Volume 54(1):33-36, 2010
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Effect of different statins on the antifungal activity of polyene antimycotics

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ABSTRACT The antifungal activities of amphotericin B/statin and nystatin/statin combinations against some opportunistic pathogenic fungi (*Candida albicans*, *Candida glabrata*, *Paecilomyces variotii*, *Aspergillus fumigatus, Aspergillus flavus* and *Rhizopus oryzae*) were investigated. The *in vitro* interactions between polyene antifungal drugs and different statins were evaluated using a standard chequerboard broth microdilution method. Most of the detected interactions were additive, though in some cases synergism was also observed. In most cases, the extents of inhibition were higher when these compounds were applied together, and as a result the concentrations of amphotericin B and a given statin, needed to prevent fungal growth, generally could be decreased by some dilution steps. **Acta Biol Szeged 54(1):33-36 (2010)**

KEY WORDS

amphotericin B
drug interaction
microscopic fungi
nystatin
statins

The number of opportunistic fungal infections is continuously increasing which creates a substantial challenge for establishing new and more efficient antifungal therapies (Singh 2001; Groll 2009). One approach could be the application of combination therapy: co-administration of different antifungal compounds might improve the efficacy of the treatment. Reduced toxicity (due to the lower effective concentration of antifungal drugs) is also an important advantage (Baddley and Pappas 2005; Nosanchuk 2006; Vazquez 2007). More and more studies have focused on the antifungal activities of non-antifungal drugs, and on the development of antifungal combination therapies based on non-antifungal compounds (Afeltra and Verweij 2003).

Nystatin (NYS) and amphotericin B (AMB) belongs to the polyene antifungals: among them AMB and its lipid complexes (Tiphine et al. 1999; Moen et al. 2009) are one of the most efficient antimycotic agents. However, AMB is quite toxic and may have serious side effects (Gallagher et al. 2003). Combined application of AMB with other effective antifungal agents would be advantageous as a basis of a less toxic therapy. Therefore, there is a substantial interest for drugs, which can act additively or synergistically with AMB, and allow decreasing its therapeutic concentration.

Statins act by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in the sterol biosynthesis pathway; therefore, they are used in human therapy to reduce the level of cholesterol in the blood. These compounds also have certain other (pleiotropic) effects, e.g. decreasing inflammation and improving the endothelial function (Liao and Laufs 2005). Recent reports described their inhibitory effect on the growth of different pathogenic fungi (Roze and Linz 1998; Lukács et al. 2004; Macreadie et al. 2006). Sun and Singh (2009) in their publication reported that statins directly attenuate the virulence of microorganisms: modulate regulatory pathways involved in the infection process. There are also sporadic new reports on the combined application of statins and different antimycotics (Lorenz and Parks 1990; Chin et al. 1997; Nash et al. 2002; Chamilos et al. 2006; Natesan et al. 2008; Nyilasi et al. 2010).

The aim of the present work was to investigate the *in vitro* antifungal activities of the most widely used polyene antimycotics (NYS and AMB), in combination with the most important, commercially available statins – lovastatin (LOV), pravastatin (PRA), simvastatin (SIM), fluvastatin (FLV), atorvastatin (ATO) and rosuvastatin (ROS) – against some opportunistic pathogenic yeast and filamentous fungi.

Materials and Methods

Fungal strains

The following fungal strains were used in this study: *Candida albicans* (*C. albicans*, American Type Culture Collection, USA; ATCC 90028), *Candida glabrata* (*C. glabrata*, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; CBS 138), *Aspergillus fumigatus* (*A. fumigatus*, Szeged Microbial Collection, Szeged, Hungary SZMC 2486), *Aspergillus flavus* (*A. flavus*, SZMC 2521), *Rhizopus oryzae* (*R. oryzae*, CBS 109939) and *Paecilomyces variotii* (*P. variotii*, ATCC 36257). All these strains were maintained on potato dextrose agar (PDA, Sigma-Aldrich, 0.4% potato starch, 2% glucose, 1.5% agar) slants at 4°C.

Accepted May 11, 2010
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Table 1. Examples of effective AMB/statin combinations.

Isolate / Combination [MIC alone (μg/ml)][a]	MIC (μg/ml) of AMB and MIC (μg/ml) of the different statins in combination [effect, IR][b]
C. albicans ATCC 90028	
AMB-ATO [1, 128]	0.5/0.391 [A, 1.13]
C. glabrata CBS 138	
AMB-ROS [1, 128]	0.5/0.391 [A, 1,02]
AMB-ATO [1, 32]	0.5/0.391 [A, 0.88]
P. variotii ATCC 36257	
AMB-SIM [0.125, 8]	0.031/1.563 [A, 0.77]
AMB-ATO [0.125, 32]	0.063/0.391 [A, 0.81]
A. fumigatus SZMC 2486	
AMB-FLV [4, 2]	2/1.563 [A, 0.57]
AMB-ATO [4, 64]	2/0.391 [A, 1.15]
A. flavus SZMC 2521	
AMB-FLV [8-16, 128]	4/12.5 [S, 1.62], 1/25 [A, 1.13]
R. oryzae CBS 109939	
AMB-SIM [2-4, 64]	0.25/25 [A, 0.79]
AMB-FLV [2-4, 2-3.125]	1/1.563 [A, 0.65]
AMB-ROS [2-4, >128]	2/25 [A, 0.91]
AMB-ATO [2-4, 32]	1/6.25 [A, 0.74], 0.5/12.5 [A, 1.20]

