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Combination of amorolfine with various antifungal drugs in dermatophytosis

Kombination von Amorolfin mit verschiedenen Antimykotika bei Dermatophytose

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Key words. *Trichophyton*, dermatophytosis, *Candida albicans*, amorolfine, azoles, griseofulvin, antimycotic chemotherapy, combination therapy, animal model.

Schlüsselwörter. *Trichophyton*, Dermatophytose, *Candida albicans*, Amorolfin, Azole, Griseofulvin, antimykotische Chemotherapie, Kombinationstherapie, Tierversuche.

Summary. The fungistatic activity of ketoconazole, griseofulvin and terbinafine alone and in combination with amorolfine was tested in casitone agar. The fungistatic activity of all three against *Trichophyton mentagrophytes* is slightly increased by the addition of amorolfine. In yeasts the results are not so clear. The combinations of amorolfine with griseofulvin, terbinafine, itraconazole and fluconazole were always more efficient in a murine model of dermatophytosis than the monotherapy, thus a clear synergism between amorolfine and other antifungals was seen.

Zusammenfassung. Auf Casitone-Agar wurde die fungistatische Aktivität von Ketoconazol, Griseofulvin und Terbinafin allein und in Kombination mit Amorolfin geprüft. Die fungistatische Aktivität aller drei Antimyzetika gegen *Trichophyton mentagrophytes* wurde durch die Zugabe von Amorolfin leicht erhöht. Bei Hefen waren die Ergebnisse nicht so klar. Die Kombination von Amorolfin jeweils mit Griseofulvin, Terbinafin, Itraconazol und Fluconazol war im Dermatophytosemodell an der Maus stets wirksamer als die Monotherapie und belegte damit einen eindeutigen Synergismus zwischen Amorolfin und den übrigen Antimyzetika.

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Introduction

Treatment of onychomycosis remains a difficult task. Both systemic and topical therapy are possible, but the treatment period is long. Many patients are concerned about the side-effects of systemic drugs when a long treatment period is necessary. The introduction of amorolfine nail lacquer opened new possibilities in the treatment of onychomycosis without removal of the nails. The lacquer is effective in onychomycosis without matrix involvement, extremely well tolerated and patient compliance is excellent. Nevertheless, heavily infected nails with matrix involvement do not respond to this topical therapy, and a combination of amorolfine lacquer with short systemic treatment could be the answer to such refractory nail infections. The best combination partner for amorolfine from a biochemical viewpoint would be a sterol biosynthesis inhibitor, since compounds or combinations which inhibit a single biosynthetic pathway at two separate steps are usually more active than those which act only on one step. So the combination of amorolfine with azoles or allylamines should be highly synergistic. Griseofulvin, the first antimycotic for the treatment of superficial mycosis, is still used for the therapy of onychomycosis and could also be envisaged as a candidate for combination therapy. The interaction of amorolfine with several antimycotics was studied *in vitro* and

in a model of dermatophytosis in the mouse, and the results of this investigation are presented in this paper.

Materials and methods

The following antifungals were used: amorolfine (Roche Basel); ketoconazole, itraconazole (Janssen, Beerse); fluconazole (Pfizer, UK); and griseofulvin.

The various strains of *Candida albicans*, one strain of *Trichophyton mentagrophytes* and the *Trichophyton mentagrophytes* var. *quinckeanum* were from the Roche culture collection; other species of dermatophytes (*Microsporum canis*) were obtained from the dermatological department of Basel University and from Professor Mensing of Hamburg University.

Synergism of fungistatic activity was investigated using an agar dilution checkerboard method. The medium contained per litre 9 g casitone (Difco, Detroit, MI, USA), 20 g glucose, 10 g yeast extract, 1 g KH_2PO_4, 1 g Na_2HPO_4, 10 g sodium citrate and 20 g agar. Compounds were dissolved in dimethylsulphoxide and gradually diluted with distilled water. The minimum inhibitory concentrations (MIC) of the individual compounds and combinations were read after an incubation period of 48 h at 37 °C for yeasts and after 4 days incubation at 30 °C for dermatophytes. The fractional inhibitory concentration (FIC) index was calculated according to the method of Elion *et al.* [1].

