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Original article

Comparison of in vitro antifungal activities of topical antimycotics launched in 1990s in Japan

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Abstract

In vitro anti-dermatophyte, anti-*Candida albicans* and anti-*Malassezia furfur* activities of amorolfine hydrochloride (AMF), terbinafine hydrochloride (TBF), butenafine hydrochloride (BTF), neticonazole hydrochloride (NCZ) and ketoconazole (KCZ), all of which were introduced for the treatment of dermatomycoses in the 1990s in Japan, were compared. Although all of the test drugs are classified as an ergosterol biosynthesis inhibitor, the antifungal properties were found to be different. TBF and BTF exerted extremely potent antifungal activity against *Trichophyton* spp. but not against *C. albicans* and *M. furfur*, whilst KCZ and NCZ showed potent antifungal activity against *C. albicans* and *M. furfur* rather than *Trichophyton* spp. AMF exhibited potent antifungal activity against all of the fungal species tested. Fungicidal activities of these antifungal agents against *T. rubrum* were determined by using neutral red staining. The fungicidal potentialities correlated with those obtained in the in vitro susceptibility test as determined by MICs against dermatophytes. TBF, BTF and AMF exerted more potent fungicidal action than NCZ and KCZ. © 2001 Elsevier Science B.V. and International Society of Chemotherapy. All rights reserved.

Keywords: Amorolfine; Terbinafine; Butenafine; Neticonazole; Ketoconazole; Dermatophytes; *Candida albicans*; *Malassezia furfur*

1. Introduction

A large number of antifungal agents have been introduced for the clinical topical treatment of dermatomycoses [1–8], and in Japan several topical agents were additionally launched in the 1990s. Included among them were imidazole, allylamine, benzylamine, and morpholine classes of antifungal agents. Although all of the agents were reported to exhibit a broad antifungal spectrum and to be highly active [9–18], direct comparative studies on antifungal activities of these agents have not been reported so far. In this report, we chose five representative antifungal agents based on their chemical structures and antifungal mechanisms, and compared their antifungal activities in vitro against *Trichophyton* spp., *Candida albicans* and *Malassezia furfur* which are major pathogenic fungi of dermatophytosis, cutaneous candidiasis and pityriasis versicolor, respectively. The chosen antifungal agents were two imidazole compounds (ketoconazole and neticonazole hydrochloride), an allylamine compound (terbinafine hydrochloride), a benzylamine compound (butenafine hydrochloride) and a morpholine compound (amorolfine hydrochloride).

Most of the antifungal agents developed in the 1990s were also reported to be characterized by having fungicidal action [9–13,16]. Recently Fukuda et al. successfully established a susceptibility test for determining fungicidal activity against dermatophytes by using neutral red staining which has been used for supravital staining [19,20]. We describe here the fungicidal activities of the five antifungal agents against *T. rubrum* measured using the neutral red assay.

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In this study, anti-*C. albicans* activity was measured according to the NCCLS M27-A protocol [21], and anti-*M. furfur* activity by an agar dilution technique with Dixon agar that is commonly used for the culture of *Malassezia* spp. Anti-dermatophyte activity was measured by a micro-broth dilution technique using Sabouraud dextrose broth instead of RPMI 1640 that is recommended by the NCCLS M38-P protocol [22]. There have been several reports of in vitro susceptibilities of *Trichophyton* spp. measured using Sabouraud dextrose broth and/or agar [10,12,14,16,17] and Fukuda et al. reported the use of Sabouraud medium in the neutral red assay for assessing fungicidal activity against *Trichophyton* spp. [19,20].

2. Materials and methods

2.1. Organisms

Twenty stock cultures of *Trichphyton* spp., five stock cultures of *C.albicans* and five stock cultures of *M.furfur* obtained from Teikyo University Institute of Medical Mycology (Tokyo, Japan), Institute for Fermentation Osaka (Osaka, Japan) or Chiba University Research Center for Pathogenic Fungi and Microbial Toxicoses (Chiba, Japan) were used in this study.