[a] In brackets, MICs of AMB and the given statin are presented, respectively. MICs for statins were determined earlier by Nyilasi et al. (2010).
[b] 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 AMB, and the second is the concentration of the given statin. In brackets, the type of the interaction (A, additive; S, synergistic) and IR values are presented, respectively.

Antifungal drugs

AMB (Sigma-Aldrich) was purchased as a stock solution (250 μg/ml in deionised water). NYS (Sigma-Aldrich) was provided by the manufacturer as standard powder and dissolved in dimethyl sulfoxide at a concentration of 16 mg/ml. The statins used in this study were FLV (Lescol, Novartis), LOV (Mevacor, Merck Sharp & Dohme), SIM (Vasilip, Egis), ROS (Crestor, AstraZeneca) and ATO (Atorvox, Richter), which were of pharmaceutical grade and PRA (Sigma-Aldrich), which was provided as standard powder. Statins stocks (12.8 mg/ml) were prepared in methanol (except PRA, which was dissolved in distilled water). Stock solutions were stored at -70°C until needed. For drug tests, dilutions were performed in RPMI 1640 medium (Sigma-Aldrich) containing L-glutamine, but lacking sodium bicarbonate, buffered to pH 7.0 with 0.165 M 3-(N-morpholino)propanesulfonic acid (Sigma-Aldrich). 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 as described by Lorenz and Parks (1990).

In vitro antifungal susceptibility testing

The antifungal activities of NYS, AMB and statins were determined using a broth microdilution method according to the CLSI guidelines (NCCLS 1997; NCCLS 2002). Assays were performed as described earlier (Galgóczy et al. 2009a; Nyilasi et al. 2010) in 96-well flat-bottomed microtitre plates by measuring the optical density of the fungal cultures at 620 nm after incubation for 48 h at 35°C. Final inocula (prepared in RPMI 1640) were 5×10^3 CFU/ml and 5×10^4 spores/ml, for yeasts and for filamentous fungi, respectively. In the wells, the final concentrations for each statin ranged from 0.25 to 128 μg/ml, and for AMB and NYS ranged from 0.0313 to 16 μg/ml. For calculation of the extents of inhibition the OD_{620} readings of the drug-free control cultures were referred to 100% growth. MICs for statins were determined earlier by Nyilasi et al. (2010). MICs for AMB and NYS were the lowest concentration of drugs that produced an optically clear well. All experiments were repeated 3 times.

Statin/polyene interactions were tested by chequerboard broth microdilution method using twofold dilutions from each drug. Fifty μl of each drug dilutions for both drugs were placed in the wells, and were mixed with 100 μl of yeast or sporangiospore suspension. The final concentrations of AMB and NYS were the same as described previously, while those of the various statins ranged from 0.391 to 25 μg/ml. Condition for chequerboard broth microdilution (inoculum preparation, initial inoculum, controls and the conditions of the incubation) were the same as described by Nyilasi et al. (2010).

Data analysis

Interaction ratio between the investigated drugs was calculated using the Abbott formula: $I_e = X+Y-(XY/100)$: I_e is the expected percentage inhibition for a given interaction, X and Y are the percent inhibitions given by each compound when used alone. If I_o is the observed percentage inhibition, the interaction ratio (IR) is given by $IR = I_o/I_e$, which corresponds to the type of the interaction between the compounds. The interaction is additive when IR is between 0.5 and 1.5, when IR>1.5 denotes synergism and when IR<0.5 denotes antagonism (Gisi 1996).

Results and discussion

Sensitivity to statins and polyene antifungals

The MICs of the involved statins and polyene antifungals against the tested fungal isolates are listed in Table 1 and 2. AMB was very effective against all of the investigated isolates in the administered concentration range. The most sensitive species were *P. variotii* (MIC: 0.125 μg/ml), *C. albicans* (MIC: 1 μg/ml) and *C. glabrata* (MIC: 1 μg/ml). Filamentous fungi were also sensitive to AMB in the range of 2-16 μg/ml (Table 1). NYS was also effective against *Candida* isolates and *P. variotii* in the range of 1-2 μg/ml. *A. fumigatus* and *A. flavus* was moderately sensitive to NYS (MICs: 8 and 16 μg/ml, respectively), whilst *R. oryzae* was not sensitive at all to NYS in the administered concentration range (Table 2).

