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Testing of antifungal combinations against yeasts and dermatophytes

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BACKGROUND: Fungal infections of the nail are a common and chronic problem. The main pathogens responsible for onychomycosis are dermatophytes, yeasts and moulds. Despite significant improvements, approximately 20% of patients with onychomycosis still fail on antifungal therapy. The successful exploitation of drug synergy may provide a useful approach to improve cure rates.
METHODS: The minimum inhibitory concentrations (MIC_{80}) were recorded for pathogens that are most frequently responsible for onychomycosis against combinations of several antifungal agents, namely, fluconazole, itraconazole, terbinafine and amorolfine. Fractional inhibitory concentrations (FICs) were then calculated from the MIC_{80} results and the FIC values for each drug in the combinations added to determine the degree of synergy. A combined value of <1 was taken to suggest synergy; a value of 1–2 indicated an additive effect or indifference; and a combined FIC value of >2 was taken to suggest antagonism.
RESULTS: Overall, 46% of amorolfine combinations showed results suggestive of synergy, with the most synergistic results seen against dermatophytes (54%) and moulds (52%).
CONCLUSIONS: Some combinations of drugs may have synergistic activity *in vitro*; however, the importance of this in a clinical setting is yet to be established, and more studies are justified. (*J Dermatol Treat* (2004) **15:** 104–107)

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Introduction

Fungal infection of the nail, or onychomycosis, is a common and usually chronic problem.[1,2] The introduction of new oral therapies have dramatically improved cure rates. Topical therapies, for example amorolfine, are especially appropriate for milder, distal disease.[2] Despite the improvements, approximately 20% of patients with onychomycosis still fail on antifungal therapy.[1] This is likely to be due to either kinetic problems or because the fungal structures found in nail (e.g. spores) are not sensitive to the antifungal concentrations that can be achieved in nail.

One approach to improving cure rates would be to exploit any synergy between drugs by using combination therapy. Synergy can be defined in this context as: two antifungal drugs in combination achieving a better effect than the sum of each acting separately. Synergy *in vitro* has been reported previously.[3–6] Furthermore, various clinical studies have revealed the increased efficacy of drug combinations in treating onychomycosis.[7,8]

Topical and systemic agents were tested in combination to determine whether synergy may occur. Care has to be taken when carrying out sensitivity testing of antifungal compounds because variations in any factor during the testing (e.g. incubation temperature, pH, inoculum size) result in changes in the apparent sensitivity of the isolate tested. The US National Committee of Clinical Laboratory Standards (NCCLS) has developed microdilution methods, which are now internationally accepted as the 'gold standard' for testing both yeasts and moulds.[9,10] We used these methods, and an accepted modification thereof, for use with dermatophytes[11] to look for synergy between amorolfine plus fluconazole or

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itraconazole or terbinafine against the common superficial fungal pathogens. We expressed the synergy test results as the fractional inhibitory concentration (FIC),[12,13] which is the interaction coefficient indicating whether the combined inhibitory effect of drugs is synergistic, additive or antagonistic. It was particularly important to be sure that the antifungal combinations tested were not antagonistic, i.e. the combination achieves poorer results than either drug used alone.

Methods

The organisms tested (see Table I) were from the UK National Collection of Pathogenic Fungi (NCPF), the American Type Culture Collection (ATCC) or from the Mycology Reference Centre (MRC) culture collection. The minimum inhibitory concentration (MIC) for all organisms was determined against each antifungal, alone or in combination, namely, fluconazole, itraconazole, terbinafine and amorolfine. The sensitivity testing method used a microtitre plate format, with RPMI 1640 as the culture medium (GIBCO, Paisley, Renfrewshire, UK). Fluconazole was dissolved in water, terbinafine and itraconazole in dimethyl sulphoxide and amorolfine in methanol.

