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In vitro synergistic interactions of the effects of various statins and azoles against some clinically important fungi

Ildikó Nyilasi,[1,2] Sándor Kocsubé,[1,2] Krisztina Krizsán,[1] László Galgóczy,[1] Miklós Pesti,[3] Tamás Papp[1] & Csaba Vágvölgyi[1]

[1]Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary; [2]PannonPharma Ltd, Pécsvárad, Hungary; and [3]Department of General and Environmental Microbiology, Faculty of Sciences, University of Pécs, Pécs, Hungary

Correspondence: Ildikó Nyilasi, Department of Microbiology, Faculty of Science and Informatics, University of Szeged, H-6726 Szeged, Közép fasor 52, Hungary. Tel.: +36 62 544 516; fax: +36 62 544 823; e-mail: nyilasiildi@gmail.com

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Abstract

The treatment of opportunistic fungal infections is often difficult as the number of available antifungal agents is limited. Nowadays, there is increasing interest in the investigation of the antifungal activity of nonantifungal drugs, and in the development of efficient antifungal combination therapy. In this study, the *in vitro* interactions of the effects of various statins (lovastatin, simvastatin, fluvastatin, atorvastatin (ATO), rosuvastatin (ROS) and pravastatin) and various azole antifungals [miconazole, ketoconazole, itraconazole and fluconazole (FLU)] against different opportunistic pathogenic fungi were investigated using a standard chequerboard broth microdilution method. When the investigated strains were sensitive to both compounds of the combination, additive interactions were frequently noticed. Synergistic interactions were observed in many cases when a strain was sensitive only to the azole compound (as in certain combinations with ATO or ROS) or the statin compound (as in certain combinations with FLU). In many combinations with an additive effect, the concentrations of drugs needed for total growth inhibition could be decreased by several dilution steps. Similar interactions were observed when the variability of the within-species sensitivities to some selected drug combinations was investigated.

Introduction

The number of immunocompromised individuals with an enhanced susceptibility to opportunistic fungal infections has increased significantly in recent decades (Singh, 2001). These mycoses are predominantly caused by *Candida* and *Aspergillus* species (Walsh & Groll, 1999), but the incidence of infections due to zygomycetous fungi has also risen (Kauffman, 2004; Chayakulkeeree *et al.*, 2006). As the treatment of these fungal infections is frequently hampered by the lack of an efficient antifungal agent, there is increasing interest in the application of combination antifungal therapy. Coadministration of two or three antifungal compounds may improve the efficacy of the treatment, and extends the spectrum of activity; furthermore, resistance also may be avoided and toxicity reduced using lower concentrations of the chemotherapeutic agents (Nosanchuk, 2006). As a result, a number of studies have focused on the antifungal activity of nonantifungal drugs, and on the development of efficient antifungal combination therapy involving such compounds (Afeltra & Verweij, 2003; Galgóczy *et al.*, 2009a).

Statins are used to reduce the cholesterol level in the blood. They are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, which catalyzes a rate-limiting step in the acetate–mevalonate pathway of the terpenoid biosynthesis (Liao & Laufs, 2005). Statins were originally identified as secondary metabolites of fungi, and various natural, chemically modified and synthetic compounds are now available commercially, including lovastatin (LOV), pravastatin (PRA), simvastatin (SIM), fluvastatin (FLV), atorvastatin (ATO) and, most recently, rosuvastatin (ROS) and pitavastatin (Schachter, 2005).

Statins are currently used for hyperlipidemia control and protection from cardiovascular events, but they have other pleiotropic properties, including anti-inflammatory,

immunomodulatory and antioxidant effects (Liao & Laufs, 2005). In addition, there is increasing evidence for the potential use of statins in preventing and treating infections (Falagas *et al.*, 2008; Galgóczy *et al.*, 2009b), as they attenuate the pathogenicity of microorganisms, modulating the signaling and other regulatory pathways involved in controlling infection (Sun & Singh, 2009). Recent studies have revealed their direct antimicrobial effect as well; statins exert substantial growth-inhibitory effects on pathogenic bacteria and fungi. The inhibitory effect of LOV has been investigated in detail. LOV induced apoptosis-like cell death in *Mucor racemosus* (Roze & Linz, 1998) and inhibited the growth of different *Rhizomucor* species (Lukács *et al.*, 2004). The fungistatic effect of LOV has been demonstrated in *Candida albicans* (Gyetvai *et al.*, 2006), and the antifungal activities of SIM and ATO have been observed against *Aspergillus fumigatus* and various *Candida* species (Macreadie *et al.*, 2006). The growth-inhibitory effect of statins is probably based on their negative influence on membrane fluidity (Gyetvai *et al.*, 2006). They also indirectly affect cell signaling (Cordle *et al.*, 2005), proliferation and differentiation through inhibition of the synthesis of important terpenoids (Miida *et al.*, 2004). Because of the fungus-specific or immunomodulating actions of statins, it has been hypothesized that the widespread use of statins by patients with diabetes has led to lower rates of zygomycoses in developed countries since the 1990s (Kontoyiannis, 2007).

