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ANTIFUNGAL ACTIVITY OF STATINS AND THEIR INTERACTION WITH AMPHOTERICIN B AGAINST CLINICALLY IMPORTANT ZYGOMYCETES

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The *in vitro* antifungal activity of different statins and the combinations of the two most effective ones (fluvastatin and rosuvastatin) with amphotericin B were investigated in this study on 6 fungal isolates representing 4 clinically important genera, namely *Absidia*, *Rhizomucor*, *Rhizopus* and *Syncephalastrum*. The antifungal effects of statins revealed substantial differences. The synthetic statins proved to be more effective than the fungal metabolites. All investigated strains proved to be sensitive to fluvastatin. Fluvastatin and rosuvastatin acted synergistically and additively with amphotericin B in inhibiting the fungal growth in clinically available concentration ranges. Results suggest that statins combined with amphotericin B have a therapeutic potential against fungal infections caused by Zygomycetes species.

Keywords: Statin – amphotericin B – Zygomycetes – drug interaction – synergism

INTRODUCTION

Various members of the class Zygomycetes are frequently isolated agents of mycotic diseases caused by non-*Aspergillus* moulds [26–28, 36]. Over the past decade, case number of zygomycosis (the opportunistic fungal infection caused by Zygomycetes fungi) has shown an increasing tendency in immunocompromised patients and persons having diabetes mellitus or burn injuries [5, 28, 30, 38]. Unfortunately, these fungi have a substantial intrinsic resistance to most of the widely used antifungal drugs (e.g. azoles) and show high MIC values for several other agents in *in vitro* tests [1, 32]. Treatment with amphotericin B (AMB) and its lipid formulations is the standard and only available effective therapy [30, 36], in spite of the fact that these are quite toxic and may have serious side-effects [12, 37]. 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 synergistically with AMB, and allow decreasing its therapeutic concentration. Statins are interesting from this respect, because earlier reports presented that they are exhibiting inhibitory potential against some Zygomycetes [34].

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Statins were originally applied as cholesterol lowering drugs in human therapy. They are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the enzyme that catalyses the conversion of HMG-CoA to mevalonate, which is a central step in the terpenoid biosynthetic pathway. Therefore, effects of statins are connected with the inhibition of the synthesis of important terpenoid derivatives, such as sterols and prenyl groups of proteins involved in different signal transduction pathways [6, 13, 17, 18, 22]. They were discovered as fungal metabolites (mevastatin, lovastatin, simvastatin and pravastatin), but more effective fully synthetic compounds (atorvastatin, cerivastatin, fluvastatin, pitavastatin and rosuvastatin) are also available.

The aim of the present study was to investigate the *in vitro* antifungal effects of statins and their combinations with AMB against clinically important Zygomycetes.

MATERIALS AND METHODS

Strains and media

To examine the effects of the statins, AMB and their interactions, the following 6 clinically important zygomycetous isolates were involved: *Absidia corymbifera* (Szeged Microbial Collection, Szeged, Hungary; SZMC 2010), *Rhizomucor miehei* (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; ETHM, Swiss Federal Institute of Technology Culture Collection, Zurich, Switzerland; CBS 360.92), *R. pusillus* (Wellcome Bacterial Collection, Beckham, Great Britain; WRL CN(M)231), *Rhizopus microsporus* var. *oligosporus* (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois USA; NRRL 514), *Rh. oryzae* (CBS 112.07) and *Syncephalastrum racemosum* (SZMC 2011). The fungal strains were maintained on malt extract slants (ME; 0.5% malt extract, 0.5% yeast extract, 0.5% glucose, 1.0% KH_2PO_4, 1.5% agar) at 4 °C. The antifungal susceptibility tests were performed in M3 medium (Difco, pH = 7.0) based on the recommendation of CLSI M38-A method [25].

Antifungal activity assays

In vitro antifungal activities were investigated in 96-well (flat bottom) microtitre plate bioassays by measuring the absorbances of the fungal cultures (inoculated and incubated media) at 620 nm.