A range of doubling dilutions were prepared in RPMI 1640 and dispensed into the microtitre plate wells. The yeast inoculum was prepared from overnight subcultures, adjusted to obtain a suspension of 2.5×10^3 colony-forming units per ml in RPMI 1640. For the dermatophyte and mould inocula, the test isolates were subcultured onto Sabouraud's agar (SAB) plates and incubated at 27°C for 7 days. The inoculum was obtained from the colony surface and spores were counted using a haemocytometer and diluted in RPMI 1640 to obtain a concentration of 2×10^3 spores per ml.

All test plates contained a number of controls. A medium-only control was included to check for contamination and an inoculum control (with no antifungal) was taken as 100% growth. For each batch of plates tested, an azole-sensitive yeast and an azole-resistant yeast were included. After inoculation with 100 µl of inoculum into relevant wells, the yeast test plates were incubated at 37°C for 48 h, and the dermatophyte test plates at 27°C for 4 days.

After incubation, the plates were examined visually. The inoculum control was taken as 100% growth and the other wells were compared with this. The MIC_{80} was taken as the first well with an 80% inhibition of growth (i.e. residual 20% growth compared with the 100% growth well).

To determine whether synergy occurred between drugs, plates consisted of checkerboard titrations of amorolfine against fluconazole, itraconazole or terbinafine. Again, all synergy test plates had appropriate control wells. Individual drug MICs were also checked in each of the plates.

After incubation, two independent observers examined the plates visually. Each well was checked to see if it contained more or less than 80% growth compared with the inoculum control. From these results, the FIC for each organism against each of the drug

Organisms	Terb	Fluc	Itra	Amor
YEASTS				
Candida albicans (2; ATCC 90028; MRC 7650467)	2.0–3.0	0.12–0.38	0.06	0.05–0.75
C. krusei (NCPF 3953)	0.02	16–32	0.06	2.0
C. parapsilosis (2; NCPF 3938; ATCC 22019)	0.02–0.06	0.5–1.0	0.06–0.19	0.05–2.0
C. guilliermondii (MRC 9967053)	0.05	2.0	0.19	0.5
DERMATOPHYTES				
Trichophyton rubrum (4; MRC 53986; MRC 50952; MRC 58322; MRC 57494)	0.007–0.01	0.05–8.0	0.03–0.25	0.045–0.12
T. mentag. var. interdigitale (2; MRC LM 43; MRC 59002)	0.007–0.015	32.0–64.0	0.19–0.5	0.25–0.38
T. mentag. var. granulare (2; MRC 54818; MRC 58001)	0.007–0.01	16.0–32.0	0.19–0.25	0.16–0.25
T. tonsurans (MRC 17404)	0.015	12.0	0.14	0.14
MOULDS				
Scytalidium dimidiatum (2; MRC LM 71; MRC 56597)	0.19	⩾64.0	1.25–16.0	0.75–2.0
Scopulariopsis brevicaulis (2; MRC 54850; MRC 54760)	0.63–0.75	⩾64.0	0.52–16.0	0.19–2.0
Aspergillus flavus (MRC 54849)	0.12	⩾64.0	0.75	16.0
Acremonium strictum (MRC 54667)	0.75	⩾64.0	16.0	2.0
Fusarium solani (MRC LM 35)	2.0	⩾64.0	16.0	16.0

Terb=terbinafine; Fluc=fluconazole; Itra=itraconazole; Amor=amorolfine.
The numbers of isolates tested are given in parentheses, together with their relevant accession number to the culture collection in which they are deposited.

Table I

Range of MIC_{80} values for species tested

combinations was calculated using the formula:

$$\text{A: FIC for drug X} = \frac{\text{MIC for combination of drugs X+Y}}{\text{MIC for drug X alone}}$$

$$\text{B: FIC for drug Y} = \frac{\text{MIC for combination of drugs X+Y}}{\text{MIC for drug Y alone}}$$

The combined FIC value for the two drugs was calculated as A+B.

A combined FIC value of <1 was taken to suggest synergy; a value of 1–2 indicated either an additive effect of the two drugs or indifference; and a combined FIC value of >2 was taken to suggest antagonism.

