

Agar sublimation test for the *in vitro* determination of the antifungal activity of morpholine derivatives

Agar-Sublimations-Test zur *In vitro*-Bestimmung der antimykotischen Aktivität von Morpholin-Derivaten

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Summary

We studied the *in vitro* antifungal activities of a wide range of antimycotic agents, including amorolfine, terbinafine, naftifine, five morpholine derivatives, ciclopiroxolamine, bifonazole, clotrimazole, ketoconazole, itraconazole, fluconazole, voriconazole, flucytosine, amphotericin B, nystatin, and caspofungin, against *Candida albicans* and *Trichophyton rubrum* by conventional agar diffusion tests and by a novel sublimation method. For the sublimation method, 6 mm filter paper disks were soaked with defined amounts of antimycotic drugs, air dried, placed in the center of the lids of 9 cm Petri dishes, and incubated upside down with inoculated agar plates 10 mm above the disks. The conventional disk diffusion tests produced inhibition zones as previously described. The disk sublimation tests produced large inhibition zones with amorolfine, five amorolfine derivatives, and terbinafine, but with none of the other antifungal agents. Possible therapeutic advantages of agents, which are able to overcome air cavities in mycotic lesions, e.g. in onychomycosis, are discussed.

Zusammenfassung

Wir untersuchten *in vitro* die antimykotische Aktivität eines breiten Spektrums von Antimykotika, einschließlich Amorolfin, Terbinafin, Naftifin, fünf Morpholin-Derivaten, Ciclopiroxolamin, Bifonazol, Clotrimazol, Ketoconazol, Itraconazol, Fluconazol, Voriconazol, 5-Fluorcytosin, Amphotericin B, Nystatin und Caspofungin, gegenüber *Candida albicans* und *Trichophyton rubrum* mit konventionellen Agardiffusionstesten und mit einer neuartigen Sublimationsmethode. Für die Sublimationsmethode wurden 6 mm-Filterpapier-Blättchen mit definierten Mengen von Antimykotika getränkt, luftgetrocknet, in die Mitte der Deckel von 9 cm-Petrischalen gelegt und mit der inokulierten Agarplatte 10 mm über den Blättchen umgedreht inkubiert. Die konventionellen Agardiffusionsteste produzierten Hemmhöfe wie früher beschrieben. Die Blättchen-Sublimationsteste produzierten große Hemmhöfe mit Amorolfin, fünf Morpholin-Derivaten und Terbinafin, nicht jedoch mit den anderen Antimykotika. Mögliche therapeutische Vorteile von Agentien, die luftgefüllte Hohlräume in mykotischen Läsionen überbrücken können, z. B. im Nagel bei Onychomykose, werden diskutiert.

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Introduction

The phenyl-propyl morpholine derivative amorolfine is a sterol biosynthesis inhibitor, which interferes with the ergosterol biosynthesis at two steps, the Δ_{14} reductase and the Δ_{7-8} isomerase. It is chemically unrelated to other classes of antifungal agents-like hydroxy-pyridones, imidazoles, and polyenes. Amorolfine has a broad antimycotic spectrum against fungi pathogenic to plants, animals, and humans. The drug does not only inhibit the fungal growth, it also shows a strong fungicidal activity against yeasts, dermatophytes, dimorphic, and dematiaceous fungi. This fungicidal effect is both time- and concentration-dependent. Its fungicidal activity against dermatophytes is in the same order of magnitude as the one of terbinafine.¹⁻⁴

Topical amorolfine nail laquer 5% (amorolfine hydrochloride in Loceryl[®] nail laquer, Galderma, Freiburg, Germany) is widely used for the therapy of human onychomycosis as mono- and combination therapy. Amorolfine readily penetrates the nail after topical application. The kinetics of penetration follows an exponential function. The concentration in the upper layer was 100 times higher than in the lowest one after 24 h.⁵ High antifungal activity was also demonstrated in subungual material from onychomycosis patients *in vivo*.⁶ The degree of penetration is dependent on the consistency of the nail. However, despite the differences in penetration of damaged nails, amorolfine levels in the nail exceed the minimum inhibitory concentration values of most fungi causing onychomycosis.

