125	0.250	0.125	0.063	0.016	0.016	0.250	0.125	0.125	0.063	0.25	0.083	>4	>4	NC
ECO	0.016	0.063	0.063	0.063	0.031	0.125	1.000	0.031	0.008	0.063	0.016	0.016	0.031	0.016	0.016	0.016	0.016	0.063	0.016	0.008	0.016	0.063	0.032	>1	>1	NC
MCO	0.25	0.50	0.50	0.50	0.50	1.00	>2.00	0.25	0.13	0.50	0.25	0.13	0.06	0.13	0.06	0.13	0.13	0.50	0.50	0.25	0.25	0.5	0.25	16	>16	NC
TIO	0.008	0.250	0.250	0.250	0.250	0.500	1.000	0.063	0.016	0.063	0.031	0.031	0.125	0.008	0.016	0.031	0.031	0.125	0.031	0.063	0.063	0.25	0.067	>4	>4	NC
AMO	0.008	0.004	0.002	0.002	0.004	0.001	0.008	0.004	0.002	0.008	0.004	0.004	0.004	0.004	0.002	0.002	0.002	0.008	0.004	0.004	0.004	0.008	0.003	0.125	0.25	NC
CPX	1.0	1.0	1.0	1.0	0.5	1.0	1.0	0.5	0.5	1.0	0.5	0.5	0.5	0.5	1.0	0.5	0.5	0.5	0.5	0.5	0.5	1	0.7	>32	>32	NC
UDA	64	64	64	64	64	64	64	64	64	64	64	64	64	64	64	128	64	32	32	32	64	64	60	>128	>128	NC

[a] Abbreviations: TER, terbinafine; ITR, itraconazole; KET, ketoconazole; VOR, voriconazole; FLU, fluconazole; GRI, griseofulvin; BUT, butenafine; NFT, naftifine; TCI, tolciclate; TLN, tolnaftate; CLT, clotrimazole; ECO, econazole; MCO, miconazole; TIO, tioconazole; AMO, amorolfine; CPX, ciclopiroxolamine; UDA, undecylenic acid; Geom., geometric; NC, not calculable. MICs are expressed in micrograms per milliliter. MIC_{50} and MIC_{90}, MIC at which 50 and 90% of organisms are inhibited, respectively.

[b] Species and NFI identification number are given.

All experiments were repeated at least twice (topical agents) or more (systemic drugs). MICs usually did not differ by more than one dilution step.

For our purpose of evaluating drugs against a defined set of dermatophytes, large-scale preparation of inoculum with well-defined CFU is advantageous. Therefore, we initially compared MICs obtained with four drugs, terbinafine, itraconazole, fluconazole, and griseofulvine, against a few dermatophytes using either fresh inocula prepared according to the method of Jessup et al. (13) or frozen inocula. The results indicated that both freezing and the presence of mycelium in the inoculum did not significantly affect MICs of antifungals, in agreement with results obtained by Manavathu et al. (15).

The ideal incubation temperature, 28 to 35°C, and time for antifungal susceptibility testing of dermatophytes are still a matter of debate. In our hands, *M. canis* and *E. floccosum* grew very poorly at 35°C, so we decreased the temperature to 30°C for these two species. Concerning the incubation time, 4 to 5 days was found to be sufficient to observe prominent growth in control wells without drug with our restricted panel of dermatophytes, which were selected from a larger panel on the basis of their abundant conidium production and robust growth properties.

There is no consensus concerning the optimal growth inhibition endpoint for MICs (5, 6, 21). We uniformly adopted a score of 1 as the MIC for all the tested drugs, as recommended by Norris et al. (20). The obtained MIC results are presented in Table 1. Among the six systemic antifungals tested, fluconazole, griseofulvine, itraconazole, ketoconazole, terbinafine, and voriconazole, the allylamine terbinafine was the most potent agent. In our assay, voriconazole was significantly more active than itraconazole, in agreement with the findings of Fernandez-Torres et al. (6) but in contrast to the results of Perea et al. (21). The reason for these differences is unknown.

We also measured MFCs with a simple but rigorous method requiring complete elimination of viable particles in the culture well during the MIC incubation time, while MFC is often defined as a ≥99% reduction of CFU (3, 4, 16, 22, 24). Amorolfine and the squalene epoxidase inhibitors, butenafine, naftifine, and terbinafine, were systematically fungicidal toward our panel of dermatophytes within the range of tested concentrations, ≥32× the MIC at which 50% of the organisms were inhibited (Table 1).

In summary, the proposed microdilution assay for dermatophytes is convenient and reproducible. While parameters such as scoring range and MIC endpoint could be harmonized, it appears that the incubation temperature cannot be uniformly set at 35°C. The test strains were selected for adequate growth and normal susceptibility to standard drugs; we suggest that a comparable set of strains could be picked from any dermatophyte collection and used to obtain similar results. Among the systemic antifungals tested, terbinafine was the most potent, while tolnaftate and amorolfine were the most active topical agents.

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