Some published work has suggested the possibility of the combined application of statins and different antimycotics (Chin *et al.*, 1997; Chamilos *et al.*, 2006; Galgóczy *et al.*, 2007; Natesan *et al.*, 2008; Nyilasi *et al.*, 2010). Azoles are a class of antifungal drugs that target the fungal cell membrane by inhibiting the cytochrome P450-dependent 14α-lanosterol demethylase, which catalyzes a critical step of ergosterol biosynthesis. Imidazoles, such as miconazole (MCZ) and ketoconazole (KET), are generally used topically, whereas triazoles, such as fluconazole (FLU), itraconazole (ITR) and voriconazole, are applied orally or intravenously against systemic mycoses.

The aim of our study was to examine the inhibition of fungal growth by pairs of drugs, in order to find effective drug combinations. Each pair contained a statin (LOV, SIM, FLV, ATO, ROS or PRA) and an azole compound (MCZ, KET, ITR and FLU). The *in vitro* interactions of the effects of these compounds against some opportunistic pathogenic yeasts and filamentous fungi were examined using a standard chequerboard broth microdilution method. Clinically important *Candida* (*C. albicans* and *Candida glabrata*) and *Aspergillus* species (*A. fumigatus* and *Aspergillus flavus*) and *Rhizopus oryzae*, the most frequent causative agent of zygomycoses (Ribes *et al.*, 2000), were included in the study.

Materials and methods

Strains

All fungal isolates were collected from clinical sources. The *A. fumigatus* and *A. flavus* strains were isolated in Indian hospitals, and the *C. albicans* and *C. glabrata* strains in Hungarian hospitals. These strains were deposited in the Szeged Microbial Collection (SZMC) at the University of Szeged, Szeged, Hungary. Eleven *C. albicans* (ATCC 1001, ATCC 10231, SZMC 1458, SZMC 1379, SZMC 1421, SZMC 1453, SZMC 1363, SZMC 1456, SZMC 1411, SZMC 1426, SZMC 1423), six *C. glabrata* (CBS 138, ATCC 35590, SZMC 1362, SZMC 1374, SZMC 1370, SZMC 1386), six *A. fumigatus* (SZMC 2486, SZMC 2394, SZMC 2397, SZMC 2399, SZMC 2406, SZMC 2422), six *A. flavus* (SZMC 2521, SZMC 2431, SZMC 2395, SZMC 2425, SZMC 2427, SZMC 2429) and one *R. oryzae* (syn. *Rhizopus arrhizus*) (CBS 109939) isolates were investigated. *Candida albicans* ATCC 90028 and *Paecilomyces variotii* ATCC 36257 were used as quality-control strains in the antifungal susceptibility and chequerboard broth microdilution tests.

Antifungal agents

The statins used in this study were FLV (Lescol; Novartis), LOV (Mevacor; Merck Sharp & Dohme), SIM (Vasilip; Egis), ROS (Crestor; AstraZeneca), ATO (Atorvox; Richter), which were of pharmaceutical grade, and PRA (Sigma-Aldrich), which was provided as standard powder. The azoles used were MCZ, KET, FLU and ITR, which were also provided by the manufacturer (Sigma-Aldrich) as standard powders. The statins were dissolved in methanol, with the exception of PRA, which was dissolved in distilled water; stock solutions were prepared to a concentration of 12.8 mg mL^{-1}. LOV and SIM were activated freshly from their lactone prodrug forms by hydrolysis in ethanolic NaOH (15% v/v ethanol, 0.25% w/v NaOH) at 60 °C for 1 h (Lorenz & Parks, 1990). Stock solutions of MCZ, KET and ITR were made in dimethyl sulfoxide (Sigma-Aldrich) at concentrations of 1.6 or 0.8 mg mL^{-1}, while FLU was dissolved in dimethylformamide (Reanal) at a concentration of 6.4 mg mL^{-1}.

Antifungal susceptibility testing

The *in vitro* antifungal activities of the various azoles and statins were determined using a broth microdilution method, which was performed in accordance with Clinical and Laboratory Standards Institute guidelines (NCCLS, 1997, 2002). Minimal inhibitory concentration (MIC) values were determined in 96-well flat-bottomed microtitre plates by measuring the OD of the fungal cultures. 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 (Sigma-Aldrich). 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 to give a final inoculum of 5×10^3 CFU mL^{-1} for yeasts and 5×10^4 spores mL^{-1} for filamentous fungi. Series of twofold dilutions were prepared in RPMI 1640 and were mixed with equal amounts of cell or sporangiospore suspensions in the microtitre plates. The final concentrations for each statin in the wells was 0.25–128 μg mL^{-1}, and for MCZ, KET, ITR and FLU, 0.031–16, 0.031–16, 0.016–8, and 0.125–64 μg mL^{-1}, respectively.