During the clinical trial of the compound, one of the authors attempted to develop a commercial disk for the *in vitro* susceptibility testing of amorolfine by agar diffusion. In diffusion tests on casitone agar, amorolfine produced large clear inhibition zones against yeasts and dermatophytes. During this experimental development an unusual quality of amorolfine was observed: amorolfine loaded disks (1 and 10 μg) did not only produce an inhibition zone in direct contact with the incubated agar, but also indirectly, when the disk was placed on the lid of the Petri dish containing inoculated agar (opposite to agar, Fig. 1). This phenomenon was repeatedly observed during the developmental work with amorolfine and other compounds of the chemical class of morpholines. Obviously, amorolfine and other morpholines were able to exert their fungistatic and fungicidal activities over a certain distance, thus spanning or bridging air-filled space, without direct contact to the target fungal cells. We assume from preliminary observations that this effect may correspond to the physicochemical phenomenon of sublimation. Sublimation is a property of a chemical substance to change the aggregate directly from solid to gaseous without transition through the liquid-phase state. This phenomenon has not yet been reported for any antifungal compound. It was never studied or published in connection with an antimycotic agent in general and amorolfine or morpholines in particular.

To investigate and characterize our former incidental observations more appropriately, we thoroughly initiated systematic experimental studies *in vitro*. This paper

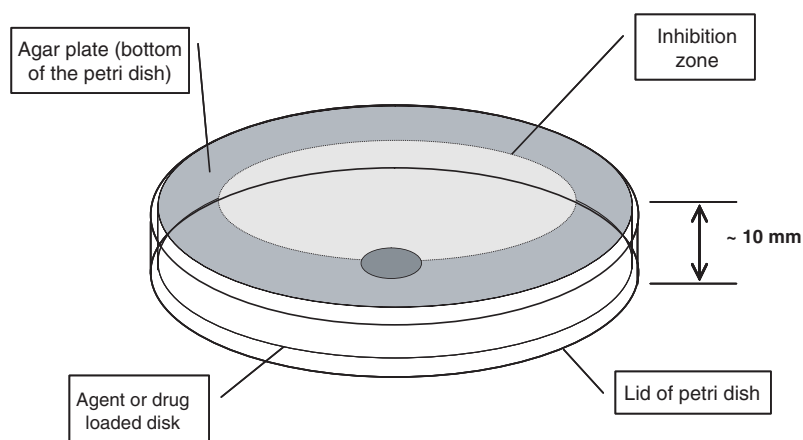


Figure 1 Diagram of the experimental setting of the novel sublimation method for the *in vitro* testing of antifungal activity. Inhibition zones were generated with the drug-loaded disk placed in the lid of the Petri dish opposite of the agar. The Petri dish was incubated upside down.

describes for the first time the specific property of amorolfine and some other morpholine derivatives to bridge air-filled space and maintain antifungal activity in comparison with other chemical classes of antifungal agents. Furthermore, we discuss the possible therapeutic advantage of this property in the topical therapy of onychomycosis as a model fungal infection.

Materials and methods

Fungi

Two fungal strains were used as test organisms: the yeast *Candida albicans* ATCC 90028 and a clinical isolate of the dermatophyte *Trichophyton rubrum*.

Antifungal agents, antimycotica disks, and galenical formulations

Amorolfine (-hydrochloride) and its commercial galenical formulations (Loceryl[®] nail laquer and Loceryl[®] creme) were gifts from Galderma Laboratorium GmbH, Freiburg i. Br., Germany. The non-commercial amorolfine solution was a result of a preclinical development by Hoffmann La Roche, Basel, Switzerland. Terbinafine, pure compound, was a gift from Novartis, Nürnberg, Germany. Naftifine was purchased from Rentschler, Laupheim, Germany. Ciclopiroxolamine and bifonazole were purchased from Sigma, Taufkirchen, Germany. Nagel Batrafen[®] (Aventis Pharma, Bad Soden, Germany) (Ciclopirox nail laquer) was purchased from a pharmacy in Switzerland. Caspofungin infusion solution was a kind gift from Dr Michael Herold (Helios Medical Center, Erfurt, Germany). The morpholine derivatives were synthesized at Dr R Maag Ltd (Dielsdorf, Switzerland) or Hoffmann La Roche. The solvents of nail laquers (ethanol, ethyl acetate, *n*-butylacetate, and isopropanol) were purchased from Sigma.

