In vitro interactions between primycin and different statins in their effects against some clinically important fungi

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The in vitro antifungal activities of primycin (PN) and various statins against some opportunistic pathogenic fungi were investigated. PN completely inhibited the growth of Candida albicans (MIC 64 μg ml⁻¹) and Candida glabrata (MIC 32 μg ml⁻¹), and was very effective against Paecilomyces variotii (MIC 2 μg ml⁻¹), but had little effect on Aspergillus fumigatus, Aspergillus flavus or Rhizopus oryzae (MICs >64 μg ml⁻¹). The fungi exhibited different degrees of sensitivity to the statins; fluvastatin (FLV) and simvastatin (SIM) exerted potent antifungal activities against a wide variety of clinically important fungal pathogens. Atorvastatin, rosuvastatin and lovastatin (LOV) had a slight effect against all fungal isolates tested, whereas pravastatin was completely ineffective. The in vitro interactions between PN and the different statins were investigated using a standard chequerboard titration method. When PN was combined with FLV, LOV or SIM, both synergistic and additive effects were observed. The extent of inhibition was higher when these compounds were applied together, and the concentrations of PN and the given statin needed to block fungal growth completely could be decreased by several dilution steps. Similar interactions were observed when the variability of the within-species sensitivities was investigated.

INTRODUCTION

There has been a significant increase in the number of opportunistic fungal infections (caused predominantly by Candida and Aspergillus species) in recent decades (Singh, 2001). Non-albicans Candida and various filamentous fungal species have also been increasingly detected (Nucci, 2003; Walsh & Groll, 1999; Walsh et al., 2004). The treatment of fungal infections is difficult, as the number of available antifungal agents is limited. The identification of new targets for new antifungal compounds is far from easy, not to mention the complication of the financial, experimental and clinical requirements necessary for the development of any new chemotherapeutic agent. Accordingly, a number of studies have focused on the antifungal activity of non-antifungal drugs, and on the development of efficient antifungal combination therapy (Afeltra & Verweij, 2003; Galgóczy et al., 2009a).

Primycin (PN) is a non-polyene macrolide lactone antibiotic complex (Blaskó et al., 1979; Vályi-Nagy et al., 1954), which has a broad antimicrobial spectrum: it is effective against Gram-positive bacteria, and at higher concentrations against yeasts and filamentous fungi. The antimicrobial action of PN is thought to be due to an increase in the ionic permeability of the cell membrane (Horvath et al., 1979). PN may act as an ionophore in many membrane systems; it can cause changes in the membrane potentials of liver mitochondrial inner membranes (Meszaros et al., 1980), and it also has effects on transmitter release at the neuromuscular junctions (Henderson & Marshall, 1984). Due to its toxicity in animal models, PN is limited to topical use in the treatment of skin infections (Vályi-Nagy et al., 1954).

Statins are used to reduce the level of cholesterol in the blood: they act by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase in the sterol biosynthesis pathway. Statins also have certain cholesterol-independent (pleiotropic) effects, such as improving endothelial function and decreasing inflammation (Liao & Laufs, 2005). Recent studies have revealed their growth-inhibitory effects on...
different pathogenic fungi (Gyetvai et al., 2006; Macreadie et al., 2006; Roze & Linz, 1998). A recent review (Sun & Singh, 2009) showed that statins directly attenuate the virulence of micro-organisms, and modulate signalling and regulatory pathways involved in controlling infection. Some publications have suggested the possibility of the combined application of statins and different antifungotics (Chamilos et al., 2006; Chin et al., 1997; Galgóczy et al., 2009b; Natesan et al., 2008).

Nowadays, various natural and chemically modified statins are commercially available, such as lovastatin (LOV), pravastatin (PRA), simvastatin (SIM), fluvastatin (FLV), atorvastatin (ATO) and rosuvastatin (ROS) (Schachter, 2005). The aim of the present work was to investigate the in vitro antifungal activities of PN and these statins, as well as their combinations, against opportunistic pathogen fungi.