Results

The MIC_{80} results recorded for the yeast, dermatophyte and mould strains tested are shown in Table I. These values are broadly in line with what would be expected for the fungi and antifungals tested, which indicates that the test system is reliable.

The range of mean FIC values calculated for the yeasts, dermatophytes and moulds are shown in Table II. Overall, 46% of amorolfine combinations showed results suggestive of synergy (FIC<1). Of these, 54% of combinations showed synergy against dermatophytes; 28% showed synergy against yeasts and 52% against moulds.

Amorolfine+terbinafine combinations showed a tendency towards synergy with 7/20 (35%) organisms tested; with amorolfine+fluconazole the figure was 12/22 (55%) organisms; and for amorolfine+itraconazole it was 10/21 (48%) organisms. No cases of antagonism (FIC>2) were seen, although some combinations gave an FIC of 2 with some moulds.

The percentages of dermatophytes, yeasts and moulds showing potentially synergistic activity (FIC<1) with amorolfine in combination with terbinafine, fluconazole or itraconazole are shown in Table III.

Results suggesting synergy between amorolfine and terbinafine, fluconazole or itraconazole were seen with yeast species, dermatophytes and, encouragingly, moulds. Because of the relatively small numbers, it was not possible to determine statistically which drug combinations gave the best results overall. However, amorolfine and fluconazole appeared to result in synergy most often, especially for dermatophytes. Differences were also seen between different strains of the same species, which is not unexpected given biological diversity.

Discussion

The use of combinations of drugs may be beneficial in the treatment of patients with onychomycosis, especially

Organisms	Terb+Amor	Fluc+Amor	Itra+Amor
Dermatophytes	29%	78%	50%
Candida species	33%	17%	33%
Selected moulds	43%	57%	57%

Terb=terbinafine; Fluc=fluconazole; Itra=itraconazole; Amor=amorolfine.

Table III

The percentage of organisms showing potentially synergistic activity (FIC<1) with the amorolfine combinations

Organisms	Terb+Amor	Fluc+Amor	Itra+Amor
YEASTS			
Candida albicans (2)	1.13–1.25	1.13–1.25	1.0–1.13
C. krusei	1.5	1.5	1.0
C. parapsilosis (2)	0.88	0.88–1.13	0.38–1.13
C. guilliermondii	1.5	1.0	0.75
DERMATOPHYTES			
Trichophyton rubrum (4)	0.55–2.0	0.69–1.13	0.56–1.15
T. mentag. var. interdigitale (2)	1.25–1.5	0.64–1.13	0.8–1.25
T. mentag. var. granulare (2)	0.78–1.25	0.75–0.88	0.81–1.13
T. tonsurans	1.25	0.75	1.0
MOULDS			
Scytalidium dimidiatum (2)	0.55–1.13	0.3–0.63	0.64–0.98
Scopulariopsis brevicaulis (2)	0.5–1.0	0.78–1.5	1.16–1.75
Aspergillus flavus	0.56	2.0	0.4
Acremonium strictum	2.0	0.88	0.62
Fusarium solani	1.0	1.75	2.0

Terb=terbinafine; Fluc=fluconazole; Itra=itraconazole; Amor=amorolfine.
The numbers of isolates are given in parentheses.

Table II

Range of mean FIC values for species tested

if the combination of drugs has a synergistic action. When testing for synergy, the activity of the combined drugs is indicated by the FIC index. The interpretation of this figure can vary,[12,13] but, in general, values lower than 1.0 suggest synergy between the two compounds tested, values of 1.0–2.0 indicate indifference or an additive effect, and values greater than 2.0 indicate antagonism. For synergy, the lower the value below 1.0 the greater the degree of synergy.