The following commercially ready-made 6 mm loaded paper disks were from Mast Diagnostika, Reinfeld, Germany: miconazole 10 µg, ketoconazole 10 µg, itraconazole 1 µg, fluconazole 25 µg, clotrimazole 10 µg, amphotericin B 20 µg, nystatin 100 units, and flucytosine 1 µg. Voriconazole E-test strips were from AB BIODISK, Solna, Sweden. Unloaded neutral paper disks (Sigma) were loaded by hand with terbinafine, naftifine, bifonazole, amorolfine, and ciclopiroxolamine. The pure compounds were dissolved in dimethylsulfoxide (DMSO) and diluted in saline. Twenty microliter of the antimycotic solutions were then pipetted on the disks and dried for 1 h at room temperature. Furthermore, neutral disks were loaded with 10–20 µl of the commercial products

creme and lacquer of Loceryl[®] and Nagel Batrafen[®]. The loading of the disks with lacquers was rather difficult and clearly not exact, despite the fact that a standardized 10 µl inoculation loop was used.

Media

All agar plates had a thickness of 4 mm. For the diffusion tests, three different media were used: (i) Yeast Morphology Agar (Remel, Santa Fe, NM, USA; 35 g l⁻¹) plus agar (Oxoid, Wesel, Germany) 5 g l⁻¹, the pH was adjusted to 5.6; (ii) casitone agar: Bacto Casitone (Becton Dickinson, Heidelberg, Germany) 9 g l⁻¹, yeast extract (Merck, Darmstadt, Germany) 5 g l⁻¹, sodium citrate 10 g l⁻¹, glucose 22 g l⁻¹ (Merck), KH₂PO₄ 0.54 g l⁻¹, Na₂HPO₄ 3.34 g l⁻¹ and agar (Oxoid) 20 g l⁻¹, pH 7.2; (iii) Sabouraud Agar: Sabouraud agar (Sifin, Berlin, Germany) 42 g l⁻¹, agar (Oxoid) 3 g l⁻¹, pH 5.7.

Inoculation and incubation

Candida albicans cultures grown for 48 h at 37 °C were harvested with a cotton swab and suspended in saline, adjusted to McFarland 0.5, and spread on the agar surface using a cotton swab according to the National Committee for Clinical Laboratory Standards (NCCLS) method⁷.

Trichophyton rubrum cultures (5–7 days at 28 °C) were gently harvested using a cotton swab, suspended in saline and adjusted to McFarland 2.0. This suspension was spread on the agar surface with a cotton swab. After inoculation of the agar with the fungus, the loaded disks were placed either directly on the agar surface (diffusion method) or opposite on the lid of the Petri dishes (sublimation method, Fig. 1). This experimental arrangement caused a distance between agar and disk of about 10 mm. *Candida albicans* plates were incubated for 24 h at 37 °C. The dermatophyte plates were incubated at 28 °C for 6 days. The inhibition zones were measured in mm using a clear ruler.

Results

Pure compounds

All tested antimycotic drugs – except bifonazole and naftifine – showed a clear inhibition zone of various ranges against *C. albicans*, when tested directly on casitone agar (Table 1). However, only amorolfine showed a clear inhibition zone, even when the disk was placed opposite to the inoculated agar surface

Table 1 Inhibitory activity of various antimycotic agents against *Candida albicans* and *Trichophyton rubrum* measured by diffusion and sublimation methods.