Murine dermatophytosis was studied using albino mice (Charles River) weighing approximately 20 g. For each experiment fresh subcultures of *T. mentagrophytes* var. *quinckeanum* ND24 were taken from the lyophilized stock and incubated on potato and carrot agar for 10 days at 30 °C. From this primary culture suspensions were made in honey using a mortar and pestle. Small quantities of the suspension containing approximately 10^5 conidia were rubbed into the shaven skin of the backs of the animals. Favus-like lesions developed regularly in untreated animals reaching maximum intensity about 6 days after the infection and healing spontaneously after 2 weeks. Antifungals were administered twice on day 0, approximately 1 h before and 5 h after the infection, and treatment continued once daily through day 4 for a total of 6 doses. The presence

Table 1. Synergism of fungistatic activity of amorolfine and various antifungals against dermatophytes

Strain	Combination partner	MIC alone (μg ml^{-1})		MIC in combination (μg ml^{-1})		FIC index
	=X	Amor.	X	Amor.	X	
T. mentagrophytes	griseofulvin	0.1	5	0.025	2.5	0.75
		0.1	2.5	0.05	1.25	1
		0.1	10	0.05	0.31	0.532
T. rubrum		0.05	0.5	0.0125	0.125	0.5
M. canis		0.1	5	0.025	2.5	0.75
		0.1	2.5	0.05	1.25	1
T. mentagrophytes	ketoconazole	0.1	10	0.025	2.5	0.5
		0.1	5	0.025	2.5	0.75
		0.1	2.5	0.05	0.3	0.625
T. rubrum		0.1	10	0.025	2.5	0.5
M. canis		0.1	5	0.025	2.5	0.75
		0.1	10	0.025	2.5	0.5
T. mentagrophytes	itraconazole	0.05	2	0.025	0.25	0.625
		0.1	0.5	0.012	0.12	0.25
		0.05	0.5	0.025	0.25	1
T. rubrum		0.05	0.5	0.012	0.12	0.25
M. canis		0.1	0.5	0.012	0.12	0.375
		0.05	1	0.012	0.12	0.375
T. mentagrophytes	terbinafine	0.1	0.005	0.05	0.0012	0.75
		0.1	0.01	0.05	0.0012	0.625
		0.1	0.01	0.025	0.0025	0.5
T. rubrum		0.05	0.005	0.006	0.0006	0.25
M. canis		0.05	0.005	0.02	0.0025	1
		0.05	0.01	0.012	0.0012	0.375

T., Trichophyton; M., Microsporum.

Table 2. Synergism of fungistatic activity of amorolfine and various antifungals against *Candida albicans*

Strain	Combination partner	MIC alone (μg ml^{-1})		MIC in combination(μg ml^{-1})		FIC index
	=X	Amor.	X	Amor.	X	
C. albicans						
H12	ketoconazole	0.06	5	0.03	1.25	0.75
H29		0.06	5	0.03	2.5	1
3153		0.03	10	0.015	5	1
B4		0.03	10	0.06	10	>1
N6		0.03	10	0.12	10	>1
C. albicans						
H12	itraconazole	0.012	0.75	0.006	0.38	1
H29		0.012	0.024	0.006	0.006	0.75
3153		0.025	0.19	0.012	0.003	0.65
B4		0.012	0.024	0.006	0.006	0.75
N6		0.012	0.024	0.006	0.006	0.75
H28		0.025	100	0.006	0.38	0.253
C. albicans						
H12	terbinafine	0.12	12	0.03	6.25	0.75
H29		0.06	25	0.075	6.25	0.375
3153		0.06	>100	0.015	12	<0.375
B4		0.06	>100	0.12	>100	>1
N6		0.03	>100	0.12	>100	>1
H28		0.12	>100	0.06	12	<0.56
H42		0.12	12	0.03	6.25	0.75
H72		0.06	25	0.015	6.25	0.5

Table 3. Chemotherapeutic activity of various antifungals in a murine dermatophytosis model

