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Original Article

Susceptibility of clinically important dermatophytes against statins and different statin-antifungal combinations

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Abstract

The investigation of the antifungal activities of drugs whose primary activities are not related to their antimicrobial potential is in the current forefront of research. Statin compounds, which are routinely used as cholesterol-lowering drugs, may also exert direct antimicrobial effects. In this study, the *in vitro* antifungal activities of various statins (lovastatin, simvastatin, fluvastatin, atorvastatin, rosuvastatin and pravastatin) were examined against one isolate each of four dermatophyte species (*Trichophyton mentagrophytes, Trichophyton rubrum, Microsporum canis* and *Microsporum gypseum*). Basically, statins were effective in inhibiting all dermatophyte studied, but were particularly active against *M. canis* and *T. mentagrophytes*. Fluvastatin and simvastatin were active against all of the tested fungi causing a complete inhibition of their growth at very low concentrations (6.25–12.5 μg/ml). Lovastatin and rosuvastatin had inhibitory effects at higher concentrations (25–128 μg/ml), while atorvastatin and pravastatin proved the less effective. The *in vitro* interactions between statins and different antifungals (ketoconazole, itraconazole, fluconazole, amphotericin B, nystatin, griseofulvin, terbinafine and primycin) were also investigated using a standard chequerboard broth microdilution method. Synergetic interactions were observed in several cases, most of them were noticed when statins were combined with terbinafine and the different azoles. Some combinations were particularly active (ketoconazole-simvastatin or terbinafine-simvastatin), as they were found to exert synergistic effect against all of the investigated isolates. The other antifungals showed synergistic interactions with statins in only certain cases. These results suggest that statins exert substantial antifungal effects against dermatophyte fungi and they should

be promising components in a combination therapy as they can act synergistically with a number of clinically used antifungal agents.

Key words: Statin, dermatophytes, antifungal susceptibility testing, drug combination, synergism.

Introduction

More and more studies have focused on the antifungal activities of drugs not routinely used in the treatment of fungal diseases, as well as on the development of antifungal combination therapies with such compounds [1]. Statins are the most frequently applied cholesterol-lowering drugs as they inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the enzyme that catalyzes the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonic acid, the rate-limiting step of the sterol biosynthetic pathway [2]. Besides hyperlipidemia control, statins have several cholesterol-independent (pleiotropic) effects as well, e.g., they improve the endothelial function by stimulating the production of nitrogen monoxide of endothelial cells, inhibit the aggregation of platelets, reduce the accumulation of β-amyloid in brain inhibiting the development of dementia [2]. They have antioxidant and anti-inflammatory effects as well, since these agents modify the inflammatory cascades by pleiotropic actions at multiple levels [3].

There is increasing evidence for the potential use of statins in preventing and treating infections as they attenuate the virulence and pathogenicity of microorganisms and modulate signaling and other regulatory pathways involved in controlling infection [4–6]. Statins exert substantial growth-inhibitory effects on the growth of different pathogenic fungi in that recent studies have revealed their direct antimicrobial effect against yeasts, as well as ascomycetous and zygomycetous molds [7–10]. There are also sporadic new reports on the combined application of statins and different antimycotics against fungi [11–19].

However, no data have been found about the antifungal effects of statins on the growth of dermatophyte fungi. Dermatophytes are a group of closely related fungi that have a high affinity to keratinized tissues such as skin, hair, body hair and nails, which are members of three genera, i.e., *Epidermophyton, Microsporum* and *Trichophyton*. Based on their natural habitats, three groups can be differentiated, i.e., geophilic (found primarily in soil) saprophytic species (*M. gypseum, T. ajelloi*), zoophilic species (routinely encountered on animals; *M. canis, T. verrucosum*) and anthropophilic species (naturally associated with humans; *T. rubrum, E. floccosum, M. audounii, T. tonsurans*) [20]. These fungi can attack the human and animal keratinized tissues and cause a wide spectrum of clinical manifestations of superficial infections. Dermatophytosis is one of the most common fungal infections worldwide and the incidence of infections has been increasing, particularly in immunocompromised patients [21]. Dermatophyte infections generally need prolonged treatment with topical and systemic antifungal agents. Although, localized non-extensive lesions respond well to topical antifungal therapy, other extensive infections, particularly involving the scalp or nails, require prolonged systemic therapy [22]. Oral treatment with antifungal agents such as terbinafine, itraconazole, ketoconazole and fluconazole constitutes the treatment of choice for dermatophytoses that fail to respond to topical therapy [23,24].