| Antimycotic agents | Disk load (µg) | Inhibition zone (mm) | | | | | |
|--------------------|----------------|-------------------------|-----------------|-----------------------|-------------|----------------------------|-------------|
| | | <i>Candida albicans</i> | | | | <i>Trichophyton rubrum</i> | |
| | | Casitone agar | | Yeast morphology agar | | Sabouraud agar | |
| | | Diffusion | Sublimation | Diffusion | Sublimation | Diffusion | Sublimation |
| Amphotericin B | 20 | 21/20 | 0/0 | 18 | 0 | 9 | 0 |
| Nystatin | 100 u | 25/25 | 0/0 | ND | ND | 11 | 0 |
| Flucytosine | 1 | ND | ND | 28 | 0 | ND | ND |
| Miconazole | 10 | 26/24 | 0/0 | ND | ND | 37 | 0 |
| Clotrimazole | 10 | 32/32 | 0/0 | ND | ND | 60 | 0 |
| Bifonazole | 100 | ND | ND | ND | ND | 0 | 0 |
| | 10 | 0 | 0 | ND | ND | 0/0 | 0/0 |
| Ketoconazole | 10 | 36/35 | 0/0 | ND | ND | 0 | 0 |
| Fluconazole | 25 | ND/26 | ND/0 | ND | ND | 0 | 0/0 |
| Itraconazole | 1 | 15/15 | 0/0 | ND | ND | 18 | 0 |
| Voriconazole | E-test | MIC 0.023/MIC 0.023 | 0/0 | ND | ND | 0 | 0 |
| Terbinafine | 10 | 18/12 | 0/0 | ND | ND | >85/>85 | 82/>85 |
| | 1 | 15 | 0 | ND | ND | >85/75 | 80/>85 |
| | 0.5 | ND | ND | ND | ND | >85 | 75 |
| | 0.25 | ND | ND | ND | ND | >85 | 70 |
| | 0.125 | ND | ND | ND | ND | 75 | 64 |
| Naftifine | 10 | 0 | 0 | ND | ND | 70 | 0 |
| Caspofungin | 10 | 26/22 | 0/0 | 0 | 0 | 50* | 0 |
| | 1 | 17 | 0 | 0 | 0 | ND | ND |
| Amorolfine | 100 | 70/75 | 70/70 | ND | ND | >85/>85 | >85/>85 |
| | 10 | 63/66/70/70/75 | >85/78/70/70/70 | 48 | 60 | 72/75 | >85/>85 |
| | | | | | | 80/>85 | 80/>85 |
| | 1 | 52/48/52 | 74/64/60 | 34 | 41 | 55/60 | 66/75 |
| | | 52/55 | 60/60 | | | 60/70 | 65/70 |
| | 0.5 | 52 | 45 | ND | ND | 55/60 | 57/60 |
| | 0.25 | 35 | 45 | ND | ND | 40/35 | 48/25 |
| | 0.125 | 33 | 40 | ND | ND | 30/20 | 16/15 |
| | 0.1 | 30/40 | 40/50 | ND | ND | 30/52 | 25/40 |
| Ciclopiroxolamine | 10 | 17 | 0 | 17 | 0 | 9 | 0 |
| | 1 | 12 | 0 | 12 | 0 | 0 | 0 |

ND, not done; MIC, minimal inhibitory concentration; u, international unit; *significantly reduced growth in inhibition zone; numbers after a slash represent results of repeat experiments.

(Table 1 and Fig. 2). Amphotericin B, flucytosine, amorolfine, and ciclopiroxolamine showed clear inhibition zones on yeast morphology agar, whereas caspofungin – at 1 and 10 µg – generated no inhibition zone (Table 1). Generally, the inhibition zone caused by sublimated amorolfine ('sublimation zone') was larger than the one caused by direct diffusion of dissolved amorolfine into the agar.

Some of the antifungal agents tested directly on agar showed also a clear inhibition zone against the slow growing dermatophyte *T. rubrum* (Table 1). Of the five tested imidazoles only clotrimazole showed a measurable inhibition zone, whereas both allylamines – naftifine and terbinafine – and the morpholine derivative amorolfine exerted an extremely large inhi-

bition zone. Ciclopiroxolamine (10 µg) showed a small inhibition zone only, and caspofungin did not show a clear inhibition zone at all. Rather, there was a reduced growth observed in the fuzzy inhibition zone round the disk. In this experimental setting not only amorolfine, but also the highly active terbinafine showed an inhibition zone opposite to the disk (Table 1 and Fig. 3).

