In vitro antimycotic activity and biological assay of nail permeation of a new climbazole/octopirox hydrolacquer

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Introduction

Onychomycosis has long been one of the most difficult fungal infections to treat. The lengthy period the nail takes to grow, the hardness of the nail plate and location of the infectious process between the nail bed and plate are major factors interfering with the eradication of fungal agents affecting these tissues. The availability of potent, orally active antifungal agents, such as itraconazole and terbinafine, has revolutionized the treatment of this condition (1, 2).

Those new drugs provide adequate therapy for a prolonged period of time, but they are not devoid of a significant, though rare, systemic toxicity. The key of these agents is that once given, they are absorbed into the nail matrix where they remain active for months. Their limitations consist in severe adverse events and drug-drug interactions. Fatal cases of heart failure have been reported after long-term oral administration of itraconazole in onychomycosis patients (3). Terbinafine resulted more effective than itraconazole, but its use was still associated with fatalities due to acute liver failure in patients with onychomycosis (3).

Topical treatments of onychomycosis are also available, in form of medicated nail lacquers. Those products show a lower rate of clinical success in patients with onychomycosis, compared to oral agents, despite their proven in vitro antimycotic activity (4). The formulations available till now have the disadvantage of poor nail permeation of the active principles, as well as the need for weekly removal of the old layers. Both mechanical removal and organic sol-

Riassunto

Sono stati effettuati studi in vitro per valutare l’attività antimycotica di un nuovo dispostivo medico, contenente climbazolooctopirox in una base di idrolacca di idrossipropilchitosano, contro alcuni patogeni responsabili di onicomicosi, Candida parapsilosis, Scopulariopsis brevicaulis, Trichophyton mentagrophytes e Trichophyton rubrum. Le concentrazioni minime inibenti (MIC), studiate per mezzo delle microdiluizioni del prodotto da testare, erano comprese tra 0,8 e 1,6 µg/ml di climbazolo e altrettanto di octopirox per tutti i ceppi testati. In confronto ai principi attivi da soli, l’associazione mostrava un’efficacia superiore all’atteso, soprattutto su C. parapsilosis e S. brevicaulis, ed almeno un effetto additivo sull’altro ceppo testato, il T. rubrum. Lo studio di permeazione ungueale in vitro, effettuato per mezzo di un saggio biologico, ha mostrato la comparsa di aloni di inibizione della crescita per tutti i ceppi, da parte del prodotto appoggiato sulla superficie di membrane di zoccolo bovino, confermando l’efficienza del film di idrossipropilchitosano nel consentire la permeazione delle unghie da parte di principi attivi.

Key words: Climbazolo, Octopirox, Idrossipropilchitosano, Idrolacca, Permeazione ungueale
vents, besides being uncomfortable to patients, may cause further potential damage to the nail structure (5), by rendering the newly growing nail less resistant to the diffusion of the dermatophytes. The use of nail penetration enhancers, besides improving nail permeation, may further damage the nail structure (6) as a result of breaking disulphide bridges or of keratolytic activity.

Thus, there is an unfulfilled medical need for safe and effective treatments characterized by a good efficacy/safety ratio.

A new hydro-lacquer technology, based on hydroxypropyl chitosan in water/ethanol solution, has recently been proposed (7) for administration of actives to the nails, its application being easy and more acceptable to subjects than the medicated drug varnishes, due to the simple (rinsing) removal procedure and no requirement for periodic nail surface filing.

In a previous study (8), recently published with our contribution, a new medical device, based on the above technology, and containing 0.5% of piroctone olamine (octopirox) as the sole active ingredient, has been reported to allow nail permeation of the active and to be provided of efficacy in in vitro onychomycosis models. In this study, we report an experience made with a product containing the same hydro-lacquer technology, and based on an octopirox 0.5% - climbazole 0.5% combination, by testing its in vitro antymycotic activity and nail permeation in a biological assay.