Inoculum preparation was performed by the slightly modified EUCAST method [29]. Fungal sporangiospore suspensions (10^5 spores/ml in final concentration) were prepared from 7-day-old cultures grown on ME slants at 37 °C. These were washed with 5 ml M3 medium, suspended with gentle vortexing than separated from mycelial remnants by filtration through sterile cotton-wool.

Atorvastatin (Atorvox, Richter), fluvastatin (Lescol, Novartis), lovastatin (Mevacor, Merck Sharp & Dohme), pravastatin (Sigma-Aldrich), rosuvastatin (Crestor, AstraZeneca) and simvastatin (Vasilip, Egis) was of pharmaceutical grade and amphotericin B (Sigma-Aldrich) was provided by the manufacturer as a stock solution (250 μg/ml in deionised water) Statin compounds were dissolved in methanol while amphotericin B was dissolved in dimethyl sulfoxide to obtain the stock solution. Stocks were stored at –20 °C until needed. Drug dilutions were performed in M3 medium to yield twice the final strength required for the tests.

To determine the antifungal effect of the drugs, 100 μl of statin or AMB was mixed with 100 μl of sporangiospore suspension and diluted in the wells of a microtitre plate: concentrations of statins ranged from 0.044 to 96 μg/ml in threefold dilutions and those of AMB ranged from 0.03 to 1.0 μg/ml in twofold dilutions. Lovastatin and simvastatin were hydrolyzed in ethanolic NaOH (15% [vol/vol] ethanol and 0.25% [wt/vol] NaOH) at 60 °C for 1 h from their less active lactone prodrug forms [19].

Standard checkerboard titration was used to reveal the interaction between pairs of drugs: statins in the concentrations ranging from 0.044 to 3.6 μg/ml, were mixed with AMB concentrations ranging from 0.03 to 1 μg/ml [9]. In this case, inocula were prepared in the appropriate AMB solutions.

Plates were incubated for 48 h at 37 °C without shaking. Absorbances were then measured with a microtitre plate reader (ASYS Jupiter HD-ASYS, Hitech); the non-inoculated medium was used as background for the spectrophotometric calibration. Determinations of MIC values and the rates of the interactions were repeated three times.

Data analysis

For calculation of the inhibition rates, the absorbances of the untreated control cultures were referred to 100% growth in each case. The interaction ratio between the antifungal agents was calculated by using the Abbott formula: $I_e = X+Y-(XY/100)$, were I_e is the expected percentage inhibition for a given interaction, X and Y are 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 reflects the nature of the interaction between the antifungal compounds. When IR is between 0.5 and 1.5, the interaction is additive, $IR > 1.5$ denotes synergism and $IR < 0.5$ denotes antagonism [24].

RESULTS

Sensitivity to statins

The MIC_{90} and MIC_{50} values determined for the different statins are summarized in Table 1. Their antifungal effects showed substantial differences. Synthetic statins (atorvastatin, fluvastatin and rosuvastatin) proved to be more effective than the fungal

metabolites (lovastatin, simvastatin and pravastatin). Atorvastatin was slightly active against most of the tested fungi, except of *Rh. microsporus* var. *oligosporus*, which proved to be completely resistant to it. MIC_{50} values of atorvastatin were between 11–96 μg/ml, depending on the sensitivity of the investigated strain. Rosuvastatin exerted potent antifungal activity against *A. corymbifera*, *R. miehei* and *R. pusillus*; its MIC_{90} and MIC_{50} values varied between 11–33 and 1.2–11 μg/ml. Other isolates proved to be slightly sensitive to rosuvastatin at the tested concentrations. Simvastatin was effective against all of the tested Zygomycetes, especially against *A. corymbifera* and *R. pusillus* (MIC_{90} = 96 and 11–33 μg/ml, respectively). Its hydrolyzed form had stronger antifungal activity, because the MIC_{90} values were 11 and 1.2–3.6 μg/ml, respectively. A similar effect was detected at lovastatin: its hydrolyzed form exerted greater antifungal activity than its prodrug form. All investigated strain proved to be sensitive to fluvastatin, it was the most efficient one among the statins tested; MIC_{90} and MIC_{50} values were 0.4 and 0.044 μg/ml, respectively, in case of *R. pusillus*. Lovastatin, its hydrolyzed form and pravastatin proved to be the less efficient in our experiments. Most of the isolates proved to be insensitive to them, and where they exerted some antifungal activity, MIC_{90} and MIC_{50} values were much higher than those determined for other statins. Four of the investigated isolates (*R. miehei*, *Rh. microsporus* var. *oligosporus*, *Rh. oryzae*, *S. racemosum*) were completely resistant to pravastatin, which generally showed the weakest antifungal activity.