2.2. Test materials

Neticonazole hydrochloride (NCZ), butenafine hydrochloride (BTF) and terbinafine hydrochloride (TBF) were extracted and purified from the commercially available 1% cream preparations, Atolant® (SSP Co., Ltd., Tokyo, Japan), Mentax® (Kaken Pharmaceutical, Tokyo, Japan), and Lamisil® (Novartis Pharma K.K., Tokyo, Japan), respectively. Their chemical structures were identified by nuclear magnetic resonance spectra; the purity of all test materials was > 99.0%. Amorolfine hydrochloride (AMF) was supplied by Kyorin Pharmaceutical (Tokyo, Japan). Ketoconazole (KCZ) was purchased from Sigma (St. Louis, MO). The chemical structures of the test drugs are shown in Fig. 1.

2.3. In vitro susceptibility testing as determined by MICs

The minimum inhibitory concentrations (MICs) for *Trichophyton* spp. were determined by a twofold microbroth dilution technique using Sabouraud dextrose broth (SDB). Each drug dissolved in dimethyl sulfoxide (DMSO) was diluted 1:49 with the same medium. A conidial suspension of each strain was prepared in sterile physiological saline containing 0.05% (v/v)

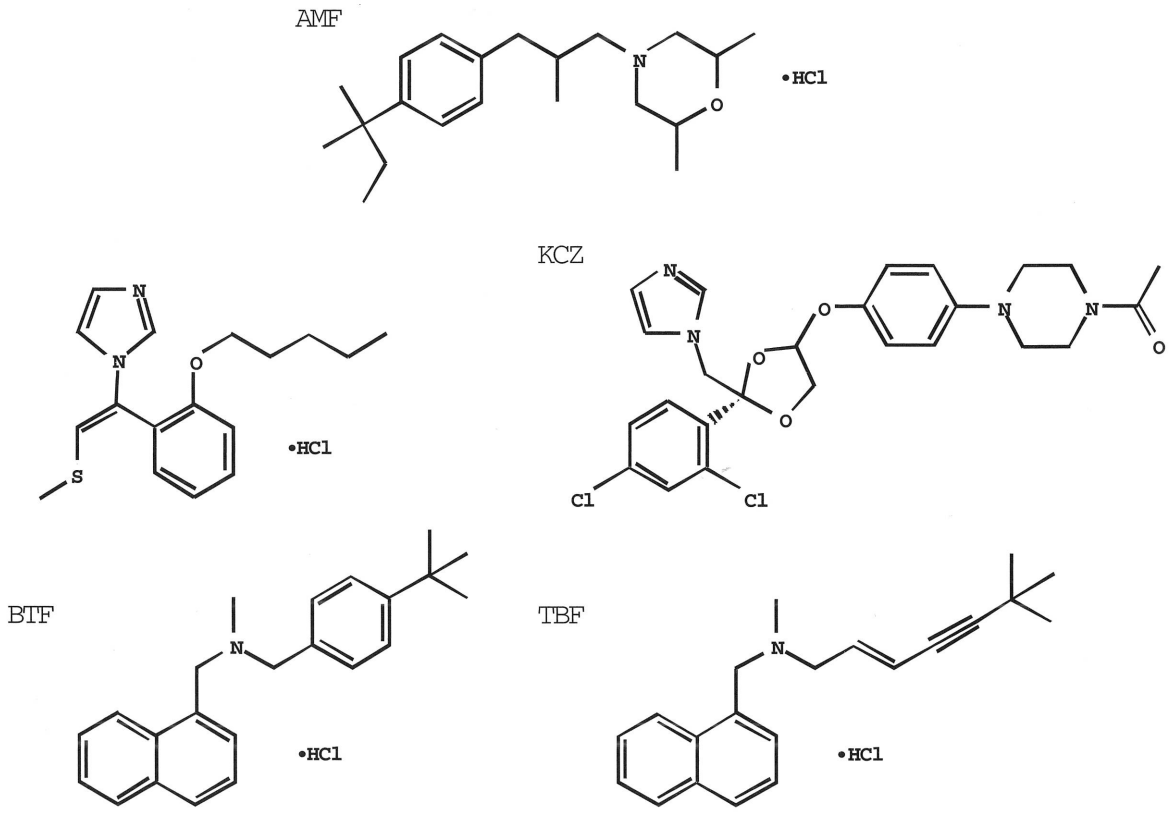

Fig. 1. Chemical structures of amorolfine hydrochloride (AMF), terbinafine hydrochloride (TBF), butenafine hydrochloride (BTF), neticonazole hydrochloride (NCZ) and ketoconazole (KCZ).

Tween 80 from cultures grown on 1/10 Sabouraud dextrose agar slants at 27 C for 1–4 weeks. Following filtration through a sterile cell strainer (pore size 40 μm, Becton Dickinson Labware, Franklin Lakes, NJ) to remove hyphal fragments and agar blocks, the final conidial suspension was adjusted to 2×10^4 conidia/ml in SDB. One hundred microliters of a conidial suspension and 100 μl of the drug-containing medium were added to each well of flat-bottomed microculture plates. After incubation at 27 C for 7 days, the MIC was determined as the lowest drug concentration that prevented visual fungal growth.

MICs for stock cultures of *C. albicans* were measured by a twofold micro-broth dilution technique with RPMI 1640 medium buffered with 0.165 M morpholinopropanesulfonic acid (pH 7.0) according to the NCCLS M27-A protocol [21]. Each drug dissolved in DMSO was diluted 1:49 with the same medium. One hundred μl of a yeast cell suspension (1×10^3 cells/ml) and 100 μl of the drug-containing medium were added to each well of flat-bottomed microculture plates. After incubation at 35 °C for 24–48 h, turbidity of each well was measured at 620 nm. The MIC was determined as the lowest drug concentration that showed 80% inhibition of control fungal growth.