The microplates were incubated for 48 h at 35 °C, and the OD was measured at 620 nm with a microtitre plate reader (Jupiter HD; ASYS Hitech). Uninoculated medium was used as the background for the spectrophotometric calibration; the growth control wells contained inoculum suspension in the drug-free medium. The solvent control wells contained inoculum suspension in the drug-free solvent-containing (1%) medium to prove that solvent had no inhibitory effect on the investigated fungi at the applied concentration. For calculation of the extent of inhibition, the $OD_{620\,nm}$ of the drug-free control cultures was set at 100% growth. The MICs for statins were the lowest concentration of drugs that produced an optically clear well, while the MICs for azoles were the lowest concentration of drugs that produced a prominent decrease in turbidity. The quality-control strains were included every time an isolate was tested. All experiments were repeated at least three times.

Chequerboard broth microdilution method

For drug interaction studies, each statin was tested with each azole by the chequerboard broth microdilution method, using twofold dilutions of both drugs. The final concentrations of the various statins in the rows were 0.391–25 μg mL^{-1}. The final concentrations of the azoles in the wells, the inoculum preparation, the initial inoculum, the controls and the conditions of the incubation were as described above for antifungal susceptibility testing. 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. The IR reflects the nature of the interaction between the antifungal compounds: if IR is between 0.5 and 1.5, the interaction is considered additive, an IR > 1.5 denotes synergism and an IR < 0.5 denotes antagonism (Gisi, 1996).

Results

In vitro susceptibility testing

The 50%, 80% and 90% growth-inhibitory concentrations (IC_{50}, IC_{80} and IC_{90}) of the various azoles against *C. albicans* ATCC 90028, *C. glabrata* CBS 138, *A. fumigatus* SZMC 2486, *A. flavus* SZMC 2521, *R. oryzae* CBS 109939 and *P. variotii* ATCC 36257 were determined (Tables 1–4). Among the azoles, ITR had the strongest inhibitory effect; it completely blocked the growth of all tested isolates at low concentration (< 1 μg mL^{-1}). MCZ and KET were equally effective, their inhibitory concentrations ranging from 0.5 to 8 μg mL^{-1} for all tested strains. Conversely, FLU only inhibited the growth of yeasts, and was ineffective against the filamentous fungi in the administered concentrations. In the case of *C. albicans*, ITR, KET and FLU showed the trailing effect, which means that the growth inhibition was only 50–60% at low azole concentrations (0.016 μg mL^{-1} for ITR, 0.031 μg mL^{-1} for KET and 0.25 μg mL^{-1} for FLU), but this inhibitory effect could not be enhanced further by the application of higher drug concentrations, and complete blockage of growth could not be achieved.

The MICs of the involved statins against the same six fungal strains (Tables 1–4) have already been reported (Nyilasi *et al.*, 2010). Those results showed that FLV and SIM exhibited potent antifungal activities and frequently a higher activity than the other statins. The natural statins (SIM and LOV) were inactive in their prodrug forms, but their active metabolites obtained by hydrolysis of the lactone ring manifested pronounced antifungal effects (Nyilasi *et al.*, 2010).

Interactions between azoles and statins

The *in vitro* interactions between the various azoles and statins were also studied against the abovementioned six fungal strains. We tested all investigated statins in combination with all investigated azoles, and in most cases, positive interactions were observed between them. Antagonistic interactions were not observed between any of the statins and azole compounds. Tables 1–4 show the data for all tested drug combinations. We could not display the results of all azole–statin combinations because of the huge amount of data. Thus, in Tables 1–4, only examples for concentrations of the combined drugs causing total growth inhibition are presented. The types of interaction, as well as IR values, are also given.

