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Original article

Synergistic antifungal activity of statin–azole associations as witnessed by *Saccharomyces cerevisiae*- and *Candida utilis*-bioassays and ergosterol quantification

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ABSTRACT

Background: Frequent opportunist fungal infections and the resistance to available antifungal drugs promoted the development of new alternatives for treatment, like antifungal drug combinations.
Aims: This work aimed to detect the antifungal synergism between statins and azoles by means of an agar-well diffusion bioassay with *Saccharomyces cerevisiae* ATCC 32051 and *Candida utilis* Pr_{1-2} as test strains.
Methods: Synergistic antifungal effects were tested by simultaneously adding a sub inhibitory concentration (SIC) of statin (atorvastatin, lovastatin, pravastatin, rosuvastatin or simvastatin) plus a minimal inhibitory concentration (MIC) of azole (clotrimazole, fluconazole, itraconazole, ketoconazole or miconazole) to yeast-embedded YNB agar plates, and a positive result corresponded to a yeast growth inhibition halo higher than that produced by the MIC of the azole alone. Yeast cell ergosterol quantification by RP-HPLC was used to confirm statin–azole synergism, and ergosterol rescue bioassays were performed for evaluating statin-induced ergosterol synthesis blockage.
Results: Growth inhibition was significantly increased when clotrimazole, fluconazole, itraconazole, ketoconazole and miconazole were combined with atorvastatin, lovastatin, rosuvastatin and simvastatin. Highest growth inhibition increments were observed on *S. cerevisiae* (77.5%) and *C. utilis* (43.2%) with a SIC of simvastatin plus a MIC of miconazole, *i.e.* 4 + 2.4 μg/ml or 20 + 4.8 μg/ml, respectively. Pravastatin showed almost no significant effects (0–7.6% inhibition increase). Highest interaction ratios between antifungal agents corresponded to simvastatin–miconazole combinations and were indicative of synergism. Synergism was also confirmed by the increased reduction in cellular ergosterol levels (*S. cerevisiae*, 40% and *C. utilis*, 22%). Statin-induced ergosterol synthesis blockage was corroborated by means of ergosterol rescue bioassays, pravastatin being the most easily abolished inhibition whilst rosuvastatin being the most ergosterol-refractory.
Conclusions: Selected statin–azole combinations might be viable alternatives for the therapeutic management of mycosis at lower administration doses or with a higher efficiency.

Actividad antifúngica sinérgica de combinaciones de estatinas y azólicos revelada mediante bioanálisis con *Saccharomyces cerevisiae* y *Candida utilis* y cuantificación de ergosterol

Palabras clave:
Estatinas
Azólico
Sinergismo
Levaduras oportunistas
Saccharomyces cerevisiae
Candida utilis

RESUMEN

Fundamento: La frecuencia de micosis oportunistas y la resistencia a los antimicóticos convencionales han fomentado la búsqueda de nuevas alternativas terapéuticas, como las combinaciones de antimicóticos.
Objetivos: El presente estudio trató de detectar el sinergismo antifúngico entre las estatinas y los azólicos mediante un bioanálisis de difusión en pocillos de agar, utilizando *Saccharomyces cerevisiae* (*S. cerevisiae*) ATCC 32051 y *Candida utilis* (*C. utilis*) PR_{1-2} como cepas de control.
Métodos: Los efectos antifúngicos sinérgicos se examinaron mediante la adición simultánea de una concentración sub-inhibitoria (CSI) de estatina (atorvastatina, lovastatina, pravastatina, rosuvastatina o simvastatina) más una concentración mínima inhibitoria (CMI) de un azólico (clotrimazol,

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fluconazol, itraconazol, ketoconazol o miconazol) a placas de agar YNB con las levaduras sembradas por inclusión. Un resultado positivo correspondió a un diámetro del halo de inhibición del crecimiento de la levadura mayor que el producido por la CMI del azólico exclusivo. Para confirmar el sinergismo estatina-azólico, se cuantificó el ergosterol de la membrana celular de las levaduras con cromatografía líquida de alto rendimiento (HPLC-RP). Para valorar la inhibición de la síntesis de ergosterol inducida por estatinas, se emplearon bioanálisis de rescate de ergosterol.