Methods

Minimal inhibitory concentrations (MICs) on several representative onychomycosis pathogens - Broth microdilution susceptibility method

Climbazole + octopirox hydro-lacquer MICs were determined for Candida parapsilosis ATCC 90018, Scopulariopsis brevicaulis ATCC 36840, Trichophyton mentagrophytes ATCC 9533 and Trichophyton rubrum DSM 4167, all obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The method was developed in accordance with the NCCLS standard reference documents M27-A2 (9) and M38-A (10). Antifungal working solutions were prepared by appropriate dilution from the tested hydro-lacquer solution device P-3063 (EcoMed, containing climbazole 0.5% and octopirox 0.5%, Manufacturer Polichem SA, Lugano, Switzerland, Distributor for Italy Sinon Pharmaceuticals S.p.A., Italy) with RPMI-1640 broth (Sigma, Buchs, Switzerland) buffered with 0.165 M MOPS (Sigma, Buchs, Switzerland) pH 7.0. The concentrations tested were in the range 0.003 μg/mL to 1.6 μg/mL of climbazole plus the same of octopirox. The broth microdilution test was performed using sterile multiwell microdilution plates, 96-wells U-shaped (Labohasi, Novazzano, Switzerland). The 2x antifungal concentrations to test were dispensed into wells in 100 μL volume well. The organisms were subcultured onto SDA, Sabouraud dextrose agar (Labohasi, Novazzano, Switzerland), and incubated at 35 ± 1°C to provide fresh and alive colonies for inocula preparation. Working suspensions were prepared in RPMI-1640 broth (Sigma, Buchs, Switzerland) buffered with 0.165 M MOPS (Sigma, Buchs, Switzerland) pH 7.0 to contain 1 - 5 x 10^5 CFU/ mL for C. parapsilosis and 0.8 - 10 x 10^6 CFU/mL for the moulds. Each well was then inoculated with 100 μL of these inocula suspensions, representing the 2X final concentrations. The final inoculum size for C. parapsilosis was 5.0 x 10^5 to 2.5 x 10^6 CFU/ mL, and the final inoculum size for the moulds was 0.4 x 10^6 to 5.0 x 10^6 CFU/ mL. Inocula quantitation was performed planting a 100 μL aliquot of the serial dilutions on SDA, incubating and counting the viable number of CFU. Each plate contained a sterility control

<table>
<thead>
<tr>
<th></th>
<th>P-3063 (climbazole+octopirox)</th>
<th>climbazole</th>
<th>octopirox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida parapsilosis</td>
<td>1.6 (0.8 + 0.8)</td>
<td>12.5</td>
<td>62.5</td>
</tr>
<tr>
<td>Scopulariopsis brevicaulis</td>
<td>3.2 (1.6 + 1.6)</td>
<td>&gt; 50</td>
<td>15.6</td>
</tr>
<tr>
<td>Trichophyton rubrum</td>
<td>3.2 (1.6 + 1.6)</td>
<td>6.25</td>
<td>≤ 3.9^*</td>
</tr>
<tr>
<td>Trichophyton mentagrophytes</td>
<td>1.6 (0.8 + 0.8)</td>
<td>*</td>
<td>*</td>
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</tbody>
</table>

*not performed; *least concentration tested

Table 1. MICs (μg/mL) on different nail pathogens.
row, drug-free medium only, and a growth control row, inoculated drug-free medium. The microdilution plates were incubated at 35 ± 1°C and observed for presence or absence of visible growth at 24 and 48 hours for C. parapsilosis 48 and 96 hours for the moulds. The MIC was defined as the lowest concentration of the antifungal that substantially inhibited the growth of the organism as detected visually.

In vitro permeation study through bovine hoof membranes – Biological assay

Candida parapsilosis ATCC 90018, Scopulariosis brevicaulis ATCC 36840, Tricho phyton mentagrophytes ATCC 9533 and Tricho phyton rubrum DSM 4167 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The organisms were subcultured onto SDA, Sabouraud dextrose agar (Labobasi, Novazzano, Switzerland), and incubated at 35 ± 1°C. After the growing was visually abundant, a stock suspension was prepared using sterile saline solution (Labobasi, Novazzano, Switzerland). For each organism SDA square plates were prepared inoculated with 0.4 - 4.0 x 10⁶ CFU/mL of agar. The test product was added on the surface of the agar plates either by adsorbing 10 mL of the solution on a 10 mm neutral disk (Labobasi, Novazzano, Switzerland), and by placing 10, 20 mL of the device, and 10 mL vehicle, hydroxypropyl chitosan solution (Polichem, Lugano, Switzerland) on 10 x 20 mm, 75 mm thickness, nail slices obtained from bovine hooves. The plates were then incubated at 35 ± 1°C for 5 days. Inhibition rings of the microorganisms growth were visually evaluated as a consequence of nail permeation and diffusion into agar of climbazole and octopirox contained in the test hydrolacquer.