The results here indicate the likelihood that synergy occurred between topical amorolfine and terbinafine for 35% of the organisms tested. The remaining organisms showed indifference or an additive effect, with none showing antagonism. For amorolfine with fluconazole, synergy was likely against 55% of the organisms. Again, the remaining organisms all showed indifference and no antagonism. Finally, for itraconazole and amorolfine, a tendency towards synergy was seen in 48% of the organisms, with the remainder indifferent. Overall, for the yeasts, moulds and dermatophytes, 46% of the amorolfine combinations showed FICs suggestive of synergy, with most synergistic combinations seen with dermatophytes (54%) and moulds (52%). These results are in keeping with previously published studies that have examined the synergy of antifungal agents. As previously suggested, the synergistic interaction between amorolfine with other sterol biosynthesis inhibitors is predictable but still compelling.[3]

This *in vitro* study set out to determine whether synergy occurred between amorolfine and other antifungals commonly used to treat mycoses orally, and to check that the drug combinations were not antagonistic. It does not mean, however, that the combinations showing the greatest *frequency* of synergistic interactions should necessarily be used to treat onychomycosis. For example, there were a greater number of instances of possible synergy between amorolfine and fluconazole against dermatophytes than with the other drugs. However, fluconazole is a relatively poor antifungal against dermatophytes and terbinafine in combination with amorolfine is likely to be better for treating onychomycosis. It may be that these effects will also be seen *in vivo* as indicated by various clinical studies.[3,7,8]

This study has suggested, in keeping with previous findings, that there is synergistic activity for some combinations of drugs against some organisms *in vitro* and adds to a growing body of evidence to suggest that clinical trials assessing the efficacy of combined topical and oral therapy are justified.

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References

1. Roberts DT, Evans EGV, Allen R, *Fungal infections of the nail*. Mosby-Wolfe: London, 1998: 88.
2. Evans EGV, Nail dermatophytosis: the nature and scale of the problem. *J Dermatol Treat* (1990) **1** (Suppl 2): 47–8.
3. Polak A, Combination of amorolfine with various antifungal drugs in dermatophytosis. *Mycoses* (1993) **36** (1–2): 43–9.
4. Meletiadis J, Mouton JW, Meis JF, Verweij PE, In vitro drug interaction modeling of combinations of azoles with terbinafine against clinical *Scedosporium prolificans* isolates. *Antimicrob Agents Chemother* (2003) **47**: 106–17.
5. Perea S, Gonzalez G, Fothergill AW et al, In vitro interaction of caspofungin acetate with voriconazole against clinical isolates of *Aspergillus* spp. *Antimicrob Agents Chemother* (2002) **46**: 3039–41.
6. Evans EG, The rationale for combination therapy. *Br J Dermatol* (2001) **145** (Suppl 60): 9–13.
7. Baran R, Feuilhade M, Combernale P et al, A randomized trial of amorolfine 5% solution nail lacquer combined with oral terbinafine compared with terbinafine alone in the treatment of dermatophytic toenail onychomycoses affecting the matrix region. *Br J Dermatol* (2000) **142**: 1177–83.
8. Lecha M, Alsina M, Torres-Rodriguez JM et al, An open-label, multicenter study of the combination of amorolfine nail lacquer and oral itraconazole compared with oral itraconazole alone in the treatment of severe toenail onychomycosis. *Curr Ther Res* (2002) **63**: 366–79.
9. Barchiesi F, Colombo AL, McGough DA, Rinaldi MG, Comparative study of broth macrodilution and microdilution techniques for in vitro antifungal susceptibility testing of yeasts by using the national committee for clinical laboratory standards' proposed standard. *J Clin Microbiol* (1994) **32**: 2494–500.
10. National Committee for Clinical Laboratory Standards. *Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi; proposed standard M38-P*. Villanova, PA: National Committee for Clinical Laboratory Standards, 1998.
11. Jessup CJ, Warner J, Isham N et al, Antifungal susceptibility testing of dermatophytes: establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates. *J Clin Microbiol* (2000) **38**: 341–4.
12. Koneman EW, Allen SD, Janda PC et al, Antimicrobial susceptibility testing. In: Koneman EW, Allen SD, Janda WM, et al, eds. *Diagnostic microbiology*. Philadelphia, PA: Lippincott, 1992.
13. Marchetti O, Moreillon P, Glauser MP et al, Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans*. *Antimicrob Agents Chemother* (2000) **44**: 2373–81.