The aim of the present work was to investigate the *in vitro* antifungal activities of the most important, commercially available statins – lovastatin (LOV), pravastatin (PRA), simvastatin (SIM), fluvastatin (FLV), atorvastatin (ATO) and rosuvastatin (ROS) alone and also in combination with the most widely used antifungal compounds – ketoconazole (KTC), itraconazole (ITC), fluconazole (FLC), amphotericin B (AMB), nystatin (NYT), griseofulvin (GRS), terbinafine (TRB) and primycin (PN) against different dermatophyte fungi.

Materials and methods

Strains and media

The following fungal strains were used in this study; *Trichophyton rubrum* (American Type Culture Collection, USA; ATCC 28188), *Trichophyton mentagrophytes* (ATCC 9533), *Microsporum gypseum* (ATCC 24102) and *Microsporum canis* (ATCC 36299) and *Candida albicans* (ATCC 90028) as the reference strain in the antifungal susceptibility tests. The isolates were maintained on potato dextrose agar slants (PDA, Difco, 0.4% potato starch, 2% glucose, 1.5% agar) at 4°C.

Antifungal agents

The statins used in this study were: fluvastatin (Lescol; Novartis), lovastatin (Mevacor; Merck Sharp & Dohme), simvastatin (Vasilip; Egis), rosuvastatin (Crestor; AstraZeneca) and atorvastatin (Atorvox; Richter), which were all pharmaceutical grade and pravastatin (Sigma-Aldrich), which was provided as standard powder. The azoles used were ketoconazole, fluconazole and itraconazole, provided by the manufacturer (Sigma-Aldrich) as standard

powders as were nystatin (Sigma-Aldrich), griseofulvin (Sigma-Aldrich), terbinafine (LGC Promochem) and primycin (PannonPharma, Pécsvárad, Hungary). In contrast, amphotericin B (Sigma-Aldrich) was purchased as a stock solution (250 μg/ml in deionized water).

Fresh stock solutions of the statins were prepared in methanol, with the exception of pravastatin, which was dissolved in distilled water. Lovastatin and simvastatin were activated 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 right before the antifungal assays [25]. Stock solutions of primycin, nystatin, ketoconazole, itraconazole and terbinafine were made in dimethyl sulfoxide (Sigma-Aldrich), while fluconazole and griseofulvin were dissolved in dimethylformamide (Reanal). Stock solutions were stored at −80°C until needed.

Antifungal susceptibility testing

The *in vitro* antifungal activities of the various statins and antimycotics were determined against dermatophyte fungi using a broth microdilution method, which was performed according to the guideline M38-A2 proposed by the Clinical and Laboratory Standards Institute (CLSI) with some modifications [26]. The *C. albicans* ATCC 90028 strain was involved as a reference strain, its antifungal susceptibility testing was performed according to the M27-A3 guideline [27].

The minimal inhibitory concentration (MIC) values were determined in 96-well flat-bottomed microtiter plates by measuring the optical density (OD) of the fungal growth in culture at 620 nm, in a test medium of RPMI 1640 (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). Inocula of dermatophyte fungi were prepared using the modified method of Santos et al. [28] which first consisted of growing the strains on PDA slants at 30°C for 14 days. The fungal colonies were then covered with 5 ml RPMI 1640 medium and suspensions prepared by scraping the agar surface with the tip of a pipette to generate a suspension containing a mixture of conidia and hyphal elements. This mixture was transferred to a sterile tube and let stand for 15–20 min at room temperature to allow the heavy particles to settle out. The upper fraction was then filtered through a membrane (pore size 8 μm, Sartorius), which retained hyphal elements but allowed for the passage of dermatophyte microconidia. The concentration of microconidia in the suspensions was determined through the use of a haemocytometer and then they were diluted in RPMI 1640 to prepare a final inoculum suspension containing 10^3–10^4 CFU/ml microconidia. Inoculum quantification was accomplished by inoculating 10 μl of each type of inoculum suspension onto PDA agar, then incubating the plates at 30°C to count the colonies as they became visible to determine the CFU/ml.