METHODS

Strains and media. Eleven Candida albicans, six Candida glabrata, one Aspergillus fumigatus, one Aspergillus flavus and five Rhizopus oryzae isolates were investigated. C. albicans ATCC 90028 and Paeclomyces variotii ATCC 36257 were used as quality control strains in the antifungal susceptibility testing. The fungal isolates were maintained on potato dextrose agar slants at 4 °C. In all experiments, the test medium was RPMI 1640 (Sigma-Aldrich) containing L-glutamine but lacking sodium bicarbonate, buffered to pH 7.0 with 0.165 M MOPS.

Antifungal agents. PN (PannonPharma) and PRA (Sigma-Aldrich) were provided by the manufacturer as standard powders. PN was dissolved in DMSO at a concentration of 6.4 mg ml⁻¹, and PRA was dissolved in distilled water at a concentration of 12.8 mg ml⁻¹. FLV (Lesclo; Novartis), LOV (Mevacor; Merck Sharp & Dohme), SIM (Vasipil; Egis), ROS (Crestor; AstraZeneca) and ATO (Atorvocax; Richter) were of pharmaceutical grade. These compounds were dissolved individually in methanol and diluted to a concentration of 12.8 mg ml⁻¹. Stock solutions were prepared and stored at −80 °C. LOV and SIM were activated freshly from their lactone pro-drug forms by hydrolysis in ethanolic NaOH [15 % (v/v) ethanol, 0.25 % (w/v) NaOH] at 60 °C for 1 h (Lorenzo & Parks, 1990).

Antifungal susceptibility testing. The in vitro antifungal activities of PN and the various statins were determined against various clinically important opportunistic pathogenic fungi using a broth microdilution method, which was performed in accordance with the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) guidelines (NCCLS, 1997, 2002). MIC values were determined in 96-well flat-bottomed microtitre plates by measuring the optical density of the fungal cultures at 620 nm.

A series of twofold dilutions was prepared from the drug stock solutions in the appropriate solvent to yield a solution that was 100 times the final strength required for the tests in the broth microdilution assays. Each intermediate solution was further diluted in RPMI 1640 to twice the final required strength. Yeast cell inocula were prepared from 1-day-old cultures, and fungal spore suspensions from 7-day-old cultures grown on potato dextrose agar slants. Yeast or spore suspensions were diluted in RPMI 1640. Aliquots of 100 μl of various concentrations of the drugs diluted in RPMI 1640 were mixed with 100 μl cell or sporangiospore suspensions in the microtitre plates. The final concentrations of PN in the wells ranged from 0.125 to 64 μg ml⁻¹, and that for each strain ranged from 0.25 to 128 μg ml⁻¹. The initial inoculum contained 5 × 10⁶ c.f.u. ml⁻¹ for yeasts and 5 × 10⁵ spores ml⁻¹ for filamentous fungi. The microplates were incubated for 72 h at 35 °C, and the optical density was measured at 620 nm with a microtitre plate reader (Jupiter HD; ASYS Hitech) after 24, 48 and 72 h of incubation. Uninoculated medium was used as the background for the spectrophotometric calibration; the growth control wells contained inoculum suspension in drug-free medium. For calculation of the extent of inhibition, the OD₆₂₀ of the drug-free control cultures was set at 100 % growth in each case. MIC values were determined at the recommended end points and time intervals. The MICs for PN and the statins were the lowest concentration of drugs that produced an optically clear well. The quality-control strains were tested in the same manner and were included each time an isolate was tested. All experiments were repeated three times.

Examination of in vitro interactions between PN and statins. The in vitro interactions of PN and the statins were evaluated by a chequerboard broth microdilution method. A series of twofold dilutions was performed from PN and the various statin stock solutions in the appropriate solvent to yield a solution 100 times the final strength required for the tests in the broth microdilution assays. Each intermediate solution was further diluted in RPMI 1640 to four times the final desired concentration. Microtitre plates containing serial twofold dilutions of the drugs were used. In brief, 50 μl of solutions of various concentrations of PN with or without statin, and 50 μl of solutions of various concentrations of different statins with or without PN diluted in RPMI 1640, were mixed with 100 μl of cell or sporangiospore suspension. The final concentration of PN in the wells ranged from 0.125 to 64 μg ml⁻¹, and those of the various statins from 0.391 to 25 μg ml⁻¹. The inoculum preparation, the initial inoculum, the controls and the conditions of the incubation were as described above for antifungal susceptibility testing.