A. corymbifera and *R. pusillus* were the most sensitive strains to the administered concentrations of statins, and fluvastatin proved to be the most effective against them. High MIC values and strong resistance to most of the tested statins were observed in case of *Rh. microsporus* var. *oligosporus*.

Sensitivity to amphotericin B

Amphotericin B was effective against all of the investigated isolates in the administered concentration range (Table 1). The most sensitive species to AMB were *Rh. microsporus* var. *oligosporus* and *S. racemosum*; MIC_{90} and MIC_{50} values were 0.5–0.25, 0.5–0.25 and 0.25, 0.13–0.06 μg/ml, respectively.

Interactions between statins and amphotericin B

The two most effective statins (fluvastatin and rosuvastatin) were involved in the interaction experiments. The investigated statin-amphotericin B concentration combinations (in the ranges 0.044–3.6 μg/ml and 0.03–1 μg/ml, respectively) showed different interactions against the tested strains in the checkerboard-titration. Only the interactions with remarkable growth inhibitions (≥25%) were considered in this study. Results are summarized in Table 2.

Synergistic interactions could be detected between fluvastatin and amphotericin B in cases of *R. miehei*, *Rh. microsporus* var. *oligosporus*, *Rh. oryzae* and *S. racemo-*

Table 1
In vitro antifungal susceptibility data for the investigated Zygomycetes fungi against statins and amphotericin B

Drug (μg/ml)/Species	ATO		ROS		SIM	
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}
A. corymbifera	96	11	33	1.2	96	3.6
R. miehei	>96	96	33–11	11	>96	33
R. pusillus	>96	96–33	11	1.2	33–11	0.4
Rh. microsporus var. *oligosporus*	>96	>96	>96	96-33	>96	33
Rh. oryzae	96	33	96	11	>96	11
S. racemosum	>96	33	>96	11	>96	33–11
Drug (μg/ml)/Species	SIMH		FLV		LOV	
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}
A. corymbifera	11	0.4	3.6	0.4	>96	96
R. miehei	>96	96–33	3.6	0.4	>96	>96
R. pusillus	3.6–1.2	0.4	0.4	0.044	3.6	1.2–0.4
Rh. microsporus var. *oligosporus*	96–33	11	96-33	11–3.6	>96	>96
Rh. oryzae	>96	33–11	11–3.6	1.2–0.4	>96	>96
S. racemosum	96–33	11	33–11	11	>96	11
Drug (μg/ml)/Species	LOVH		PRA		AMB	
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}
A. corymbifera	11	3.6	>96	11	>1	0.25
R. miehei	11	3.6	>96	>96	1	1–0.5
R. pusillus	11	1.2	>96	11	1	0.25
Rh. microsporus var. *oligosporus*	>96	96–33	>96	>96	0.5–0.25	0.5–0.25
Rh. oryzae	>96	11	>96	>96	>1	0.5
S. racemosum	>96	96	>96	>96	0.25	0.13–0.06

Abbreviations: AMB, amphotericin B; ATO, atorvastatin; FLV, fluvastatin, LOV, lovastatin; LOVH, hydrolyzed lovastatin; PRA, pravastatin; ROS, rosuvastatin; SIM, simvastatin; SIMH, hydrolyzed simvastatin.