Galenical formulations

The galenical forms Loceryl® nail lacquer, creme and non-commercial amorolfine-solution, as well as Nagel Batrafen®, were also tested in various diffusion experiments. The quantification of these galenical forms

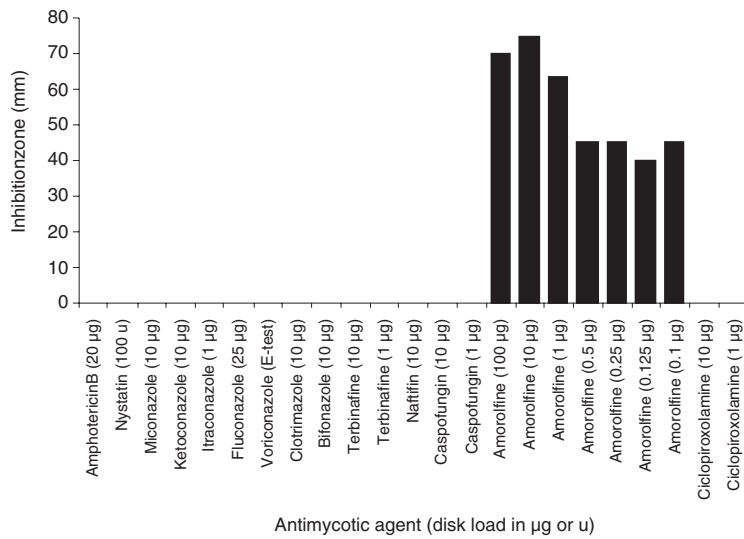


Figure 2 Inhibitory activity and dose-dependence of various antimycotic agents against *Candida albicans* as tested by the sublimation method on Casitone agar. Values denote arithmetic means of up to five repeat experiments (see also Table 1).

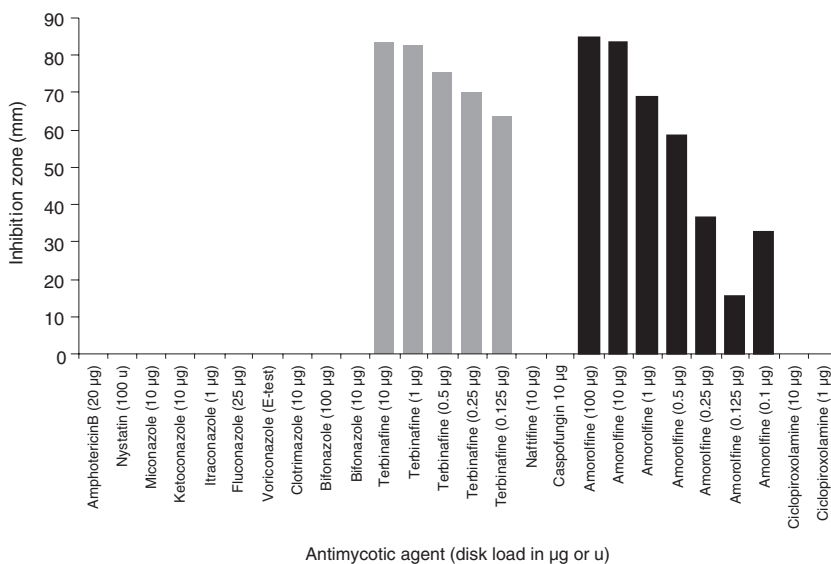


Figure 3 Inhibitory activity and dose-dependence of various antimycotic agents against *Trichophyton rubrum* as tested by the sublimation method. Values denote arithmetic mean of up to five repeat experiments (see also Table 1).

on the disks was technically difficult. Therefore, the measured inhibition zones were not as reproducible as with the pure compounds.

Loceryl® nail lacquer, creme and amorolfine-solution showed the same phenomenon of sublimation as the pure compound amorolfine. In contrast to the experiments with the pure compound, the extent of the indirect inhibition zones was smaller than the direct one. Nagel Batrafen® with a load of 10 µl also produced a small fuzzy inhibition zone opposite of the disk (Table 2).