Treatment	Dose (mg kg^{-1})	Infection score (% of mycosis-free animals in parentheses)			Significance to control
		Day 4	Day 7	Day 11	
0		0.89 (0)	1.9 (0)	1.44 (0)	
Itraconazole	0.3	0.83 (0)	2.08 (0)	1 (0)	NS
	1	0.91 (0)	2 (0)	15 (0)	NS
	3	0.58 (20)	1 (11)	0.46 (33)	$P \leqslant 0.05$
	10	0.33 (58)	0.29 (58)	0.21 (66)	$P \leqslant 0.01$
	30	0.25 (66)	0 (100)	0 (100)	$P \leqslant 0.01$
Fluconazole	1	0.83 (0)	1.58 (0)	0.58 (50)	NS
	3	0.17 (66)	0.75 (16)	0.63 (58)	$P \leqslant 0.01$
	10	0.25 (58)	0.25 (83)	0.04 (91)	$P \leqslant 0.01$
	30	0.25 (66)	0.17 (66)	0.17 (66)	$P \leqslant 0.01$
	50	0.42 (50)	0.17 (83)	0 (100)	$P \leqslant 0.01$
Terbinafine	3	0.96 (0)	1.29 (0)	0.88 (16)	$P \leqslant 0.05$
	10	0.42 (77)	0.25 (50)	0.21 (58)	$P \leqslant 0.01$
	30	0 (100)	0 (100)	0 (100)	$P \leqslant 0.01$
Griseofulvin	50	1.46 (0)	1.38 (8)	1.13 (33)	NS
	100	1 (0)	1.6 (0)	1.1 (0)	NS
Amorolfine	10 s.c.	0.33 (83)	1.13 (0)	0.63 (25)	$P \leqslant 0.05$
	30 s.c.	0.17 (66)	0 (100)	0 (100)	$P \leqslant 0.01$
	30 p.o.	0.42 (41)	1 (0)	0.71 (41)	$P \leqslant 0.05$
	100 p.o.	0.42 (50)	0.17 (66)	0.33 (66)	$P \leqslant 0.01$

NS, not significant.

or absence of mycotic foci and their intensity was scored on days 4, 7 and 11 after infection. The intensity of the mycotic foci was measured on a scale of 0.5–2; in untreated control animals the intensity was between 1 and 2 during the observation period. Two parameters were used, the score of infection and the number of mycosis-free animals.

Results

Synergy between amorolfine and other antifungals (fungistatic activity)

The MIC of amorolfine and various antifungals alone and in combination against dermatophytes are seen in Table 1. The FIC indices calculated are also listed. The fungistatic activity of griseofulvin, ketoconazole, itraconazole and terbinafine against all dermatophytic species tested is slightly increased by the addition of amorolfine. The effect is generally additive, and in some strains a synergistic effect is observed, even if slight. Antagonism was never observed.

In yeast (Table 2) the findings are not so clear-cut. Griseofulvin was not tested since it is totally inactive against yeasts *in vitro*. Amorolfine and ketoconazole showed additive or even weak synergistic interaction in some strains but a mild antagonism was seen in two, where high ketoconazole levels (> 10 μg ml^{-1}) increased the MIC of amorolfine by a factor of 2–4. No antagonism is seen with itraconazole, but the combination of terbinafine with amorolfine showed a slight antagonistic effect in two strains, the MIC being increased in the presence of a high concentration of terbinafine from 0.03 μg ml^{-1} or 0.06 μg ml^{-1} to 0.12 μg ml^{-1}.

Synergy between amorolfine and other antifungals in murine dermatophytosis

Dermatophytosis of mice is self-healing, in contrast to the infection in man. The course of infection in mice reaches a plateau on day 7 and