Antifungal potentials of the involved statins were reported in a previous study (Nyilasi et al. 2010). Among the statins, FLV and SIM exhibited potent antifungal activities and frequently showed higher activity than the other statins. The natural statins (SIM and LOV) were inactive in their pro-drug forms, but their active metabolites obtained by hydrolysis of the lactone ring manifested pronounced antifungal effects.

Interactions between AMB and statins

The *in vitro* interactions between AMB and the different statins were studied using a standard chequerboard broth microdilution method. The interaction ratios between the compounds were calculated using the Abbott formula. Table 1 and Table 2 show data of the effective drug combinations for the fungi tested, which indicates the combined drugs in the lowest concentrations causing total growth inhibition.

Positive drug interactions were observed for every investigated strain. The majority of these interactions were found in the case of *R. oryzae*: when AMB was combined with SIM, FLV, ROS or ATO additive effects were observed, so the concentrations of AMB and the given statin needed to block fungal growth completely could be decreased by some dilution steps. AMB-FLV and AMB-ATO combinations were effective in the case of most isolates, moreover, AMB and FLV acted synergistically in inhibiting the growth of *A. flavus*. AMB and FLV inhibited the growth of this fungus at relatively high concentrations, (MICs: 8-16 µg/ml and 128 µg/ml, respectively), but in combination 4 µg/ml AMB and 12.5 µg/ml FLV or 1 µg/ml AMB and 25 µg/ml FLV already inhibited the fungal growth (IRs: 1.62 and 1.13, respectively). In contrast, AMB-LOV, AMB-SIM and AMB-ATO combinations were antagonistic in the case of *A. flavus* . However, antagonistic interactions were not observed at other fungal strains.

Interactions between NYS and statins

The *in vitro* interactions between NYS and the different statins were also studied, Table 2 shows data of the effective drug combinations. NYS-LOV and NYS-FLV combinations were effective in the case of several isolates. PRA did not inhibit the fungal growth alone, but NYS-PRA combination was effective at *A. flavus*, so the concentrations needed to block fungal growth could be reached with lower concentrations in combination. However, NYS-LOV, NYS-SIM and NYS-FLV combinations were also antagonistic in the case of *A. flavus*, whilst antagonistic interactions were not observed at any other fungal strains.

NYS and ATO acted additively (near to the synergistic values) in inhibiting the growth of *R. oryzae*: NYS did not inhibit the growth of this fungus in the administered concentration range, but in combination 4 µg/ml NYS and 12.5 µg/ml ATO or 1 µg/ml NYS and 25 µg/ml ATO already inhibited the fungal growth (IRs: 1.43 and 1.47, respectively).

Table 2. Examples of effective NYS/statin combinations.

Isolate / Combination [MIC alone (µg/ml)][a]	MIC (µg/ml) of NYS and MIC (µg/ml) of the different statins in combination [effect, IR][b]
C. albicans ATCC 90028	
NYS-LOV [2, 64]	0.031/25 [A, 0.94]
NYS-SIM [2, 8]	0.031/6.25 [A, 0.96]
NYS-FLV [2, 25]	0.063/12.5 [A, 1.06]
C. glabrata CBS 138	
NYS-LOV [1, 128]	0.063/50 [A, 1.31]
NYS-ROS [1, 128]	1/12.5 [A, 0.91]
P. variotii ATCC 36257	
NYS-LOV [1, 64]	0.5/12.5 [A, 1.11]
NYS-SIM [1, 8]	0.5/0.391 [A, 0.56]
NYS-FLV [1, 25]	0.5/12.5 [A, 0.84]
A. flavus SZMC 2521	
NYS-PRA [16, >128]	4/1.563 [A, 1.27]
R. oryzae CBS 109939	
NYS-LOV [>16, 128]	8/50 [A, 1.23]
NYS-FLV [>16, 2-3.125]	16/1.563 [A, 1.08]
NYS-ATO [>16, 32]	4/12.5 [A, 1.43], 1/25 [A, 1.47]

[a] In brackets, MICs of NYS and the given statin are presented, respectively.
[b] 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 NYS, and the second is the concentration of the given statin. In brackets, the type of the interaction (A, additive; S, synergistic) and IR values are presented, respectively.

In contrast to the co-administration of azoles and statins, polyene antifungal/statin combinations could be used without serious drug interactions (Herman 1999; Schachter 2004). Therefore, they are potential agents for the treatment of fungal infections (Galgóczy 2009b). Based on the accumulating data on these potential, Kontoyiannis (2007) hypothesized that the widespread use of statins has led to the decreasing number of reported cases of zygomycosis in patients with diabetes mellitus in developed countries since the 1990s, despite the increase in the prevalence of diabetes in those populations.

Acknowledgements

This investigation was financially supported by RET-08/2005 (OMFB-00846/2005) and NKTH IPARJOG-08-1-2009-0026.

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