Additive interactions were generally noticed when the investigated strains were sensitive to both of the combined compounds. Such effects were observed in yeasts when KET and ITR were combined with any of the statins (Tables 1 and 4). In the case of *C. albicans*, sole application of ITR, KET and FLU caused a trailing effect, but complete blockage of

Table 1. Effect of antifungal activity of KET combined with different statins

Isolate/statin [MIC alone (μg mL^{-1})]*	ICs of KET (μg mL^{-1})†			MIC (μg mL^{-1}) of KET and MIC (μg mL^{-1}) of the different statins in combination [effect, IR]‡
	IC_{50}	IC_{80}	IC_{90}	
Candida albicans ATCC 90028	0.031	> 16	> 16	
LOV [50–64]				0.031+6.25 [A, 1.31], 16+3.125 [A, 1.16]
SIM [8]				0.031+1.563 [A, 1.44]
FLV [25]				0.031+1.563 [A, 1.11], 0.063+0.781 [A, 1.26], 8+0.391 [A, 1.32]
ROS [128]				0.031+12.5 [A, 1.07], 0.25+6.25 [A, 0.99], 8+3.125 [A, 1.25]
ATO [128]				0.031+25 [A, 1.29], 0.063+12.5 [A, 0.92], 16+3.125 [A, 1.10]
PRA [>128]				[I]§
Candida glabrata CBS 138	0.063–0.125	0.25	0.5–2	
LOV [128]				0.25+25 [A, 1.49], 0.5+1.563 [A, 1.13]
SIM [16–32]				0.125+25 [A, 0.80], 0.25+3.125 [A, 0.73], 0.5+0.391 [A, 0.81]
FLV [64]				0.125+25 [A, 1.0], 0.25+0.781 [A, 0.95], 0.5+0.391 [A, 0.88]
ROS [128]				0.25+ 12.5 [A, 1.08], 0.5+3.125 [A, 1.22]
ATO [32]				0.125+ 12.5 [A, 1.38], 0.25+0.781 [A, 0.89]
PRA [>128]				0.5+3.125 [A, 0.73], 1+0.391 [A, 1.05]
Paecilomyces variotii ATCC 36257	0.125–0.25	0.5	1	
LOV [64]				0.125+50 [A, 0.73], 0.5+25 [A, 0.59]
SIM [8]				[I]§
FLV [25]				0.063+12.5 [A, 1.08], 0.5+6.25 [A, 0.64]
ROS [32]				0.5+3.125 [A, 1.05]
ATO [32]				[I]§
PRA [>128]				[I]§
Aspergillus fumigatus SZMC 2486	1–2	2–4	4–8	
LOV [25]				0.5+3.125 [A, 0.78], 1+1.563 [A, 0.70]
SIM [6.25]				1+1.563 [A, 1.46], 4+0.391 [A, 0.73]
FLV [2]				0.5+1.563 [A, 0.81], 2+0.781 [A, 0.74]
ROS [128]				1+25 [A, 1.36], 2+12.5 [A, 1.09]
ATO [64]				1+25 [S, 2.70], 4+0.781 [A, 1.16]
PRA [>128]				[I]§
Aspergillus flavus SZMC 2521	1–2	2	4	
LOV [>128]				2+1.563 [A, 0.80]
SIM [>128]				2+6.25 [A, 1.15]
FLV [128]				0.5+12.5 [S, 1.79], 1+3.125 [A, 1.35], 2+0.391 [A, 0.74]
ROS [>128]				[I]§
ATO [>128]				2+25 [A, 1.23]
PRA [>128]				2+0.391 [A, 1.09]
Rhizopus oryzae CBS 109939	1–2	1–4	2–4	
LOV [128]				[I]§
SIM [64]				[I]§
FLV [2–3.125]				0.25+1.563 [A, 1.06], 0.5+0.781 [S, 1.65], 1+0.391 [S, 2.61]
ROS [>128]				0.25+25 [A, 1.37], 1+12.5 [S, 2.14]
ATO [32]				0.5+12.5 [A, 1.22], 1+3.125 [A, 1.43], 2+0.391 [S, 3.05]
PRA [>128]				[I]§

*The MICs of the statins are shown in parentheses.

†IC_{50}, IC_{80} and IC_{90} values are the concentrations required for 50%, 80% and 90% growth inhibition.

‡Examples of effective concentrations of the combined drugs causing total growth inhibition are presented; the first number indicates the concentration of KET, and the second the concentration of the given statin. The type of the interaction (A, additive; S, synergistic; I, indifferent) and IR values are presented in parentheses.

§Interaction was considered indifferent if no difference in the inhibition rates was detected (i.e. the MIC or IC values of the drugs could not decreased by their combined applications).

growth could be achieved with almost all azole–statin combinations at very low concentrations. Moreover, synergistic interaction was observed when ITR was combined with ROS (IR = 1.79). In some cases, synergistic interactions were observed when the investigated strain was sensitive to both compounds. For example, FLU and FLV acted