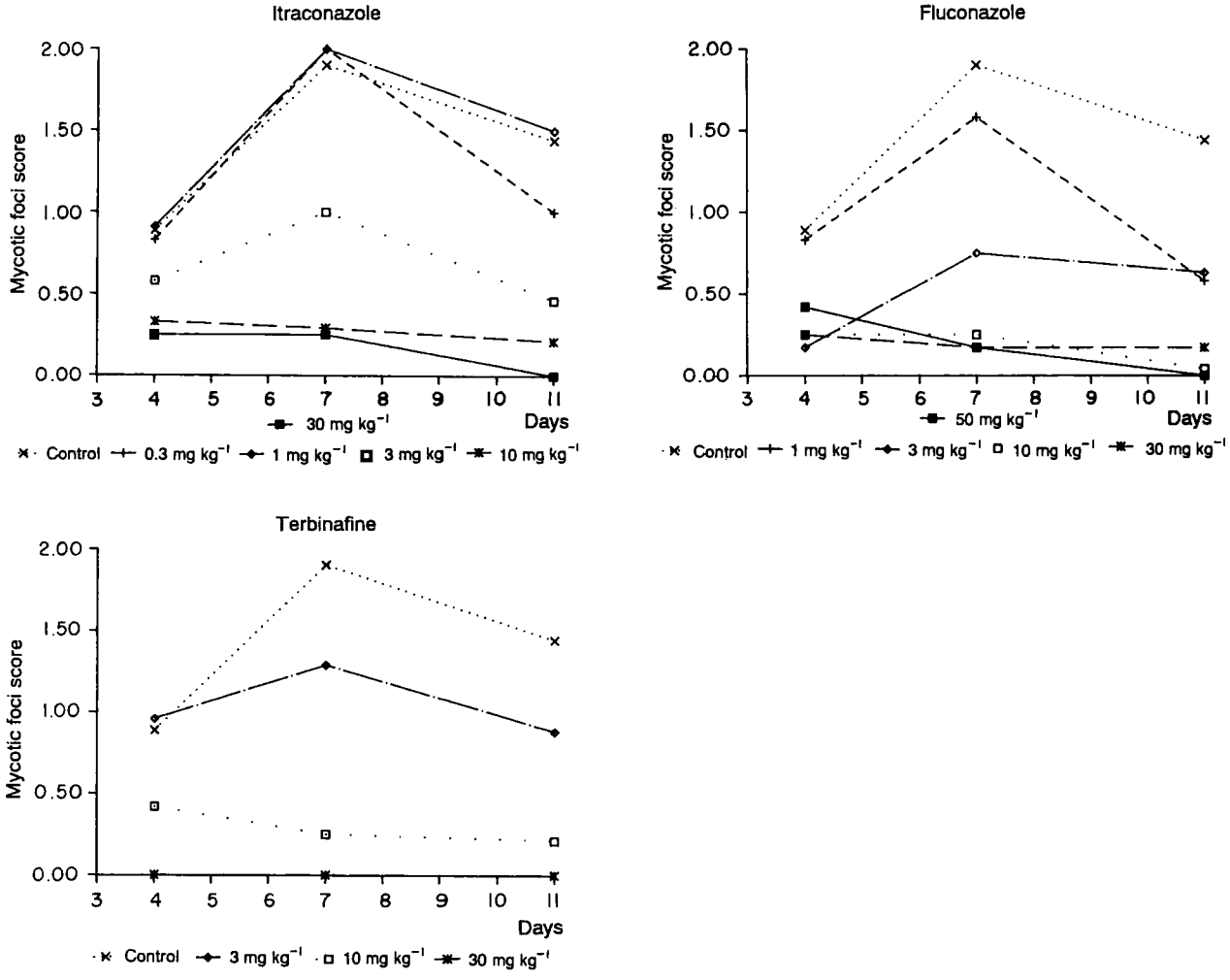

Figure 1. The course of murine dermatophytosis infection under various antifungal therapies.

declines thereafter throughout the second week. Mice infected with *Trichophyton* and treated with various antifungal drugs generally respond to the treatment in a dose-dependent manner. All antifungals induced a significant reduction in the appearance of mycotic foci on days 4, 7 and 11 after inoculation in comparison to the untreated animals (Fig. 1). The severeness of mycotic foci is reduced and the number of mycosis-free animals is increased depending on the dose applied (Table 3, Fig. 1). Amorolfine showed a systemic activity after subcutaneous and oral application in this model, whereas the drug proved to be systemically inactive against other fungal infections [2]. Ketoconazole was not studied *in vivo* since this drug is not favoured by dermatologists for the treatment of onychomycosis due to its liver toxicity.

The combinations of amorolfine with griseofulvin, terbinafine, itraconazole and fluconazole are always more efficient than the individual drug treatment alone provided that the single therapy is not overwhelmingly efficient *per se*. For instance, fluconazole at 10 mg kg^{-1} cured most of the animals and this cannot be increased by addition of amorolfine. An increase in efficacy is expressed by a lower score for the mycotic foci (Table 4; Figs 2 and 3) as well as a higher number of mycosis-free animals. If the number of mycosis-free animals is observed, the combination is always synergistic on day 7. On day 11 additive effect and synergistic effect can be seen. Antagonism is never seen.