The main solvents of the commercial lacquers did not show any activity – neither in the direct and

indirect tests against *C. albicans* nor indirectly against *T. rubrum*. However, ethanol and ethyl acetate, directly applied on the agar, did produce a small inhibition zone against *T. rubrum* in one of the two experiments (Table 3).

Derivatives of the morpholine class

Morpholine derivatives with various chemical structures were also tested. All five morpholine derivatives displayed the same property as amorolfine (Table 4). They all produced inhibition zones – not only in the direct test but also when applied on the opposite of the

Table 2 Inhibitory activity of various galenical forms of antimycotic agents against *Candida albicans* and *Trichophyton rubrum* measured by diffusion and sublimation methods.

| Galenical form of antimycotic agents | Volume per disk (μ l) | Inhibition zone (mm) | | | | | | | |
|--|----------------------------|-------------------------|-------------|-----------------------|-------------|----------------|-------------|----------------------------|-------------|
| | | <i>Candida albicans</i> | | | | | | <i>Trichophyton rubrum</i> | |
| | | Casitone agar | | Yeast morphology agar | | Sabouraud agar | | Sabouraud agar | |
| | | Diffusion | Sublimation | Diffusion | Sublimation | Diffusion | Sublimation | Diffusion | Sublimation |
| Loceryl [®] nail lacquer (5%) | Drop | 65 | 60 | 55 | 47 | ND | ND | ND | ND |
| Loceryl [®] nail lacquer (5%) | 10 | 63 | 58/54 | ND | ND | ND | ND | >85/>85 | 70/58 |
| Loceryl [®] nail lacquer (5%) | 5 | 78 | 74 | ND | ND | ND | ND | >85/>85 | 70/63 |
| Loceryl [®] nail lacquer (5%) | 2 | 70 | 58 | ND | ND | ND | ND | >85/>85 | 70/70 |
| Amorolfine solution (0.25% fluid) | 10 | 84 | 80 | ND | ND | ND | ND | >85/>85 | >85/80 |
| Amorolfine solution (0.25% fluid) | 5 | 75 | 62/64 | ND | ND | ND | ND | >85/>85 | 75/75 |
| Amorolfine solution (0.25% fluid) | 2 | 72 | 70 | ND | ND | ND | ND | >85/>85 | 78/70 |
| Loceryl [®] creme | Drop | 42/38/43/48 | 42/40/37/38 | ND | ND | 42/32/41/37 | 25/32/25/30 | ND | ND |
| Nagel Batrafen [®] | Drop | 32 | 20 (fuzzy) | 32 | 20 (fuzzy) | ND | ND | ND | ND |
| Nagel Batrafen [®] | 10 | 33 | 17 | ND | ND | ND | ND | 55/59 | 0/0 |
| Nagel Batrafen [®] | 5 | 34/35 | 18 | ND | ND | ND | ND | 53/57 | 0/0 |
| Nagel Batrafen [®] | 2 | 33 | 6 | ND | ND | ND | ND | 53/54 | 0/0 |

ND, not done; Drop, c. 20 μ l; numbers after a slash represent results of repeat experiments.

Table 3 Antimycotic activity of solvents of the two commercial nail lacquers measured by diffusion and sublimation methods.

| Solvent | Volume per disk (μ l) | Inhibition zone (mm) | | | |
|---|----------------------------|--------------------------------------|-------------|---|-----------------------------|
| | | <i>Candida albicans</i> ¹ | | <i>Trichophyton rubrum</i> ² | |
| | | Diffusion | Sublimation | Diffusion experiments 1/2 | Sublimation experiments 1/2 |
| Ethanol | 10 | 0 | 0 | 15/0 | 0/0 |
| Ethyl acetate | 10 | 0 | 0 | 12/0 | 0/0 |
| <i>n</i> -Butyl acetate | 10 | 0 | 0 | 0/0 | 0/0 |
| Isopropanol | 10 | 0 | 0 | 0/0 | 0/0 |
| Ethanol + Isopropanol | 10+10 | 0 | 0 | 0/0 | 0/0 |
| Ethanol + Ethyl acetate + <i>n</i> -Butyl acetate | 10+10+10 | 0 | 0 | 0/0 | 0/0 |

¹Tested on casitone agar, ²tested on Sabouraud agar.

inoculated agar – against *C. albicans* and in most cases also against *T. rubrum*.