Table 2. Effect of antifungal activity of MCZ combined with different statins

Isolate/statin [MIC alone (μg mL^{-1})]*	ICs of MCZ (μg mL^{-1})†			MIC (μg mL^{-1}) of MCZ and MIC (μg mL^{-1}) of the different statins in combination [effect, IR]‡
	IC_{50}	IC_{80}	IC_{90}	
Candida albicans ATCC 90028	0.063–0.125	2–4	8	
LOV [50–64]				0.125+12.5 [A, 0.88]
SIM [8]				0.031+6.25 [A, 0.86], 0.063+3.125 [S, 1.65], 0.125+1.563 [A, 1.0]
FLV [25]				0.031+1.563 [A, 1.17]
ROS [128]				0.125+12.5 [S, 1.66], 0.5+6.25 [A, 1.45]
ATO [128]				0.25+25 [S, 1.54], 1+12.5 [A, 1.16]
PRA [>128]				[I]§
Candida glabrata CBS 138	0.063–0.125	0.125–0.25	0.5–2	
LOV [128]				0.125+50 [A, 1.13], 0.25+3.125 [A, 1.11]
SIM [16–32]				0.125+12.5 [A, 0.68], 0.25+1.563 [A, 1.14], 0.5+0.391 [A, 0.80]
FLV [64]				0.125+25 [A, 1.01], 0.25+1.563 [A, 0.80]
ROS [128]				0.25+6.25 [A, 0.94], 0.5+3.125 [A, 0.91], 1+1.563 [A, 0.94]
ATO [32]				0.063+12.5 [A, 1.13], 0.125+0.781 [A, 0.74]
PRA [>128]				0.5+3.125 [A, 1.02], 1+0.781 [A, 1.04]
Paecilomyces variotii ATCC 36257	0.25–0.5	4	8–16	
LOV [64]				0.5+50 [A, 0.53], 2+25 [A, 0.53], 4+12.5 [A, 0.57]
SIM [8]				[I]§
FLV [25]				1+12.5 [A, 0.63], 8+6.25 [A, 0.59]
ROS [32]				8+12.5 [A, 0.59]
ATO [32]				8+0.391 [A, 0.62]
PRA [>128]				[I]§
Aspergillus fumigatus SZMC 2486	1–2	2	4	
LOV [25]				1+12.5 [A, 0.72], 2+6.25 [A, 1.05]
SIM [6.25]				0.063+1.563 [A, 1.07], 1+0.781 [A, 1.28], 2+0.391 [A, 0.86]
FLV [2]				0.5+1.563 [A, 0.82], 2+0.391 [A, 0.81]
ROS [128]				2+6.25 [A, 1.29]
ATO [64]				1+25 [S, 2.12], 2+0.781 [A, 1.37]
PRA [>128]				[I]§
Aspergillus flavus SZMC 2521	1–2	2	4	
LOV [>128]				[I]§
SIM [>128]				2+1.563 [A, 0.96]
FLV [128]				0.5+25 [S, 2.43], 1+6.25 [S, 2.46], 2+0.781 [A, 0.85]
ROS [>128]				[I]§
ATO [>128]				[I]§
PRA [>128]				2+0.391 [A, 1.07]
Rhizopus oryzae CBS 109939	2–4	2–4	4	
LOV [128]				2+50 [A, 1.01]
SIM [64]				2+0.781 [A, 1.10]
FLV [2–3.125]				0.125+3.125 [A, 1.28], 2+1.563 [A, 0.78]
ROS [>128]				2+12.5 [A, 1.02]
ATO [32]				[I]§
PRA [>128]				[I]§

*The MICs of the statins are shown in parentheses.

†IC_{50}, IC_{80} and IC_{90} values are the concentrations required for 50%, 80% and 90% growth inhibition.

‡Examples for effective concentrations of the combined drugs causing total growth inhibition are presented; the first number indicates the concentration of MCZ, and the second the concentration of the given statin. The type of the interaction (A, additive; S, synergistic; I, indifferent) and IR values are presented in parentheses.

§Interaction was considered indifferent if no difference in the inhibition rates was detected (i.e. the MIC or IC values of the drugs could not decreased by their combined applications).

synergistically against *C. albicans* (IR = 1.70), KET and SIM against *A. fumigatus* (IR = 1.46), and ITR and FLV against *R. oryzae* (IR = 2.24).