The statins and the antifungal agents were tested in series of two-fold dilutions at concentrations ranging from: 0.25–128 μg/ml for statins; 0.125–64 μg/ml for fluconazole, primycin and griseofulvin; 0.031–16 μg/ml for ketoconazole, nystatin and amphotericin B; and 0.016–8 μg/ml for itraconazole and terbinafine. The microtiter plates were incubated for 4 days at 30°C, and the endpoint determination was done by visually and spectrophotometrically to quantify the growth of the fungal cultures. The OD was measured at 620 nm with a microtiter 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 demonstrate that the solvent had no inhibitory effect on the investigated fungi at the concentration investigated. For calculation of the extent of inhibition, the OD_{620} readings of the drug-free control cultures were set at 100% growth. The MICs for the statins and the antifungals were determined as the lowest concentration of drugs that produced an optically clear well after four days incubation. The quality-control strain was included on each occasion that an isolate was tested and all experiments were performed in duplicate.

Chequerboard broth microdilution method

For drug interaction studies, each statin was tested with each antifungal compound by the chequerboard broth microdilution method. The statins and the antifungal agents were tested at series of two-fold dilutions at concentrations ranging from 0.391–25 μg/ml for statins and from 0.004–2 μg/ml for terbinafine. The concentrations of other antifungals, the inoculum preparation, the initial inoculum, the controls and the conditions of the incubation were the same 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. The IR reflects the nature of the interaction between the antifungal compounds: an IR > 1.5 denotes synergism and an IR < 0.5 denotes antagonism [29]. If IR is between 0.5 and 1.5, the interaction is considered additive by Gisi [29]; however, we designated these interactions as indifferent since additivism is not a valid category. The interaction ratio between the antifungal agents was also calculated by using the fractional inhibitory concentration index (FICI), 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 indexes were interpreted as follows: ≤0.5, synergy; >0.5 to <4.0, indifference; and ≥4.0, antagonism [30,31].

Results

In vitro susceptibility testing

The *in vitro* antifungal activities of lovastatin, simvastatin, fluvastatin, rosuvastatin, atorvastatin and pravastatin were determined against the four indicated dermatophytes strains in the range of 0.25–128 μg/ml by the broth microdilution method. The MICs of the statins were regarded as the lowest concentration of drugs that produced greater than 90% growth inhibition, but it should be noted that 50% growth-inhibitory concentrations (IC_{50}) were also determined. Since we had established the MIC values of the investigated statins against *C. albicans* ATCC 90028 in a previous study we used it as a control in these tests [16]. The MICs of the different statins against dermatophyte strains are reported in Table 1.

Dermatophyte fungi were sensitive to the statins and the different species showed similar patterns of susceptibility to each statin tested. However, the antifungal effects of the different statins varied what with fluvastatin and simvastatin displaying the strongest antifungal activity, followed in sequence by lovastatin, rosuvastatin, atorvastatin and pravastatin. The natural statins (lovastatin and simvastatin) 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. Fluvastatin and simvastatin were active against all of the tested fungi completely inhibiting their growth at very low concentrations (6.25–12.5 μg/ml). Lovastatin and rosuvastatin had inhibitory effects at higher concentrations (25–128 μg/ml), atorvastatin inhibited the growth of only *T. mentagrophytes* and *M. canis* at the highest applied concentration, whilst pravastatin proved completely ineffective against all isolates at the concentrations used in these studies. Fungi were equally sensitive to the statins, although *M. canis* and *T. mentagrophytes* proved to be the most sensitive strains. *Trichophyton rubrum* and *M. gypseum* were also sensitive to statins, but their MIC values were higher with one or two dilutions.

The *in vitro* MICs of the investigated antifungal agents were also determined (Table 2), which were regarded, mainly for the azoles, as the lowest concentrations that produced prominent inhibition of growth (approximately

Table 1. MICs of the investigated statins against dermatophyte strains.