Generally, the antifungal activity of morpholine derivatives against human pathogenic fungi strongly depends on the chemical structure of the side chain and the presence of a double bond in the chain between the phenyl/piperidine- and the morpholine-ring; the *cis* is more active than the *trans*-configuration in the 2,6-dimethyl-morpholine series.⁸

Under the present experimental conditions – measuring the antifungal activity by agar diffusion – the largest diameter of the inhibition zones even at low concentrations was seen with amorolfine. A phenyl moiety in the side chain did reduce the overall antifungal activity

against yeasts and drastically reduced the sublimation property (measured by *T. mentagrophytes*). The *cis*- and *trans*-configurations did not differ significantly. The morpholine derivative with a piperidine ring was still highly active and showed a good sublimation activity, whereas the double bond in the chain between the morpholine derivative and the phenyl ring drastically reduced the antifungal activity against yeasts and dermatophytes.

Discussion

The management of onychomycosis remains a problem despite the significant progress seen during the

Table 4 Inhibitory activity of some morpholine derivatives against *Candida albicans* and *Trichophyton rubrum* measured by diffusion and sublimation methods.

| Morpholine derivative ³ | Volume per disk (µl) | Inhibition zone (experiments 1/2, mm) | | | |
|---|----------------------|---------------------------------------|-------------|---|-------------|
| | | <i>Candida albicans</i> ¹ | | <i>Trichophyton rubrum</i> ² | |
| | | Diffusion | Sublimation | Diffusion | Sublimation |
| Amorolfine | 100 | 70/75 | 70/70 | >85/>85 | >85/>85 |
| C ₂₁ H ₃₅ NO | 10 | 70/75 | 70/70 | 80/>85 | 80/>85 |
| | 1 | 52/55 | 60/60 | 60/70 | 65/70 |
| | 0.1 | 30/40 | 40/50 | 30/52 | 25/40 |
| Derivative 1 C ₂₅ H ₃₅ NO <i>cis</i> | 100 | 30/33 | 25/21 | 60/65 | 0/0 |
| | 10 | 28/32 | 20/21 | 50/57 | 0/0 |
| | 1 | ND/30 | 12/20 | 30/32 | 0/0 |
| Derivative 1 C ₂₅ H ₃₅ NO <i>trans</i> | 100 | 20/23 | 0/10 | 0/0 | 0/0 |
| | 10 | 30/35 | 22/25 | 55/65 | 30/40 |
| | 1 | 30/32 | 20/20 | 45/55 | 0/0 |
| Derivative 2 C ₁₉ H ₃₃ NOSi | 100 | 25/30 | 8/15 | 20/35 | 0/0 |
| | 10 | 15/22 | 0/0 | 0/0 | 0/0 |
| | 1 | 80/>85 | 70/>85 | >85/>85 | >85/85 |
| Derivative 3 C ₂₀ H ₃₉ NO | 100 | 75/<85 | 80/>85 | 80/>85 | >85/>85 |
| | 10 | 45/70 | 60/60 | 65/70 | 65/>85 |
| | 1 | 20/30 | 30/30 | 0/30 | 25/40 |
| Derivative 4 C ₂₀ H ₃₁ NO | 100 | >85/>85 | >85/85 | >85/>85 | >85/>85 |
| | 10 | >85/>85 | >85/85 | >85/>85 | >85/>85 |
| | 1 | 55/70 | 65/65 | 65/70 | 65/>85 |
| Derivative 4 C ₂₀ H ₃₁ NO | 100 | 13/0 | 25/15 | 0/30 | 25/40 |
| | 10 | 15/20 | 0/0 | 55/60 | 20/25 |
| | 1 | 17/16 | 0/0 | 35/40 | 0/15 |
| Derivative 4 C ₂₀ H ₃₁ NO | 1 | 0/0 | 0/0 | 0/0 | 0/0 |
| | 0.1 | 0/0 | 0/0 | 0/0 | 0/0 |

¹Casitone agar, ²Sabouraud agar.