When the investigated strain was sensitive to only the azole compound, but insensitive to the given statin (or the statin inhibited its growth only in high concentrations), the

Table 3. Effect of antifungal activity of FLU combined with different statins

Isolate/statin [MIC alone (μg mL^{-1})]*	ICs of FLU (μg mL^{-1})†			MIC (μg mL^{-1}) of FLU and MIC (μg mL^{-1}) of the different statins in combination [effect, IR]‡
	IC_{50}	IC_{80}	IC_{90}	
Candida albicans ATCC 90028	0.125–0.25	4–64	> 64	
LOV [50–64]				0.125+12.5 [S, 25.2], 0.25+6.25 [S, 2.67], 0.5+3.125 [A, 1.32]
SIM [8]				0.125+6.25 [A, 0.95], 0.25+1.563 [A, 1.15]
FLV [25]				0.125+6.25 [S, 1.70], 0.25+1.563 [A, 1.0]
ROS [128]				16+25 [A, 1.30], 64+12.5 [A, 1.26]
ATO [128]				0.125+12.5 [A, 1.25]
PRA [>128]				[I]§
Candida glabrata CBS 138	2–4	4–8	8–16	
LOV [128]				0.25+50 [A, 1.17], 4+25 [S, 1.65]
SIM [16–32]				4+25 [A, 0.99], 8+0.391 [A, 1.25]
FLV [64]				1+25 [A, 1.12], 4+12.5 [A, 0.96], 8+0.781 [A, 0.96]
ROS [128]				8+25 [A, 1.12]
ATO [32]				1+25 [S, 1.51], 4+6.25 [A, 0.81]
PRA [>128]				[I]§
Paecilomyces variotii ATCC 36257	> 64	> 64	> 64	
LOV [64]				0.125+50 [A, 1.09]
SIM [8]				[I]§
FLV [25]				[I]§
ROS [32]				¶
ATO [32]				¶
PRA [>128]				¶
Aspergillus fumigatus SZMC 2486	> 64	> 64	> 64	
LOV [25]				16+6.25 [S, 1.60]
SIM [6.25]				0.125+3.125 [A, 1.20], 8+1.563 [S, 2.20]
FLV [2]				0.25+1.563 [A, 0.79]
ROS [128]				¶
ATO [64]				8+25 [S, 2.88]
PRA [>128]				¶
Aspergillus flavus SZMC 2521	> 64	> 64	> 64	
LOV [>128]				¶
SIM [>128]				¶
FLV [128]				¶
ROS [>128]				¶
ATO [>128]				¶
PRA [>128]				¶
Rhizopus oryzae CBS 109939	> 64	> 64	> 64	
LOV [128]				64+50 [A, 0.96]
SIM [64]				¶
FLV [2–3.125]				[I]§
ROS [>128]				¶
ATO [32]				¶
PRA [>128]				¶

*The MICs of the statins are shown in parentheses.

†IC_{50}, IC_{80} and IC_{90} values are the concentrations required for 50%, 80% and 90% growth inhibition.

‡Examples for effective concentrations of the combined drugs causing total growth inhibition are presented; the first number indicates the concentration of FLU, and the second the concentration of the given statin. The type of the interaction (A, additive; S, synergistic; I, indifferent) and IR values are presented in parentheses.

§Interaction was considered indifferent if no difference in the inhibition rates was detected (i.e. the MIC or IC values of the drugs could not decreased by their combined applications).

¶Complete growth inhibition was not detected in the administered concentration range.

combined administration of azoles and statins decreased the concentrations needed to achieve the complete blockage of growth by several dilution steps. Such synergistic effects were observed, for example, in the case of *C. albicans*, when MCZ was combined with ROS (IR = 1.66) or LOV was combined with FLU (IR = 25.2). The combination of KET