Strain	Statin	MIC values (μg/ml) IC_{50}	MIC
Trichophyton rubrum (ATCC 28188)			
	LOV	16–25	25–32
	SIM	3.125	6.25–12.5
	FLV	1.56–3.125	6.25
	ROS	64	128
	ATO	64–128	>128
	PRA	>128	>128
Trichophyton mentagrophytes (ATCC 9533)			
	LOV	12.5–25	25–32
	SIM	1.56–3.125	6.25
	FLV	3.125	6.25–12.5
	ROS	32	64
	ATO	32	64–128
	PRA	128	>128
Microsporum gypseum (ATCC 24102)			
	LOV	25–32	32–64
	SIM	3.125–6.25	6.25–12.5
	FLV	3.125–6.25	8
	ROS	128	>128
	ATO	128	>128
	PRA	>128	>128
Microsporum canis (ATCC 36299)			
	LOV	12.5–25	32
	SIM	1.56	6.25–12.5
	FLV	1.56	6.25–12.5
	ROS	16–32	64
	ATO	32	128
	PRA	64	>128

IC_{50}, 50% growth-inhibitory concentration; MIC, minimal inhibitory concentration; LOV, lovastatin; SIM, simvastatin; FLV, fluvastatin; ROS, rosuvastatin; ATO, atorvastatin; PRA, pravastatin.

Table 2. The statin-antifungal agent combinations displaying synergistic interactions against the investigated dermatophytes strains.

Strain	Statin [MIC alone (μg/ml)][a]	Antifungal agent [MIC alone (μg/ml)][b]	MIC in combination (μg/ml)[c]	IR	I[d]	FICI	I[e]
Trichophyton rubrum							
ATCC 28188	LOV [25–32]	FLC [1–2]	12.5 + 0.25	1.50	S	0.64	I
		TRB [0.016–0.03]	6.25 + 0.008	0.94	I	0.50	S
	SIM [6.25–12.5]	KTC [0.06–0.5]	0.39 + 0.06	0.86	I	0.09	S
			3.125 + 0.03	0.86	I	0.28	S
		ITC [0.06–0.25]	0.39 + 0.06	0.88	I	0.28	S
			3.125 + 0.03	1.11	I	0.38	S
		FLC [1–2]	3.125 + 0.5	1.75	S	0.50	S
		TRB [0.016–0.03]	0.78 + 0.008	0.86	I	0.38	S
		PN [16–32]	3.125 + 2	1.18	I	0.31	S
	FLV [6.25]	KTC [0.06–0.5]	0.39 + 0.03	0.83	I	0.31	S
		FLC [1–2]	1.56 + 0.25	1.64	S	0.50	S
		TRB [0.016–0.03]	1.56 + 0.008	0.89	I	0.50	S
	ROS [128]	KTC [0.06–0.5]	0.39 + 0.03	0.69	I	0.504	S
		FLC [1–2]	0.39 + 2	0.91	I	0.504	S
			6.25 + 1	0.93	I	0.30	S
			25 + 0.5	2.70	S	0.32	S
		TRB [0.016–0.03]	25 + 0.004	1.03	I	0.45	S
	ATO [>128]	KTC [0.06–0.5]	0.39 + 0.06	0.87	I	<0.502[f]	S
			0.78 + 0.03	0.96	I	<0.25[f]	S
		ITC [0.06–0.25]	0.39 + 0.03	1.22	I	<0.25[f]	S
			6.25 + 0.016	2.28	S	<0.15[f]	S
		FLC [1–2]	0.39 + 1	0.94	I	<0.502[f]	S
			12.5 + 0.5	1.32	I	<0.30[f]	S
		TRB [0.016–0.03]	0.39 + 0.008	0.69	I	<0.501[f]	S
			12.5 + 0.004	0.80	I	<0.30[f]	S
	PRA [>128]	AMB [2]	0.39 + 1	0.78	I	<0.504[f]	S
Trichophyton mentagrophytes							
ATCC 9533	LOV [25–32]	TRB [0.016]	3.125 + 0.008	1.05	I	0.38	S
	SIM [6.25]	KTC [0.5–1]	1.56 + 0.03	1.15	I	0.31	S
		FLC [>64]	1.56 + 32	1.00	I	<0.5[f]	S
		TRB [0.016]	0.78 + 0.004	0.99	I	0.50	S
	FLV [6.25–12.5]	KTC [0.5–1]	3.125 + 0.125	1.49	I	0.38	S
	ATO [64–128]	TRB [0.016]	25 + 0.004	1.48	I	0.45 < x < 0.64[f]	S/I
Microsporum gypseum							
ATCC 24102	LOV [32–64]	KTC [1–2]	25 + 0.5	1.69	S	0.64	I
		FLC [> 64]	12.5 + 1	1.08	I	<0.21[f]	S
			25 + 0.125	0.66	I	<0.39[f]	S
		TRB [0.06–0.125]	6.25 + 0.03	1.42	I	0.50	S
	SIM [6.25–12.5]	AMB [2–4]	0.78 + 2	1.61	S	0.56	I
		NYT [4–8]	1.56 + 4	1.53	S	0.63	I
		KTC [1–2]	3.125 + 0.06	2.28	S	0.56	I
		ITC [0.06–0.125]	3.125 + 0.016	1.27	I	0.38	S
		FLC [> 64]	0.78 + 32	2.60	S	<0.38[f]	S
			1.56 + 16	1.96	S	<0.38[f]	S
			3.125 + 0.5	1.53	S	<0.504[f]	S
		TRB [0.06–0.125]	3.125 + 0.016	1.37	I	0.50	S
		GRS [0.5–1]	3.125 + 0.125	0.88	I	0.50	S
	FLV [8]	KTC [1–2]	1.56 + 0.125	1.74	S	0.38	S
		FLC [> 64]	3.125 + 32	3.39	S	<0.50[f]	S
			6.25 + 0.125	1.09	I	<0.50[f]	S
		TRB [0.06–0.125]	3.125 + 0.03	0.94	I	0.50	S