MICs against stock cultures of *M. furfur* were measured by a twofold agar dilution technique with Dixon agar (malt extract 4%, oxgall 2%, Tween 40 1%, glycerol 0.25%, agar 1.5%). The yeast cell suspension of *M. furfur* was prepared in Dixon broth from cultures grown in the same medium at 37 °C for 3 days, and the final suspension was adjusted to 10^6 cells/ml. Approximately 5 μl of each suspension was inoculated onto the agar plates containing a drug using a micro-plantar (MIT-P, Sakuma Seisakusho, Tokyo, Japan), and fungal growth was observed after incubation at 25 °C for 5 days. The MIC was determined as the lowest drug concentration that prevented visual fungal growth.

2.4. Neutral red assay for measuring fungicidal activity in vitro

To evaluate the fungicidal activity of antifungal agents against dermatophytes, neutral red (NR) assay was performed basically according to the method of Fukuda et al. [19,20]. A conidial suspension of *T. rubrum* TIMM 3866 was prepared in the same way as in the in vitro susceptibility study to obtain the final conidial suspension of 10^4 conidia/ml. Five hundred μl each of the conidial suspension was incubated at 27 °C for 4 days, and then the drug solution in DMSO was added to be a 1% (v/v). After further incubation at 27 °C for 24, 72 and 168 h, NR (3-amino-7-dimethylamono-2-methylphenazine hydrochloride; Merck, Darmstadt, Germany) dissolved in phosphate buffered saline (PBS) was added to be a concentration of 50 μg/ml. The cultures were subsequently incubated at 27 °C for 1 h, and incorporated NR into the living fungal cells was measured. The cultures were centrifuged ($14\,000 \times g$, 5 min), and the precipitate obtained was fixed in formaldehyde/$CaCl_2$ solution (4% formaldehyde and 1% $CaCl_2$) following washing twice in PBS. Neutral red was then extracted in a mixture of acetic acid and ethanol (1% anhydrous acetic acid and 50% ethanol). After centrifugation ($14\,000 \times g$, 2 min), the optical density of the supernatant was measured at 540 nm.