Table 4. Effect of antifungal activity of ITR combined with different statins

Isolate/statin [MIC alone (μg mL^{-1})]*	ICs of ITR (μg mL^{-1})† IC_{50}	IC_{80}	IC_{90}	MIC (μg mL^{-1}) of ITR and MIC (μg mL^{-1}) of the different statins in combination [effect, IR]‡
Candida albicans ATCC 90028	0.016	> 16	> 16	
LOV [50–64]				0.031+12.5 [A, 1.43]
SIM [8]				0.016+0.781 [A, 1.05]
FLV [25]				0.031+1.563 [A, 1.02]
ROS [128]				0.031+25 [S, 1.79], 1+12.5 [A, 1.46]
ATO [128]				0.031+12.5 [A, 1.27]
PRA [>128]				[I]§
Candida glabrata CBS 138	0.063–0.125	0.25–0.5	0.5–1	
LOV [128]				0.5+0.781 [A, 1.05]
SIM [16–32]				0.063+25 [A, 0.90], 0.25+12.5 [A, 0.71], 0.5+0.781 [A, 0.77]
FLV [64]				0.125+6.25 [A, 0.95], 0.25+1.563 [A, 0.82], 0.5+0.391 [A, 0.79]
ROS [128]				0.25+6.25 [A, 1.08]
ATO [32]				0.063+25 [A, 1.17], 0.125+12.5 [A, 1.14], 0.25+0.781 [A, 0.70]
PRA [>128]				[I]§
Paecilomyces variotii ATCC 36257	0.016–0.031	0.063–0.125	0.125–0.25	
LOV [64]				0.031+25 [A, 0.59], 0.125+12.5 [A, 0.78]
SIM [8]				[I]§
FLV [25]				0.031+12.5 [A, 0.88], 0.063+6.25 [A, 0.86]
ROS [32]				0.063+0.781 [A, 1.05]
ATO [32]				0.016+12.5 [A, 0.72], 0.031+0.781 [A, 0.68]
PRA [>128]				[I]§
Aspergillus fumigatus SZMC 2486	0.031–0.125	0.125–0.25	0.25–0.5	
LOV [25]				0.125+12.5 [A, 1.43], 0.25+3.125 [A, 0.77]
SIM [6.25]				0.031+0.781 [A, 0.67], 0.125+0.391 [A, 0.67]
FLV [2]				0.031+1.563 [A, 0.84], 0.063+0.391 [A, 1.01]
ROS [128]				0.031+25 [S, 2.81], 0.063+12.5 [S, 1.68], 0.125+0.391 [A, 0.90]
ATO [64]				0.016+25 [S, 1.99], 0.063+6.25 [S, 2.62], 0.125+0.391 [S, 46.5]
PRA [>128]				[I]§
Aspergillus flavus SZMC 2521	0.125	0.125–0.25	0.25–0.5	
LOV [>128]				0.25+12.5 [A, 1.15]
SIM [>128]				0.25+0.781 [A, 1.41]
FLV [128]				0.031+12.5 [A, 1.23], 0.063+6.25 [S, 1.56], 0.125+0.391 [A, 0.92]
ROS [>128]				0.125+12.5 [A, 1.38]
ATO [>128]				0.125+3.125 [S, 1.65]
PRA [>128]				[I]§
Rhizopus oryzae CBS 109939	0.25–0.5	0.25–1	0.5–2	
LOV [128]				1+0.781 [A, 1.25]
SIM [64]				[I]§
FLV [2–3.125]				0.031+3.125 [A, 1.13], 0.125+1.563 [S, 1.94], 0.25+0.391 [S, 2.24]
ROS [>128]				0.063+25 [A, 1.18], 0.25+12.5 [A, 1.27], 0.5+3.125 [S, 2.15]
ATO [32]				0.016+25 [A, 1.13], 0.125+6.25 [A, 1.31], 0.25+0.781 [S, 1.50]
PRA [>128]				[I]§

*The MICs of the statins are shown in parentheses.

†IC_{50}, IC_{80} and IC_{90} values are the concentrations required for 50%, 80% and 90% growth inhibition.

‡Examples for effective concentrations of the combined drugs causing total growth inhibition are presented; the first number indicates the concentration of ITR, and the second the concentration of the given statin. The type of the interaction (A, additive; S, synergistic; I, indifferent) and IR values are presented in parentheses.

§Interaction was considered indifferent if no difference in the inhibition rates was detected (i.e. the MIC or IC values of the drugs could not decreased by their combined applications).

and ATO also acted synergistically against *R. oryzae* (IR = 3.05), while the combinations of MCZ and ATO (IR = 2.12) and ITR and ATO (IR = 46.5) acted synergistically against *A. fumigatus*. Filamentous fungi were completely insensitive to FLU; however, FLU acted synergistically against *A. fumigatus* in combination with LOV, SIM and

ATO (IR = 1.60, 2.20 and 2.88, respectively). *Aspergillus flavus* was sensitive to FLV only at high concentration (128 μg mL^{-1}), but acted synergistically in combination with KET, MCZ and ITR (IR = 1.79, 2.46 and 1.56, respectively). No complete inhibition of *A. flavus* was observed with any FLU–statin combination. Although FLU and FLV acted synergistically against this fungus (IR = 3.88), only 50% growth inhibition could be achieved at the highest applied concentrations (64 μg mL^{-1} FLU combined with 25 μg mL^{-1} FLV). The high values of IR appear when the combination of drugs caused total growth inhibition at a certain concentration, but the compounds alone had no inhibitory effect at that concentration.

Some experiments were carried out to acquire preliminary information concerning the variability of the sensitivities within species to these drugs and their combinations. A summary of these results is presented in Table 5. Two of the promising synergistic combinations, FLU–FLV and FLU–LOV, were tested against 12 *C. albicans* isolates. All investigated strains proved to be sensitive to the FLU–FLV combination; moreover, some clinical strains were more sensitive than normal. Synergism was observed in the case of five isolates; otherwise, additive effects were noted. At the same time, *C. albicans* strains were diversely sensitive to the FLU–LOV combination, which derived from their different sensitivities to LOV. Some clinical strains were also more sensitive than average, so synergistic interactions could be achieved with low concentrations. FLU was efficient against all isolates, and the interaction between the two drugs was always positive (synergistic or additive effect). KET–FLV interactions were synergistic against almost every *A. flavus* isolate, but their sensitivities to FLV differed by one or two dilution steps. The effects of MCZ–SIM combination against *C. glabrata* and the KET–SIM and ITR–ATO combination against *A. fumigatus* were also similar to those observed previously, but the sensitivities to the given azole compound differed by one or two dilution steps between the isolates. In general, these drugs proved to be more effective against all tested strains in combination than alone; however, the sensitivities to the statin or the azole compound sometimes varied in a narrow range among the isolates of a species.