RESULTS AND DISCUSSION

Sensitivity to PN

The inhibitory potential of PN was studied in the range 0.125–64 μg ml⁻¹ by broth microdilution. PN inhibited the growth of C. albicans and C. glabrata in the range 32–64 μg ml⁻¹, depending on the sensitivity of the isolates. P. variotii was more sensitive to PN than the yeasts: 2 μg PN
ml\textsuperscript{-1} caused 100% growth inhibition. *A. fumigatus* was moderately sensitive to this compound: the presence of 4 µg PN ml\textsuperscript{-1} caused 50% growth inhibition, but complete inhibition was not achieved in the given concentration range. PN at the administered concentrations was ineffective against *A. flavus* and *Rhizopus oryzae*. The MICs of PN are listed in Table 1.

The antifungal activity of PN has long been known (Uri & Actor, 1979), and our observations suggest that PN, which is applied primarily as an antibacterial therapy, may also be used as an antifungal treatment. PN is a complex of various agents, in which the individual compounds themselves exert antimicrobial activity, but when used together they act synergistically. PN exhibits synergism in combination with oxytetracyclines, neomycin, streptomycin, doxycycline, sisomycin and actinomycin (Szabo et al., 1993), but it has not previously been combined with statins.

### Sensitivity to statins

The inhibitory potentials of statins were studied in the range 0.25–128 µg ml\textsuperscript{-1} by broth microdilution. The investigated fungi exhibited different degrees of sensitivity to the statins, and the antifungal effects of the different statins also varied. In these tests, FLV and SIM displayed the strongest antifungal activity, followed in sequence by ATO, ROS and LOV, whilst PRA proved completely ineffective against *P. variotii*. PN was not equally effective against the *C. albicans* strains: FLV and SIM were effective at low concentrations, and ROS, which was effective against the other strains only at 128 µg ml\textsuperscript{-1}, completely blocked the growth of *P. variotii* at 32 µg ml\textsuperscript{-1}. In contrast, PRA did not affect the growth of this fungus, even at higher concentrations. *A. flavus* proved to be insensitive to all of the statins with the exception of FLV, which was effective only at high concentration (128 µg ml\textsuperscript{-1}). The MICs of the different statins are shown in Table 1. These observations suggested that fungal colonization could be affected by statin therapy, and that these compounds may also be used as antifungal agents. FLV and SIM exhibited potent antifungal activities against a wide variety of clinically important fungal pathogens and were frequently more active than the other statins.

Some experiments were carried out with other isolates of the investigated species in order to acquire preliminary information concerning the variability of the sensitivities within species against these drugs. The sensitivities of these strains against statins were similar to those of the previously tested strains, but sometimes they differed by one or two dilution steps. For example, all *Rhizopus oryzae* isolates were sensitive to FLV in the concentration range 1.56–6.25 µg ml\textsuperscript{-1}. FLV also inhibited the growth of all *C. albicans* isolates in the concentration range 12.5–25 µg ml\textsuperscript{-1}. Moreover, one *C. albicans* isolate was extremely sensitive to FLV with an MIC value of 6.25 µg ml\textsuperscript{-1}. In contrast, PN was not equally effective against the *C. glabrata* isolates: MICs were >64 µg ml\textsuperscript{-1} for most of the isolates, but a concentration of 32–64 µg PN ml\textsuperscript{-1} caused 50% growth inhibition in almost every case. A summary of these results is presented in Table 2.