Table 2 (Continued)

Strain	Statin [MIC alone (μg/ml)][a]	Antifungal agent [MIC alone (μg/ml)][b]	MIC in combination (μg/ml)[c]	IR	I[d]	FICI	I[e]
	GRS [0.5–1]		3.125 + 0.25	1.87	S	0.50	S
	ROS [>128]	NYT [4–8]	25 + 2	2.09	S	<0.70[f]	I
		KTC [1–2]	25 + 0.25	2.70	S	<0.70[f]	I
		ITC [0.06–0.125]	25 + 0.03	1.73	S	<0.70[f]	I
	ATO [>128]	KTC [1–2]	0.78 + 1	0.98	I	<0.503[f]	S
			25 + 0.5	2.04	S	<0.35[f]	S
		ITC [0.06–0.125]	0.39 + 0.06	1.00	I	<0.502[f]	S
			0.78 + 0.03	1.37	I	<0.25[f]	S
			12.5 + 0.016	1.81	S	<0.17[f]	S
		FLC [> 64]	3.125 + 64	1.90	S	<0.51[f]	I
			6.25 + 32	2.97	S	<0.27[f]	S
			12.5 + 16	1.57	S	<0.17[f]	S
			25 + 4	1.37	I	<0.13[f]	S
		TRB [0.06–0.125]	25 + 0.016	1.55	S	<0.35[f]	S
Microsporum canis							
ATCC 36299	SIM [6.25–12.5]	AMB [1–2]	0.78 + 1	2.71	S	0.63	I
		NYT [4–8]	0.39 + 0.5	1.09	I	0.19	S
			0.78 + 0.25	0.70	I	0.19	S
			1.56 + 0.125	0.76	I	0.28	S
		KTC [0.25–1]	0.39 + 0.03	0.82	I	0.28	S
		TRB [0.03–0.06]	1.56 + 0.016	0.77	I	0.31	S
			6.25 + 0.008	0.69	I	0.38	S
	PRA [>128]	ITC [0.016–0.125]	25 + 0.06	0.85	I	<0.45[f]	S

MIC, minimal inhibitory concentration; IR, interaction ratio; FICI, fractional inhibitory concentration index; LOV, lovastatin; SIM, simvastatin; FLV, fluvastatin; ROS, rosuvastatin; ATO, atorvastatin; PRA, pravastatin; AMB, amphotericin B; NYT, nystatin; KTC, ketoconazole; ITC, itraconazole; FLC, fluconazole; TRB, terbinafine; GRS, griseofulvin; PN, primycin.

[a,b]The MICs of the statins and the antifungals are shown in brackets. [c]The effective concentrations of the combined drugs causing total growth inhibition are presented; the first number indicates the concentration of the given statin, and the second number indicates the concentration of the given antifungal agent. [d]The type of the interaction (I, indifferent; S, synergistic) as inferred from the interaction ratio (IR) values calculated with the Abbott formula are presented. [e]The type of the interaction (I, indifferent; S, synergistic) as inferred from fractional inhibitory concentration index (FICI) are presented. [f]FICI could not be precisely calculated, when the MIC of the given statin or the given antifungal agent could not be determined, but FICI was presumed in the calculated range.