3. Results

3.1. In vitro susceptibility of Trichophyton spp., C. albicans and M. furfur to antifungal agents

In vitro susceptibility distributions of twenty strains of *Trichophyton* spp., five strains of *C. albicans* and five strains of *M. furfur* to AMF, NCZ, KCZ, BTF and TBF are summarized in Table 1. The lowest MIC against each strain of *Trichophyton* spp. was obtained with terbinafine hydrochloride ranging from 0.008 to 0.063 mg/l and the highest MIC with ketonazole, ranging from 4.0 to 16.0 mg/l. The antifungal activity as determined by MICs were in order of potency: TBF, BTF, AMF, NCZ and KCZ. These in vitro susceptibility data of *Trichophyton* spp. are similar to those reported previously [10,12,14,16,17]. In contrast to the antifungal activities against *Trichophyton* spp., KCZ and AMF exhibited the lowest MICs against each strain of *C. albicans* and *M. furfur*, respectively, while antifungal activities of BTF and TBF against these two fungal species were low.

TBF and BTF showed potent antifungal activity against *Trichophyton* spp. but not against *C. albicans* and *M. furfur*, whilst KCZ and NCZ showed potent antifungal activity against *C. albicans* and *M. furfur* rather than *Trichophyton* spp. AMF exhibited potent antifungal activity against all fungal species tested.

3.2. In vitro fungicidal activity against T. rubrum using the neutral red assay

In vitro fungicidal activities of AMF, NCZ, KCZ, BTF and TBF against *T. rubrum* were measured by the incorporation of neutral red (NR) into the living fungal cells (Fig. 2). In the control cultures, the incorporated NR (as indicated by the optical density at 540 nm) increased with time, indicating that living fungal elements increased time-dependently. All of the test drugs reduced the NR-uptake in a concentration- and in a time-dependent manner, but the reduced patterns were different among the drug-treated cultures. TBF and BTF did not reduce the NR-uptake after 24 h of incubation, but thereafter the NR-uptake was progres-