Recent studies have also revealed the antifungal activity of different statins. It has been hypothesized (Kontoyiannis, 2007) that the widespread use of statins has led to a tendency for the number of reported cases of zygomycosis in patients with diabetes mellitus in developed countries to decrease since the 1990s, despite the rapid increase in the prevalence of diabetes in those countries. The growth-inhibitory effect of the statins is probably based on their negative influence on membrane fluidity (Gyetvai et al.,

### Table 1. MICs (µg ml\textsuperscript{-1}) of PN and various statins

<table>
<thead>
<tr>
<th>Drug</th>
<th><em>C. albicans</em> ATCC 90028 (reference strain)</th>
<th><em>C. glabrata</em> CBS 138</th>
<th><em>P. variotii</em> ATCC 36257 (reference strain)</th>
<th><em>Rhizopus oryzae</em> CBS 109.939</th>
<th><em>A. fumigatus</em> 79/06 (clinical isolate)</th>
<th><em>A. flavus</em> 1846/05 (clinical isolate)</th>
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<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50}</td>
<td>MIC</td>
<td>IC\textsubscript{50}</td>
<td>MIC</td>
<td>IC\textsubscript{50}</td>
<td>MIC</td>
</tr>
<tr>
<td>PN</td>
<td>32–64</td>
<td>64</td>
<td>16–32</td>
<td>32</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>LOV</td>
<td>25</td>
<td>64</td>
<td>32</td>
<td>128</td>
<td>4–8</td>
<td>64</td>
</tr>
<tr>
<td>SIM</td>
<td>2–4</td>
<td>8</td>
<td>2–4</td>
<td>32</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>FLV</td>
<td>4–8</td>
<td>25</td>
<td>16</td>
<td>64</td>
<td>6.25–12.5</td>
<td>25</td>
</tr>
<tr>
<td>ROS</td>
<td>64–128</td>
<td>128</td>
<td>64</td>
<td>128</td>
<td>2–4</td>
<td>32</td>
</tr>
<tr>
<td>ATO</td>
<td>32–64</td>
<td>128</td>
<td>16–32</td>
<td>32</td>
<td>0.25</td>
<td>32</td>
</tr>
<tr>
<td>PRA</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
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</tbody>
</table>

IC\textsubscript{50}: Concentration required for 50% growth inhibition.
2006), and because they also indirectly affect cell signalling (Cordle et al., 2005), proliferation and differentiation through inhibition of the synthesis of important isoprenoids (Miida et al., 2004). The inhibitory effect of LOV has been investigated in detail: in Mucor racemosus, LOV induced apoptosis-like cell death (Roze & Linz, 1998). The fungistatic effect of LOV has likewise been demonstrated in C. albicans, but a similar apoptotic process has not been detected (Gyetvai et al., 2006). The antifungal activities of SIM and ATO have been observed against Mucor racemosus and various Candida species (Macreadie et al., 2006). Our results indicated that the degree of sensitivity to the statins often differed within a given genus, e.g. A. fumigatus was quite sensitive to most of the statins, whereas A. flavus proved to be insensitive to almost all of them. Similar differences in susceptibility to LOV were seen in Rhizomucor pusillus and Rhizomucor miehei species, which afforded an opportunity to elaborate a species-level differentiation method based on the higher sensitivity of Rhizomucor pusillus to LOV (Lukács et al., 2004). In our tests, some variances in the MIC values were also detected for the different isolates of a given species, but these values varied only in a narrow concentration range.

Interactions between PN and statins

The in vitro interactions between PN and the different statins were also studied using a standard checkerboard titration method against one isolate of each investigated species. The IRs between the compounds were also calculated using the Abbott formula and FIC index. The best results were achieved when the investigated strains were sensitive to both compounds. When PN was combined with FLV, LOV or SIM, both synergistic and additive effects were observed, so the concentration of PN and the given statin needed to block fungal growth completely could be decreased by several dilution steps.

The majority of the positive interactions were found in the cases of Candida species and P. variotii. Table 3 shows the data for the effective drug combinations for these fungi, which indicates the combined drugs in the lowest concentrations causing total growth inhibition. The type of interaction, as well as FIC indices and IR values, are also presented. PN and LOV acted synergistically in inhibiting the growth of C. glabrata, and additively against C. albicans and P. variotii. Similar effects for the combinations of PN and SIM were observed: they acted synergistically against C. glabrata, and additively in the cases of C. albicans and P. variotii. When PN was combined with FLV, additive interactions were observed for C. albicans, C. glabrata and P. variotii. PN combined with ATO showed also additive interactions in inhibiting the growth of C. glabrata.