Results

Minimal inhibitory concentrations (MICs) on several representative onychomycosis pathogens – Broth microdilution susceptibility method

C. parapsilosis MIC on 16 replicates was 0.8 µg/mL climbazole plus 0.8 µg/mL octopirox, with a MIC range 0.4 - 0.8 µg/mL; 50% reduction endpoint was observed at 0.4 µg/mL climbazole plus 0.4 µg/mL octopirox. S. brevicaulis MIC on 16 replicates was 1.6 µg/mL climbazole plus 1.6 µg/mL octopirox, with a MIC range 0.8 – 1.6 µg/mL; 50% reduction endpoint was observed at 0.8 µg/mL climbazole plus 0.8 µg/mL octopirox. T. mentagrophytes MIC on 16 replicates was 0.8 µg/mL climbazole plus 0.8 µg/mL octopirox, with a MIC range 0.4 - 0.8 µg/mL; 50% reduction endpoint was observed at 0.4 µg/mL climbazole plus 0.4 µg/mL octopirox. T. rubrum MIC on 16 replicates was 1.6 µg/mL climbazole plus 1.6 µg/mL octopirox, with a MIC range 0.8 – 1.6 µg/mL; 50% reduction endpoint was observed at 0.4 µg/mL climbazole plus 0.4 µg/mL octopirox and 75% reduction endpoint was observed at 0.8 µg/mL climbazole plus 0.8 µg/mL octopirox. No growth was detected in the sterility control wells, vigorous fungal growth always occurred in the growth control wells.

To allow an indirect comparison with previous investigations available on octopirox and respectively climbazole alone, made in our Institution by the broth dilution susceptibility method, the data have been reported in Table 1 as MICs (µg/mL) of both active ingredients as resulting from the 3 investigations. It is evident a synergistic effect on growth inhibition of C.parapsilosis and of S.brevicaulis, and an additive effect of the two antymycotics agents on T.rubrum.

In vitro permeation study through bovine hoof membranes – Biological assay

At the end of the incubation period the plates showed good growth of the inoculated organisms. The growth resulted inhibited in the zones near the disk and the nails treated with the test device, depending on the permeation of the product through the nail slice and its diffusion onto the agar medium. The device caused dose-dependent inhibition rings at the 2 doses tested for each of the 4 organisms: C. parapsilosis, S. brevicaulis, T. mentagrophytes and T. rubrum. The inhibition rings were narrower for nail slices than for disks and were dose-dependent. The vehicle alone applied on nail fragments did not cause any inhibition of growth. The results are depicted in Figures 1-4. The same test performed for octopirox alone (8) on T. rubrum revealed an inhibitory ring, mainly evident at the higher dose level (20 µL). No investigation was performed on the other strains. Climbazole alone caused dose-depen-
dent (in the range of 10 and 20 µL) inhibitory rings on C. parapsilosis and T. rubrum, while it did not affect the growth of S. brevicaulis. No investigation was performed on T. mentagrophytes.

Discussion

Our results showed that the climbazole/octopirox combination is able to inhibit the growth of the tested strains and confirmed the literature data on an antifungal activity of climbazole and octopirox. Moreover, even if resulting from different investigational method, broth dilution susceptibility test versus broth microdilution susceptibility test, our data suggest a more efficient antifungal effect of the two agents when combined in the same product, compared to the effect of the two agents alone in the same formulation. A synergistic effect of the two agents may be due to the different antifungal site and mechanism of action. Octopirox is a hydroxyl-pyridone derivative with a broad spectrum of activity against dermatophytes, yeasts, actinomycetes, moulds and other fungi and bacteria (11). Its primary site of action is the cell membrane, where it inhibits the transport of essential substrates like leucine, causing membrane alteration and rupture. The inhibiting mechanism of octopirox is fungicidal. Climbazole is an azole antifungal with
activity against a broad spectrum of fungi. Azoles act primarily by inhibiting the sterol 14-
\(\alpha\)-demethylase, which leads to the accumulation of 14-\(\alpha\)-methylsterols, impairing the func-
tions of membrane bound enzyme systems like ATPase and inhibiting fungal growth (12). The
resulting inhibiting mechanism is fungistatic. Both agents are classified on INCI (13, 14) and
are commonly used in cosmetic products, mainly in anti-dandruff shampoos.

By confirming previous in vitro nail permeation data (7, 8) on active agents carried by the
hydrolacquer technology, the tested product showed good permeation properties through
bovine hoof membranes, used as a model of in vivo conditions in human nails. The biological
assay confirmed the ability of the product to release active concentrations of the drugs
through nail membrane.

The tested product offers an alternative to drug treatment of onychomycosis, or to prevent
infection or recurrence in subjects at risk.

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