sum, as well as between rosuvastatin and amphotericin B against *A. corymbifera, R. miehei*, *Rh. oryzae* and *S. racemosum*. Apart from the interactions presented in Table 2, other concentration combinations of the investigated drugs acted additively in each case (data not shown). Complete blockage of growth could not be achieved in our interaction experiments, but more than 90% growth inhibition was observed in cases of *R. miehei*, *Rh. oryzae* and *S. racemosum* with the application of fluvastatin-amphotericin B and/or rosuvastatin-amphotericin B combinations; concentrations of the drugs in these combinations were lower than their own MIC_{90} values separately.

Table 2
In vitro synergistic interactions with ≥25% growth inhibition between fluvastatin, rosuvastatin and amphotericin B against the investigated Zygomycetes fungi

Species/Statin	Inh. (%)	FLV+AMB (μg/ml)	Inh. (%)	ROS+AMB (μg/ml
A. corymbifera	–	–	82±0.1 64±3.3 25±2.9	0.44+0.25 (IR=1.77±0.13) 0.44+0.125 (IR=1.87±0.02) 0.44+0.03 (IR=3.17±0.34)
R. miehei	95±2.0 94±2.0 92±0.9 91±1.0	0.133+0.5 (IR=2.36±0.78) 0.133+0.25 (IR=3.03±1.54) 0.44+0.5 (IR=2.37±0.72) 0.44+0.25 (IR=3.08±1.35)	95–96±1.5–2.0 91–94±0.0–1.5 87–89±1.0 36–40±2.0–7.5	3.6-0.044+0.5 (IR=2.09–2.40±0.42–0.59) 3.6-0.133+0.25 (IR=2.65–3.07±0.76–1.06) 3.6-0.44+0.125 (IR=2.65–3.07±0.36–1.41) 1.2-0.44+0.06 (IR=1.72–1.77±0.54–0.76)
Rh. microsporus var. *oligosporus*	65–76±1.5–6.0 62–71±1.5–3.5	3.6+0.25–0.03 (IR=1.61–3.01±0.09–0.24) 1.2+0.25–0.125 (IR=1.75–1.89±0.76–1.46)	–	–
Rh. oryzae	37±5.0 27±8.5 26±6.0	0.133+0.25 (IR=5.29±2.87) 0.133+0.125 (IR=3.00±1.85) 0.044+0.25 (IR=3.71±1.96)	91–92±1.0–4.5 82–86±0.0–4.5 32–39±2.0–3.5 27–34±1.0–4.5	0.44–0.044+1 (IR=1.51–1.53±0.03–0.09) 3.6–0.44+0.5 (IR=1.90–1.97±0.04–0.14) 3.6–0.044+0.25 (IR=3.27–4.71±1.18–1.90) 3.6+0.125–0.06 (IR=2.51–3.44±1.38–1.52)
S. racemosum	45–91±0.0–9.5 87–91±0.5–2.5	3.6–0.133+0.06 (IR=1.57–4.20±0.15–1.53) 3.6–0.44+0.03 (IR=1.70–5.94±0.10–0.82)	73±9.5	3.6+0.06 (IR=1.57±0.04)

Abbreviations: AMB, amphotericin B; FLV, fluvastatin; Inh., inhibition; IR, interaction ratio inferred from Abbott formula; ROS, rosuvastatin.

DISCUSSION

The aim of this study was to investigate the antifungal effect of different statins and their interactions with amphotericin B against clinically important Zygomycetes.