³The four morpholine derivatives differ slightly from amorolfine in the residue attached to the nitrogen atom of the morpholine ring.

last 10 years. New antifungals – in systemic or topical use – have proven their activity in onychomycosis, and new treatment schedules combining systemic with topical therapy have shown their good efficacy.^{9–11} However, the optimal treatment schedule is still unclear, despite the broader armamentarium. There is still a considerable non-response or relapse rate associated with the established therapy regimens. Two of the main problems are poor or non-responders to antimycotic therapy with recurrence or relapse of the infection. Besides other reasons, causes could include air-filled cavities in the nail plate generated by the proteolytic activity of fungal proteases, e.g. keratinases, or by the so-called dermatophytoma. A dermatophytoma is an air-rich thick mass of fungal hyphae and necrotic keratin between the nail plate and the nail bed.¹² Grimmer recognized as early as 1961 that subungual hyperkeratosis was responsible for the therapeutic failures observed under griseofulvin monotherapy.¹³ Seebacher explained these treatment

failures as follows: ‘As demonstrated on histological sections, subungual hyperkeratoses exhibit numerous air-filled cavities in which fungal arthrospores can remain viable for many weeks and months.¹⁴ Since dormant arthrospores in cavities are not in contact with the surrounding keratin, they cannot be attacked by antifungals, as no antifungal is capable of diffusing through air’.¹⁵

This statement may no longer be true. In this paper we show – with a microbiological method measuring not only the presence but the real antifungal activity of a chemical compound – that some antifungals are indeed able to overcome air-filled spaces by sublimation. The morpholine derivatives and especially amorolfine are clearly able to sublimate. They produce inhibition zones even at low concentrations after they have passed distances at least up to 10 mm through the air.

Sublimation seems to be a class property of morpholines, depending on the specific chemical structures.

The effect is still present in topical galenical forms of amorolfine (creme, lacquer, and solution). The small difference in the size of the inhibition zones between the pure substance amorolfine and the galenical forms may be explained by the properties of the vehicles, e.g. for the lacquer. It contains film-forming substances (methacrylate-polymer) and lipophilic compounds, which may interfere with the 'sublimation' characteristics of the pure compound.

The same phenomenon was observed in this system with the allylamine terbinafine – at least when the antifungal activity was measured with the highly susceptible dermatophyte *T. rubrum*.

No other tested antimycotic agent (pure substance) showed any antifungal activity when placed opposite of the agar (including ciclopiroxolamine). The fuzzy appearance of a small inhibition zone only, when testing *C. albicans* and Nagel Batrafen® (Table 2) remains unclear. It may have been due to alcoholic solvents in the lacquer, since ethanol and ethyl acetate slightly inhibited *T. rubrum*, when tested directly on the agar (Table 3), or a cause of unknown interrelations with the film-forming polymer in the lacquer. It is also possible that it is real sublimation, which becomes apparent only at very high concentrations (c. 800 µg) of pure substance of Nagel Batrafen® on the disk.

The clearly outstanding physicochemical property of amorolfine may be one explanation for the good penetration – horizontally through the nail plate. In addition, this may contribute to its topical efficacy in onychomycosis. Air-filled cavities (e.g. in the nail plate and in dermatophytoma) are apparently no obstacle for this molecule. It can easily pass through them, reach tissue layers on the other side of the cavity, and still retain its microbiological activity. The molecule appears to be able to diffuse steadily not only through nicely layered healthy nails, but also through diseased nails with their air cavities. Antifungals with this special physicochemical property – diffusing through air in concentrations which are still microbiological active – should clinically have an advantage over other compounds, e.g. in topical therapy of onychomycosis. Both terbinafine and amorolfine, which have high activity against dermatophytes, proved their good efficacy in the treatment of onychomycosis. Especially the combination of the two shows high clinical and mycological cure rates. Besides the intrinsic high fungicidal activity of these two drugs against dermatophytes, the physicochemical property of diffusing through air may contribute to the good clinical results.

To characterize the property of sublimation of antifungal agents in more physicochemical detail, further

qualitative and quantitative investigations in a pharmaceutical analytical laboratory have been initiated.

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