For A. flavus, A. fumigatus and Rhizopus oryzae, interactions between PN and the statins could be detected only in some cases. A. fumigatus and Rhizopus oryzae proved to be sensitive to statins, and the addition of PN generally did not enhance the growth-inhibitory effects. One of the exceptions was the combination of PN and ATO, which exerted an additive effect (IR=1.07) in inhibiting the growth of Rhizopus oryzae: in the presence of 25 μg ATO ml⁻¹ and 64 μg PN ml⁻¹ complete growth inhibition was observed. Similarly, PN and FLV acted additively (IR=0.68) against A. fumigatus, and 0.78 μg FLV ml⁻¹ combined with 64 μg PN ml⁻¹ inhibited fungal growth. Although PN and LOV acted synergistically against Rhizopus oryzae (IR=2.77), 50 μg LOV ml⁻¹ combined with 0.25 μg PN ml⁻¹ caused only 50% growth inhibition, and this inhibitory effect could not be increased by elevation of PN concentration (even 64 μg PN ml⁻¹ combined with LOV had the same effect). A. flavus was completely insensitive to PN and to the majority of statins, as well as to their combinations. However, FLV, which was effective alone only at high concentration (128 μg ml⁻¹), acted synergistically (IR=2.29) with PN, and a complete block of growth could be achieved in the presence of 32 μg PN ml⁻¹ and 12.5 μg FLV ml⁻¹. When the investigated strain proved to be insensitive to both compounds, application of the combination did not lead to any inhibitory effect, except in the case of Rhizopus oryzae.

Table 2. Variability of sensitivity within species

<table>
<thead>
<tr>
<th>Organism (no. of isolates)</th>
<th>IC₅₀ (μg ml⁻¹)</th>
<th>MIC (μg ml⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>MIC range</td>
<td>Mode</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. oryzae (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLV</td>
<td>0.39–0.78</td>
<td>0.78</td>
</tr>
<tr>
<td>ATO</td>
<td>1.56–12.5</td>
<td>3.125</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. glabrata (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PN</td>
<td>8–&gt;64</td>
<td>32–64</td>
</tr>
</tbody>
</table>

IC₅₀, Concentration required for 50% growth inhibition.
where 64 μg PN ml$^{-1}$ combined with 25 μg ROS ml$^{-1}$ resulted in 66% growth inhibition (IR = 1.74).

Some researchers have combined statins with different antifungal drugs, and observed additive and synergistic interactions in some cases (Chamilos et al., 2006; Chin et al., 1997; Galgóczy et al., 2007; Natesan et al., 2008). Chin et al. (1997) detected only minimal activity of FLV against Candida species and Cryptococcus neoformans, but noted synergistic and additive effects when it was combined with fluconazole or itraconazole. We found that FLV displayed significant antifungal activity when used alone, and its combination with PN enhanced this effect. Chin et al. (1997) also investigated the inhibitory effects of LOV, SIM and PRA, but observed no antifungal activities of these compounds. However, they used the pro-drug forms of LOV and SIM, which also proved ineffective in our work. Natesan et al. (2008) confirmed the in vitro inhibitory activity of FLV against A. fumigatus, with an MIC (2 μg ml$^{-1}$) in accord with our result. In contrast, other statins were reported to exhibit no activity in their study, whereas, in our work, ATO (MIC 64 μg ml$^{-1}$) and ROS (MIC 128 μg ml$^{-1}$) completely inhibited the growth of A. fumigatus. Chamilos et al. (2006) demonstrated significant in vitro synergism between LOV and voriconazole against several zygomycete fungi, though voriconazole itself was ineffective. We observed a similar effect in the case of PN, which had a slight effect against filamentous fungi alone, whereas in combination with FLV or ROS, noteworthy growth inhibition was achieved.

The activities observed for certain PN/statin combinations highlight the promise of these compounds as candidates for the treatment of opportunistic human and animal mycosis, with combination therapy offering advantages over monotherapy. The effective PN/statin combinations might be applicable as topical therapy for patients with mucocutaneous infections. PN and statins are commercially available, and a combination product appears attainable in the near future.

### ACKNOWLEDGEMENTS

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