Table 5. Variability of the within species sensitivities against some statin–azole combinations

Microorganisms [no. of isolates]	Combinations	Interactions [no. of cases]
Candida albicans [12]	LOV+FLU	A [8], S [4]
	FLV+FLU	A [7], S [5]
Candida glabrata [6]	SIM+MCZ	A [5], S [1]
Aspergillus fumigatus [6]	SIM+KET	A [6]
	ATO+ITR	S [3], A [3]
Aspergillus flavus [6]	FLV+KET	S [5], A [1]

A, additive interaction; S, synergistic interaction.

Discussion

The treatment of *Candida* infections is generally based on azole therapy, whereas azoles and amphotericin B are primarily used against filamentous fungi. Azoles inhibit the fungal growth even at low concentrations; however, their endpoint determination is of major importance, especially for isolates exhibiting trailing growth. Azoles do not cause cessation of growth soon after the exposure to the drug; fungal growth begins to slow down after one doubling time and is fully arrested only some time later (Rex *et al.*, 1993). Some turbidity may persist for all drug concentrations tested and only partial inhibition of growth can be achieved, which results in the phenomenon of the trailing endpoint. So the endpoint for azoles has been defined as the point at which there is prominent reduction in growth. This endpoint could also be referred to as 80% reduction in growth relative to the growth control (IC_{80}); however, for microdilution testing 50% inhibition of growth (IC_{50}) measured by spectrophotometry best approximates the visual endpoint (Pfaller *et al.*, 1995).

It is known that statins have antifungal effect, although it is worth mentioning that they only inhibit the fungal growth at relatively high concentrations, well above the maximum achievable serum levels in humans (Kivistö *et al.*, 1998). In the present study, we detected additive or synergistic interactions between statins and azoles in many cases at concentrations clinically achievable in the human serum. Some earlier publications also reported *in vitro* interaction studies between certain statins and azoles (Chin *et al.*, 1997; Nash *et al.*, 2002; Chamilos *et al.*, 2006); however, in these studies, only one or two statins combined with one or two other antimycotics were involved, and systematic screening of the efficient statin–azole combinations was not performed. Chin *et al.* (1997) detected synergistic and additive effects of FLV combined with FLU or ITR against different *Candida* species and *Cryptococcus neoformans*; however, FLV was used at a higher concentration than is clinically achievable (4–8 μg mL^{-1}). Nash *et al.* (2002) investigated the *in vitro* activity of FLU in combination with clinically relevant concentrations of FLV and PRA (1 and 0.25 mg L^{-1}, respectively) against *C. albicans*, but did not observe any synergistic effect. On the other hand, Chamilos *et al.* (2006) demonstrated significant *in vitro* synergism between LOV and voriconazole against several Zygomycetes when both drugs were applied in the range of clinically achievable concentrations.

The activities observed for certain azole–statin combinations highlight the promise of these compounds as candidates for the treatment of opportunistic human and animal

mycoses. However, the application of the azole–statin combinations is substantially limited because severe drug interactions can arise when these drugs are coadministered. As these agents are metabolized by the same cytochrome P450 enzyme in the liver (CYP3A4), azoles have an effect on the pharmacokinetics of certain statins by reducing their metabolic clearance (Kivistö *et al.*, 1998). The increased concentration of the coadministered statins in the serum may cause severe side effects in the patients, such as myositis and rhabdomyolysis (Herman, 1999; Mazzu *et al.*, 2000). This limits their systemic administration, but the azole–statin combinations may be applicable as topical therapy for patients with oropharyngeal candidosis or other mucocutaneous infections. Furthermore, FLV and PRA have a lower potential than other statins for metabolic drug–drug interactions, as FLV is predominantly metabolized by the CYP2C9 isoenzyme (Fischer *et al.*, 1999), whereas PRA is excreted by the renal mechanism and does not undergo significant metabolism via the cytochrome P450 system (Triscari *et al.*, 1995). In our work, PRA alone proved to be ineffective against the investigated isolates; but it decreased the MICs of KET and MCZ fourfold in the cases of *C. glabrata*. At the same time, FLV had a strong inhibitory effect against all investigated fungi, and interacted synergistically with the azoles in several cases.

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