Discussion

The beneficial interaction of amorolfine, whose mode of action is based on the inhibition of ergosterol biosynthesis, with other sterol biosynth-

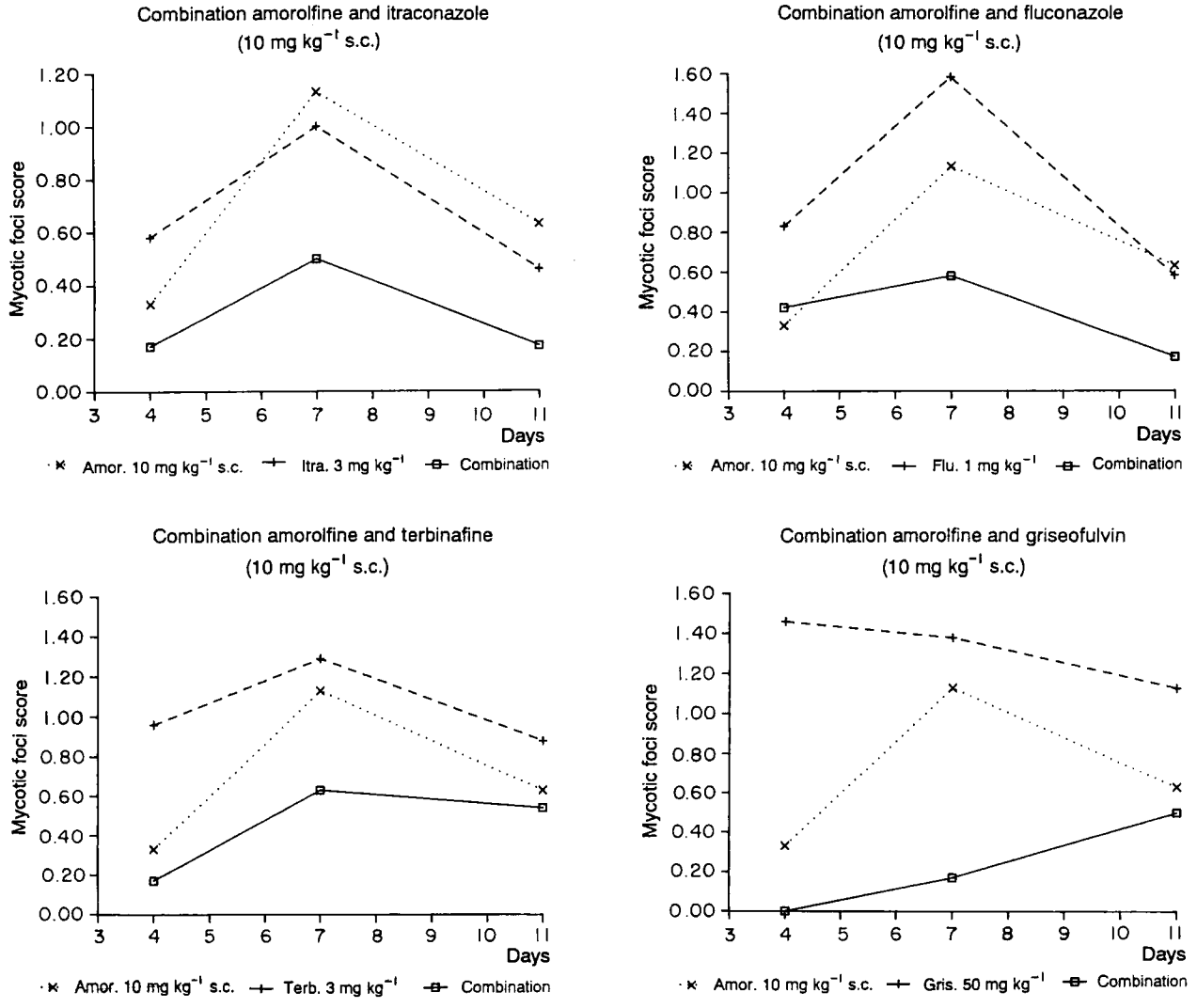

Figure 2. The course of murine dermatophytosis infection under combination therapy with amorolfine and various antifungals. Amorolfine 10 mg kg^{-1} s.c.