Antifungal activities of statins on Zygomycetes have been demonstrated previously in the cases of lovastatin, simvastatin, rosuvastatin, atorvastatin and fluvastatin [11, 34]. The antifungal effect of lovastatin against 7 clinical isolates of Zygomycetes was also tested by Chamilos et al.: all strains were sensitive to lovastatin, with MICs of 32–56 μg/ml [4]. In the present study, we observed substantial differences among the two species of *Rhizomucor* in their susceptibilities to lovastatin. A similar phenomenon was also demonstrated by Lukács et al. [20] previously. Simvastatin proved to be active against *S. racemosum* and *Rhizopus stolonifer* in a previous study: The germination of *S. racemosum* spores was blocked by ≥8 μg/ml. MIC_{50} of simvastatin was detected at ≥32 μg/ml in the case of *Rh. stolonifer*, and complete growth inhibition was achieved at 128 μg/ml [10]. Our experimental data are comparable with these observations. Two synthetic statins (atorvastatin and rosuvastatin) caused complete blockage of growth of *S. racemosum* at ≥32 μg/ml and ≥64 μg/ml, respectively, in the same study [10], and the inhibitory effects of these drugs were observed at 64 μg/ml in the case of *Rh. stolonifer*. It is noteworthy that in our study, the growth of the investigated strains was not inhibited completely even in the presence of 96 μg/ml atorvastatin. The antifungal action of fluvastatin against Zygomycetes recently furnished similar results: MICs were found in the range from 3.125 to 100 μg/ml, depending on the sensitivities of the species investigated [11]. Some of the above-mentioned data are not easily comparable with our results, because of the differences in the applied test methods and the involved organisms. The observed MICs of statins are much higher than the concentrations available in the human blood-serum; the differences are about one order of magnitude [2, 3, 7, 8, 15, 33].

One of the assumption for the different levels of fungal resistance to statins that it can be connected with the different copy numbers of the HMG-CoA reductase gene (*hmgR*). In a previous study, heterologous expression of *R. miehei hmgR* gene was carried out in *Mucor circinelloides* [21]. Transformants expressing the *R. miehei hmgR* gene showed even less sensitivity to statins compared to the untransformed *M. circinelloides* strain. The antifungal effect of statins may be based on their inhibitory effect on the HMG-CoA reductase [17]. Effects of statins are therefore connected with the inhibition of the synthesis of different prenyl groups, which are important lipid attachments for the γ subunit of heterotrimeric G-proteins [18], and guanosine triphosphate-binding protein Ras and Ras-like proteins [13, 17, 18]. Thus statins act as inhibitors of some G-protein actions and Ras or Ras-like signalling [6], which are vital processes of cell proliferation and differentiation [22]. This mechanism was also supported by the observation that lovastatin induced apoptosis-like cell death in *Mucor racemosus* [31].

Previously, MIC values of AMB against Zygomycetes were found in the range of 0.5–4 μg/ml [23]. In our study, MIC_{90}s of AMB were comparable with the above-mentioned results.

Synergistic interactions between statins and amphotericin B have not been described yet. It has been previously demonstrated by *in vitro* susceptibility tests that statins may act synergistically with other antimicrobial drugs against Zygomycetes. Combination of lovastatin with voriconazole was found to be significantly synergistic against Zygomycetes in the range of the clinically achievable concentrations of both drugs [4]. Fluvastatin and suramine (a non-antifungal drug, with antifungal activity) have also proved to act synergistically [11]. We presume that fluvastatin and rosuvastatin are able to interact with amphotericin B generating a significant antifungal effect against Zygomycetes. Concentrations of amphotericin B, fluvastatin and rosuvastatin tested in this study are reachable in the human plasma and serum [2–3, 7–8, 14–15, 33].

It is important to take into account that administration of AMB is contraindicated with drugs that are predominately metabolized by the same cytochrome P450 (CYP) isoenzymes in the liver in the interest of avoiding serious side-effects. Fluvastatin is metabolized by CYP2C9 and rosuvastatin by CYP2C9 and –2C19, whereas AMB is metabolized by CYP 3A1 [2, 3, 7, 8, 15, 16, 33], thus, in principle, they can be administered together more safely, than drugs that are metabolized at the same pathway.

The observed activities of AMB combined with fluvastatin or rosuvastatin would create new potentials in the treatment of zygomycosis without serious side-effects. Further studies are needed to evaluate the practical efficiency of these combinations, for example, *in vivo* animal model experiments.

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