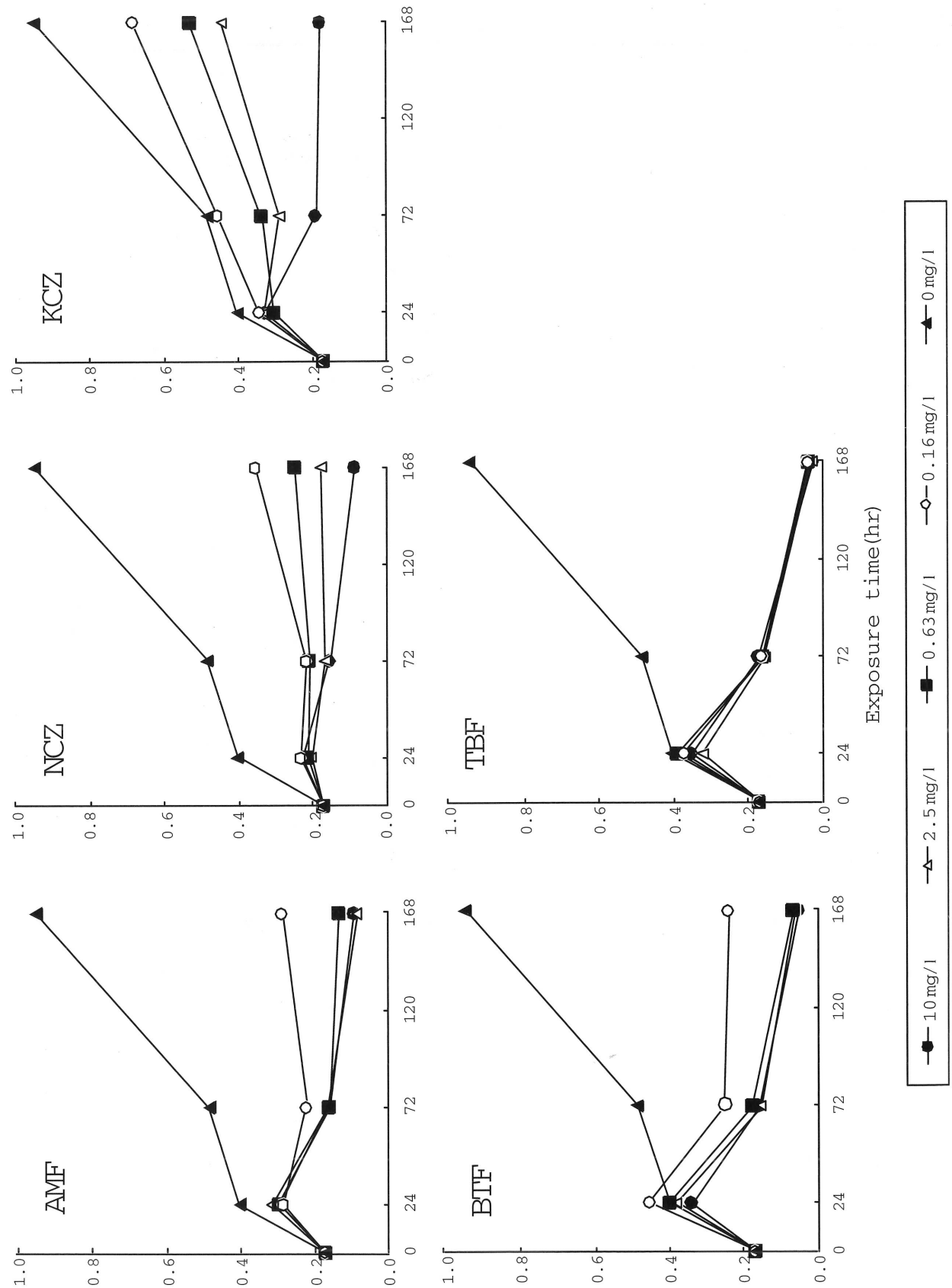

Fig. 2. Inhibitory effects of amorolfine hydrochloride (AMF), terbinafine hydrochloride (TBF), butenafine hydrochloride (BTF), neticonazole hydrochloride (NCZ) and ketoconazole (KCZ) on the growth curves of *Trichophyton rubrum* TIMM 3866 as indicated by the incorporation of neutral red.

Table 1
In vitro susceptibility distributions of *Trichophyton* spp., *Candida albicans* and *Malasezia furfur* to amorolfine hydrochloride (AMF), terbinafine hydrochloride (TBF), butenafine hydrochloride (BTF), neticonazole hydrochloride (NCZ) and ketoconazole (KCZ)

	The number of strains inhibited at drug concentration (MIC: mg/l)												
Anti-mycotic	*T. mentagrophytes* (10)[a] and *T. rubrum* (10)[a]												
	0.004	0.008	0.016	0.031	0.063	0.13	0.25	0. 5	1.0	2.0	4.0	8.0	16.0
AMF				2	14	4							
TBF		11	8		1								
BTF		1	12	7									
NCZ					2	5	13						
KCZ											4	8	8
Anti-mycotic	*C. albicans* (5)[a]												
	0.016	0.031	0.063	0.13	0.25	0. 5	1.0	2.0	4.0	8.0	16.0	>16.0	
AMF			1	2	1						1		
TBF									1		1	3	
BTF												5	
NCZ		1	1	2	1								
KCZ	4		1										
Anti-mycotic	*M. furfur* (5)[a]												
	0.063	0.13	0.25	0. 5	1.0	2.0	4.0	8.0	16.0	>16.0			
AMF		2	3										
TBF								1	2	2			
BTF									1	4			
NCZ						2	1	2					
KCZ			1	1	2	1							

[a] The number of strains tested.

sively decreased. NCZ and AMF inhibited NR-uptake at all measured points. Although NCZ strongly inhibited NR-uptake at 24 h, at and after 72 h the degree of inhibition by AMF was more marked than that by NCZ. The inhibitory pattern of NR-uptake by KCZ was the weakest.