Table 4. Chemotherapeutic activity of combinations in a murine dermatophytosis model due to *Trichophyton mentagrophytes* var. *quinckeanum*

Therapy	Dose (mg kg^{-1})	Infection score (% of mycosis-free animals)	
		Day 7	Day 11
Amorolfine	10 s.c.	1.13 (0)	0.63 (25)
Griseofulvin	50	1.38 (8)	1.13 (33)
Amorolfine + Griseofulvin	10 + 50	0.17 (50)	0.5 (66) A
Itraconazole	0.3	2.08 (0)	1.0 (0)
Amor. + Itra.	10 + 0.3	1.08 (16) S	0.25 (33) S
Itraconazole	1	2.0 (0)	1.5 (0)
Amor. + Itra.	10 + 1	0.58 (16) S	0.75 (33) S
Itraconazole	3	1.0 (16)	0.46 (33)
Amor. + Itra.	10 + 3	0.5 (25) S	0.17 (66) S
Itraconazole	10	0.29 (58)	0.21 (66)
Amor. + Itra.	10 + 10	0 (100) S	0 (100) S
Fluconazole	1	1.58 (0)	0.58 (20)
Amor. + Flu.	10 + 1	0.58 (16) S	0.17 (60)
Fluconazole	3	0.75 (16)	0.63 (58)
Amor. + Flu.	10 + 3	0.21 (58) S	0.13 (83) A
Fluconazole	10	0.25 (83)	0.04 (91)
Amor. + Flu.	10 + 10	0.25 (83) NS	0 (100) NS
Terbinafine	3	1.29 (0)	0.88 (16)
Amor. + Terb.	10 + 3	0.63 (16) S	0.54 (41) S
Terbinafine	10	0.25 (50)	0.21 (58)
Amor. + Terb.	10 + 10	0.22 (83) s	0.17 (66)
Amorolfine	30 p.o.	1.0 (0)	0.71 (41)
Griseofulvin	50	1.38 (8)	1.13 (33)
Amorolfine + Griseofulvin	30 + 50	0.33 (50)	0.42 (66) H
Itraconazole	0.3	2.08 (0)	1 (0)
Amor. + Itra.	30 + 0.3	0.75 (33) S	0.42 (50) S
Itraconazole	1	2.0 (0)	1.5 (0)
Amor. + Itra.	30 + 1	0.6 (16) S	0.2 (50) S
Itraconazole	3	1.0 (16)	0.46 (33)
Amor. + Itra.	30 + 3	0.46 (41) S	0.08 (83) S
Itraconazole	10	0.29 (58)	0.21 (66)
Amor. + Itra.	30 + 10	0.08 (82) S	0 (100)
Fluconazole	1	1.58 (0)	0.58 (50)
Amor. + Flu.	30 + 1	0.42 (16) S	0.17 (66) NS
Fluconazole	3	0.75 (16)	0.63 (58)
Amor. + Flu.	30 + 3	0.29 (38) S	0.21 (66) NS
Fluconazole	10	0.25 (83)	0.04 (91)
Amor. + Flu.	30 + 10	0.08 (83) NS	0 (100) NS
Terbinafine	3	1.29 (0)	0.88 (16)
Amor. + Terb.	30 + 3	0.35 (16) S	0.25 (58) A
Terbinafine	10	0.25 (50)	0.21 (58)
Amor. + Terb.	30 + 10	0.25 (50) NS	0.25 (66) NS

S, synergistic effect with respect to both drugs; A, additive effect with respect to both drugs; NS, not significant.

esis inhibitors such as ketoconazole, itraconazole and terbinafine, was predictable, but the synergy between amorolfine and griseofulvin is surprising from the biochemical viewpoint.

The results with *Candida* strains are less clear-cut but the antagonistic effect seen in some strains is weak, and most probably not therapeutically relevant. The interesting, and for the clinical use important, finding is the fact that the synergy between amorolfine and other antifungal drugs seen *in vitro* against dermatophytes is translated into chemotherapeutic activity, and is in fact generally more pronounced *in vivo* than *in vitro*. From our animal data it can be concluded that