80% inhibition). However, in Table 2 the lowest concentrations of drugs that produced total (above 90%) growth inhibition were presented to make it easier to compare the MIC values of the drug combinations.

Among the antifungals, terbinafine and itraconazole had the strongest inhibitory effect completely blocking the growth of all tested isolates at low concentration (0.016–0.125 μg/ml). Griseofulvin and ketoconazole were also active at low concentrations in that their MIC values ranged from 0.125–1.0 μg/ml and from 0.06–2 μg/ml, respectively. Amphotericin B, nystatin and primycin were less effective as growth inhibition was only observed at higher concentrations (1–4 μg/ml, 4–8 μg/ml and 16–32 μg/ml, respectively). Fluconazole did not equally inhibit the development of the investigated strains as the growth of *T. rubrum* and *M. canis* was inhibited at low concentration (1–2 μg/ml and 0.125–0.5 μg/ml, respectively), but the antifungal had no effect on the other two isolates at the administered concentrations.

Interactions between statins and antifungals

The *in vitro* interactions between the statins and the antifungals against dermatophyte fungi were also studied using a standard chequerboard broth microdilution method. We investigated the inhibition of fungal growth through the use of pairs of drugs, in order to find effective drug combinations. All investigated statins were tested in combination with all investigated antifungal compounds, and positive interactions were observed in most situations. The data for those drug combinations, which resulted synergistic interactions are presented in Table 2. The interaction ratio (IR) between the statins and the antifungal agents was calculated using the Abbott formula, as well as the fractional inhibitory concentration index (FICI) [29,30]. In Table 2 IR values calculated with both methods and the types of the interactions (according to the calculated IR) are also given, since the IR values calculated with the two methods reflects different interactions in some cases.

Synergistic interactions were observed in several cases, most of them occurring when statins were combined with terbinafine and the different azoles. Ketoconazole and fluconazole combined with most of the statins showed synergistic activity against *T. rubrum* and *M. gypseum*, and the concentrations needed to total growth-inhibition could be decreased by several dilutions. The ketoconazole-simvastatin combination was stronger than other combinations since it was synergistic against all of the investigated isolates. In contrast, the combination of ketoconazole and fluconazole with other statins had no effect against *T. mentagrophytes* and *M. canis*. Terbinafine also acted synergistically with statins against *T. mentagrophytes* besides *T. rubrum* and *M. gypseum*. Maximum inhibition was achieved with terbinafine-simvastatin combination since it exerted synergistic effect against all found dermatophytes. Itraconazole-simvastatin and itraconazole-atorvastatin acted synergistically against only *T. rubrum* and *M. gypseum*. While itraconazole-rosuvastatin combination was synergistic against *M. gypseum*, itraconazole-pravastatin had a synergistic *in vitro* antifungal effect against *M. canis*. Interactions between statins and the other antifungals were noted only in some cases. Amphotericin B-simvastatin combination was synergistic against *Microsporum* species, while amphotericin B acted synergistically against *T. rubrum* in combination with pravastatin. Similarly, nystatin-simvastatin combination was synergistic against *Microsporum* species but nystatin acted synergistically in combination with rosuvastatin against only *M. gypseum*. Since griseofulvin caused complete growth inhibition of dermatophytes at low concentration (0.125–1 μg/ml), improved results could not be expected in combination with statins. The only exception was the synergistic interactions against *M. gypseum* when griseofulvin was combined with simvastatin and fluvastatin. The interactions were mostly indifferent between primycin and statins, the only synergistic interaction was detected between primycin and simvastatin at *T. rubrum*.

Although synergistic interactions were observed with every investigated strain, the majority were found with *T. rubrum* and *M. gypseum*. While each statin could form synergistic interactions with antifungals, the most frequent interactions were observed with simvastatin. Dermatophyte fungi were completely insensitive to pravastatin, even so its combination with amphotericin B was synergistic against *T. rubrum*, and its combination with itraconazole was synergistic against *M. canis*.

Discussion

Antifungal activities of statins against dermatophytes has not been previously demonstrated *in vitro* but the evaluation of the data from the present investigations clearly suggest that these fungi are susceptible to these drugs. This may be a class effect of statins as antimicrobial activity has been demonstrated for fluvastatin, atorvastatin, rosuvastatin, lovastatin and simvastatin, a collection of synthetic and fungal derived drugs. However, pravastatin was found to be completely ineffective in this and previous studies. At the same time, pravastatin displayed antifungal activity *in vitro* against *C. albicans* when YM broth rather than RPMI was used as the test medium [32].