4. Discussion

As shown in Table 1, although all of the test drugs are classified as ergosterol biosynthesis inhibitor [18,23–26], these antifungal properties were proved to be different. What causes the differences in antifungal properties is not clear, but it may be related to their chemical structures and/or target enzymes in the ergosterol biosynthesis pathway. TBF and BTF, both of which are members of allylamine and benzylamine classes of antifungal compounds, and are known to be squalene epoxidase-inhibitors and are very active against *Trichophyton* spp. but only have feeble action against *C. albicans* and *M. furfur*. In contrast, KCZ and NCZ, both imidazoles and known to be CYP51 ($P450_{14DM}$)-inhibitors, are potent antifungal agents against *C. albicans* and *M. furfur*. Of the antifungal agents tested, we noted in particular that AMF, a morpholine antifungal compound and an inhibitor of Δ^{14}-reductase and Δ^{8}–Δ^{7}-isomerase, exhibited potent antifungal activity against all of the fungal species tested. It possesses well-balanced antifungal profiles.

Fungicidal effect is also an important factor in evaluating the potency of antifungal agents. In this study, *T. rubrum* was chosen as a test fungal species because it is detected most frequently in patients with dermatophytosis in Japan. TBF had the most potent fungicidal effect although it required more than 24 h to induce fungal death. In regard to fungicidal action of TBF against dermatophytes, Ryder suggested that squalene accumulation subsequent to the inhibition of squalene epoxidation might play an important role in causing cell death [27]. Thus, one of the possibilities is that it takes time for squalene accumulation to cause fungal death. BTF and AMF also exhibited potent fungicidal action against the test fungus. As for BTF, fungicidal action is probably attributable to the squalene accumulation since the antifungal target is the same as that of TBF. In the case of AMF, Polak et al. reported that the drug showed greatest fungicidal activity against *T. mentagrophytes*. Of the organisms tested (*T. mentagrophytes*, *C. albicans*, *Histoplasma capsulatum* and *Cryptococcus neoformans*) concentrations as low as 0.001 mg/l of the drug was enough to kill 90% or more of *T. mentagrophytes* when cultured on casitone agar for 48 h [9]. We used different culture conditions from those reported by Polak et al., but AMF also exhibited potent fungicidal action against *T. rubrum*. The fungici-

dal mode of action of AMF has not been clarified, but direct membrane destruction might cause fungicidal effect, at least in part, as reported by Nishiyama et al. in an electron microscopic study of *T. mentagrophytes* exposed to AMF [28]. Of the imidazole drugs, NCZ showed potent inhibition of NR-uptake but the inhibition did not fall below the initial level (at 0 h of incubation) except the highest concentration (10 mg/l)-treated cultures, suggesting that antifungal action of NCZ against *T. rubrum* was possibly fungistatic rather than fungicidal. The inhibition of NR-uptake by KCZ was weakest among the test drugs as seen also in the MICs, indicating that the antifungal effect of KCZ against *T. rubrum* was poor.

The antifungal agents tested in this study have different antifungal properties which seem to be related to their chemical structures and/or target enzymes in the ergosterol biosynthesis pathway. Of the test drugs, deserving special mention is the broad spread of antifungal activity of AMF and the extremely potent antifungal activities of TBF and BTF against dermatophytes. To further examine the causes of these differences in antifungal properties, we intend to investigate drug-incorporation into fungal cells and/or interaction of stereochemical structure of each drug and corresponding target enzyme.

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