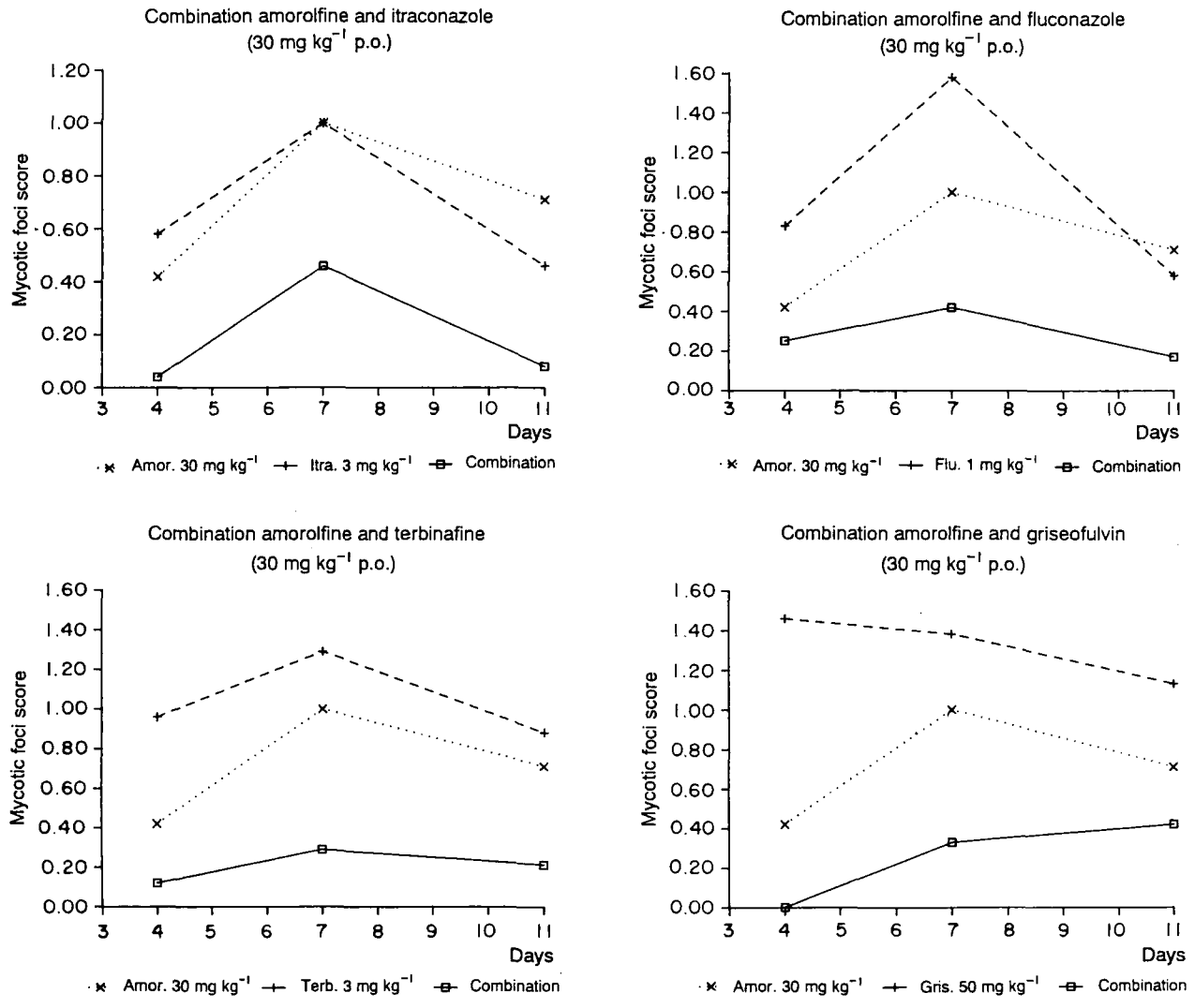

Figure 3. The course of murine dermatophytosis infection under combination therapy with amorolfine and various antifungals. Amorolfine 30 mg kg^{-1} s.c.

combinations between a systemic application of terbinafine, itraconazole or another antifungal and a topical application of amorolfine may well be synergistic in human chemotherapy.

In fact, the beneficial effect of a combination of amorolfine with griseofulvin in extensive onychomycosis was recently proven in a clinical trial. Monotherapy with griseofulvin for 12 months is being compared with combined amorolfine and griseofulvin for 2 months followed by 10 months of amorolfine lacquer. After 6 months it is already clear that the combination leads to more rapid sterilization of the infectious foci than monotherapy with griseofulvin and gives at least the same clinical response [3]. This positive finding makes it most likely that the synergy between amorolfine and other antifungal drugs seen in our animal models will be transferable to human chemotherapy and this may be especially helpful for very severe nail infections in which 100% of the nail is infected. Above all, the duration of therapy of such infections may be significantly reduced by a combination therapy between an oral and a topically applied antifungal. This would be a real step forward in the therapy of extensive onychomycosis.

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