Since we used the M38-A2 *in vitro* test protocol of the CLSI, RPMI 1640 was employed in our investigations [26]. However, some modifications were made as for example the microtiter plates were incubated at 30°C instead of the recommended 35°C. Since the MIC values were the same at both temperatures, we selected 30°C as it is the optimal growth temperature of the slower growing dermatophyte fungi [28,33]. The inoculum was also slightly different since we used microconidia without any hyphal elements prepared using the modified method of Santos et al. [28]. The growth of fungi from microconidial inocula was homogeneous which allowed for easier interpretation of results and improved reproducibility of the tests.

In our study, substantial differences were observed among statins relative to their antifungal properties with fluvastatin and simvastatin were the most effective, followed by lovastatin, rosuvastatin, atorvastatin and pravastatin. A similar phenomenon was demonstrated in our previous work where the antifungal effects of statins were investigated *in vitro* against different yeasts and filamentous fungi [16]. In these tests, fluvastatin and simvastatin both displayed the strongest antifungal activity but, atorvastatin and rosuvastatin were found to be more effective than lovastatin. In other studies, statins also acted differently on the growth of fungi, their antifungal effects against different yeast, ascomycetes and zygomycetes have been comprehensively reviewed [34].

Ergosterol is an essential component of the fungal plasma membranes, and the inhibition of its synthesis negatively influences the membrane fluidity [9]. The antifungal effect of statins are due in part to their role on ergosterol levels, as well as to their indirect effect on cell signaling, proliferation and differentiation through inhibition of the synthesis of important terpenoids [35,36].

The antifungal activity of statins cannot be exploited in current clinical practice as the high concentrations required to achieve a reliable antimicrobial effect are well above the maximum achievable serum levels in humans [37,38]. At the same time, they should be promising agents in combination therapy as they can act synergistically with a number of clinically used antifungal agents, allowing substantial decreases in the latter therapeutic

concentrations [11,12,14–19]. Treatment of dermatophyte infection generally involves oral and/or topical formulations of the two main antifungal drug families, the azoles and the allylamines, particularly itraconazole and terbinafine [39]. Griseofulvin is also in use up to this day, mainly for the treatment of pediatric patients [24]. The introduction of new therapeutic agents is rare and restricted to those with a wide-action spectrum, topical agents with anti-inflammatory as well as antifungal actions, and use of combination of existing oral/topical antifungal agents [39]. In our study, the presently employed antifungals were combined with the lipid-lowering statins, and the numerous synergistic interactions noted during the study demonstrated that statins that were originally non-antifungal might be of use in combination therapy. In some cases, statins may act synergistically with antifungal drugs even if the statins are ineffective when they used alone [11,12]. The same phenomenon was observed in our study of the use of atorvastatin and pravastatin. Atorvastatin inhibited the growth of the investigated dermatophyte fungi only at the highest applied concentration, but it showed significant antifungal activity in combination with itraconazole, ketoconazole, fluconazole or terbinafine. The administration of statins together with azole antifungals that are predominantly metabolized by the same cytochrome P450 enzyme in the liver (CYP3A4) is substantially limited, because azoles reduce the metabolic clearance of statins, hereby the increased concentration of the co-administered statins in the serum may cause severe side-effects in the patients, such as myositis and rhabdomyolysis [37,40,41]. Griseofulvin co-administration with atorvastatin, and likely the whole class of similar drugs should be avoided [24]. These drug interactions could limit their systemic administration, but it should be noted that fluvastatin and pravastatin have a lower potential than other statins for such interactions. Fluvastatin is predominantly metabolized by the CYP2C9 isoenzyme, whereas pravastatin is excreted by the renal mechanism and does not undergo significant metabolism via the cytochrome P450 system [42,43]. Other statin-azole combinations may be applicable as topical therapy for dermatophyte infections, as serum absorption are supposed to be negligible with topical dermatophytosis therapy causing only mild and transient skin reactions at the application site [39].

In the present study, we detected synergistic interactions between statins and antifungal agents in many cases. The application of these combinations in the clinical practice for the prevention or treatment fungal infections require further studies, including *in vivo* animal experiments and prospective controlled trials in at-risk human populations, which can evaluate the practical efficiency of the statin-antifungals combinations.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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