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.

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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).

Abbreviations: ATO, atorvastatin; FIC, fractional inhibitory concentration; FLV, fluvastatin; IR, interaction ratio; LOV, lovastatin; PN, primycin; PRA, pravastatin; ROS, rosuvastatin; SIM, simvastatin.

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 antimycotics (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 *Paecilomyces 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 (Lescol; Novartis), LOV (Mevacor; Merck Sharp & Dohme), SIM (Vasilip; Egis), ROS (Crestor; AstraZeneca) and ATO (Atorvox; 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 (Lorenz & 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 statin ranged from 0.25 to 128 µg ml^{-1}. The initial inoculum contained 5×10^3 c.f.u. ml^{-1} for yeasts and 5×10^4 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 OD_{620} 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.

Data analysis. A calculation matrix was created to convert OD_{620} readings into measurements of growth as percentages of control readings. In the chequerboard broth microdilution method, the interaction ratio (IR) between the antifungal agents was calculated using the Abbott formula: $IR=I_o/I_e$, where I_o is the observed percentage inhibition and I_e is the expected percentage inhibition for a given interaction. I_e was calculated using the formula $I_e=x+y-(xy/100)$, where x and y are the percentage inhibitions observed for each compound when applied alone. IR reflects the nature of the interaction between the antifungal compounds: if the IR is between 0.5 and 1.5, the interaction is additive, whilst an $IR>1.5$ denotes synergism and an $IR<0.5$ denotes antagonism (Gisi, 1996). The IR between the antifungal agents was also calculated by using the fractional inhibitory concentration (FIC) index, according to the equation $A/MIC_A+B/MIC_B=FIC_A+FIC_B=$FIC index, where A and B are the MICs of drug A and drug B in the combination, MIC_A and MIC_B are the MICs of drug A and drug B alone, and FIC_A and FIC_B are the FICs of drug A and drug B. FIC indices were interpreted as follows: $\leqslant$0.5, synergy; >0.5 to 1.0, addition; >1 to <4, indifference; $\geqslant$4, antagonism.

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^{-1} caused 100 % growth inhibition. *A. fumigatus* was moderately sensitive to this compound: the presence of 4 μg PN ml^{-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^{-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 all isolates. The natural statins (SIM and LOV) were inactive in the form of the pro-drugs, but their active metabolites (obtained by hydrolysis of the lactone ring at pH 10) manifested pronounced antifungal effects.

The fungi were not equally sensitive to the statins: SIM exhibited the strongest antifungal activity against the yeasts, and FLV was most effective against the filamentous fungi. FLV was active against all of the tested fungi; it completely inhibited the growth of *Rhizopus oryzae* and *A. fumigatus* at a very low concentration (2 μg ml^{-1}). *P. variotii* ATCC 36257 was the most sensitive strain: FLV and SIM were effective at low concentrations, and ROS, which was effective against the other strains only at 128 μg ml^{-1}, completely blocked the growth of *P. variotii* at 32 μg ml^{-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^{-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^{-1}. FLV also inhibited the growth of all *C. albicans* isolates in the concentration range 12.5–25 μg ml^{-1}. Moreover, one *C. albicans* isolate was extremely sensitive to FLV with an MIC value of 6.25 μg ml^{-1}. In contrast, PN was not equally effective against the *C. glabrata* isolates: MICs were >64 μg ml^{-1} for most of the isolates, but a concentration of 32–64 μg PN ml^{-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^{-1}) of PN and various statins

Drug	*C. albicans* ATCC 90028 (reference strain)		*C. glabrata* CBS 138		*P. variotii* ATCC 36257 (reference strain)		*Rhizopus oryzae* CBS 109.939		*A. fumigatus* 79/06 (clinical isolate)		*A. flavus* 1846/05 (clinical isolate)	
	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
PN	32–64	64	16–32	32	1	2	>64	>64	4	>64	>64	>64
LOV	25	64	32	128	4–8	64	32	128	8	25	>128	>128
SIM	2–4	8	2–4	32	1	8	16–32	64	1–2	6.25	>128	>128
FLV	4–8	25	16	64	6.25–12.5	25	0.5	2–3.125	1	2	32–64	128
ROS	64–128	128	64	128	2–4	32	16–32	>128	64	128	>128	>128
ATO	32–64	128	16–32	32	0.25	32	4	32	16–32	64	>128	>128
PRA	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128

IC_{50}, Concentration required for 50 % growth inhibition.

Table 2. Variability of sensitivity within species

Organism (no. of isolates)	IC_{50} (μg ml^{-1})			MIC (μg ml^{-1})		
	MIC range	Mode	Median	MIC range	Mode	Median
***R. oryzae* (5)**						
FLV	0.39–0.78	0.78	0.78	1.56–6.25	6.25	3.125
ATO	1.56–12.5		3.125	25–>25	>25	>25
***C. albicans* (12)**						
LOV	12.5–>25	>25	>25	25–>25	>25	>25
FLV	3.125–12.5	6.25	6.25	6.25–25	25	25
***C. glabrata* (6)**						
PN	8–>64	32–64	48	32–>64	>64	>64
SIM	3.125–>25	6.25	6.25	>25	>25	>25

IC_{5o}, 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 *A. fumigatus* 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 chequerboard 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 3. Examples of effective PN–statin combinations

Isolate/combination [MIC alone (μg ml^{-1})]*	MIC (μg ml^{-1}) of PN/statin combination†
***C. albicans* ATCC 90028**	
PN/LOV [64, 64]	32/6.25 [A, 0.59, 0.76]; 16–2/50 [A, 1.03–0.81, 0.76–1.11]
PN/SIM [64, 8]	32/1.56 [A, 0.7, 0.78], 16/3.125 [A, 0.64, 1.65], 8–4/6.25 [A, 0.91–0.84, 1.05–1.17]
PN/FLV [64, 25]	32/0.39 [A, 0.52, 0.9], 16–8/12.5 [A, 0.75–0.625, 1.81–1.85]
***C. glabrata* CBS 138**	
PN/LOV [32, 128]	16/6.25 [A, 0.55, 1.49], 8/12.5 [S, 0.35, 1.87], 4/25 [S, 0.32, 2.37], 2/50 [S, 0.45, 1.31]
PN/SIM [32, 32]	16/0.39 [A, 0.51, 0.81], 8/3.125 [S, 0.35, 1.65], 4/6.25 [S, 0.32, 1.28], 2/25 [A, 0.84, 1.94]
PN/FLV [32, 64]	16/0.39 [A, 0.51, 0.97], 8/25 [A, 0.64, 1.55]
PN/ATO [32, 32]	16–8/25 [A, 1.28–0.91, 1.11–1.50]
***P. variotii* ATCC 36257**	
PN/LOV [2, 64]	1/50 [A, 1.28, 0.64]
PN/SIM [2, 8]	1/6.25 [A, 1.28, 0.59]
PN/FLV [2, 25]	1–0.5/12.5 [A, 1–0.75, 1.26–1.04]

*The MICs of PN and the given statin, respectively, are shown in brackets.
†Effective drug combinations are presented as the lowest concentrations of the combined drugs that caused total growth inhibition together; the first number indicates the concentration of PN, and the second is the concentration of the given statin. In brackets, the type of the interaction (A, additive; S, synergistic) are indicated as inferred from the FIC index, and the FIC index and IR values are presented, respectively.

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 antimycotics, 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.

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