Resultados: La inhibición del crecimiento aumentó significativamente cuando se combinaron clotrimazol, fluconazol, itraconazol, ketoconazol y miconazol con atorvastatina, lovastatina, rosuvastatina y simvastatina. Los mayores incrementos de la inhibición del crecimiento se observaron en *S. cerevisiae* (77,5%) y *C. utilis* (43,2%) con una CSI de simvastatina y una CMI de miconazol de 4 + 2,4 μg/ml o 20 + 4,8 μg/ml, respectivamente. Para pravastatina apenas se identificaron efectos significativos (incremento de la inhibición del 0-7,6%). Los mayores cocientes de interacción correspondieron a la combinación de simvastatina y miconazol y fueron indicativos de sinergismo. Este también se confirmó por la mayor disminución de los niveles celulares de ergosterol (*S. cerevisiae*, 40% y *C. utilis*, 22%). La inhibición de la síntesis de ergosterol inducida por estatinas se corroboró mediante bioanálisis de rescate de ergosterol, donde la inhibición por pravastatina se abolió con facilidad, mientras que la de rosuvastatina fue la más refractaria.

Conclusiones: Las combinaciones seleccionadas de estatinas y azólicos podrían ser alternativas viables para el manejo terapéutico de las micosis, en dosis más bajas o con una mayor eficiencia.

The incidence of systemic mycoses has dramatically increased over last years, a fact particularly favoured by the raising prevalence of acquired immune deficiency syndrome (AIDS) and the unrestrained use of immunocompromising drugs.[9] Additionally, a number of different predisposing factors have been associated with the prevalence of infections caused by yeasts, even for immunocompetent patients.[21,25] As already emphasized, the administration of azoles for treating fungal infections has encountered certain limitations, such as their low water solubility, low bioavailability, and frequent side effects consequent on the requirement of high doses and/or long-term administration.[21,42]

Rare fungal species of low pathogenic potential, or even species never described before as a cause of disease, are being more commonly detected in hospital settings as etiological agents of infections.[39] Numerous cases of superficial or mild systemic infections caused by *Saccharomyces cerevisiae*, a yeast "generally regarded as safe" (GRAS) for industrial applications, have been up today reported, particularly in immunocompromised patients. So, safety status traditionally adjudicated to this microorganism has been upgraded to Biosafety level one in Europe.[20,26] In this way, although not as virulent as *C. albicans*, the classical identification of *S. cerevisiae* as a non-pathogenic yeast has changed to opportunistic pathogen. As a further complication, the resistance of *S. cerevisiae* to certain antifungal agents has been also repeatedly observed.[2,26,36,38]

On the other hand, most of the reported candidosis have *Candida albicans* as the causative agent.[4] However, although less frequently, the emergence of other species of the genus has also been described over last decades.[28,39] Among those unconventional opportunistic pathogen *Candida* species, *C. utilis*, a yeast commonly used with biotechnological purposes such as single-cell protein production, has been reported in opportunistic fungemia.[1,6,17]

Considering the increased incidence of opportunist fungal infections and the development of fungal drug resistances, a great deal of attention has been focused on the investigation of new alternatives for treatment.[9,14] Combination of antifungal agents with other drugs in order to improve the efficacy and/or decrease the toxicity has been described early as one of the possibilities.[21,29,33] In this context, studies on the interaction between azoles and statins have gained renewed attention.[10,11,27,32,37]

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the key enzyme which catalyzes the rate limiting step of sterols biosynthesis.[24] This inhibition affects fungal propagation by decreasing ergosterol levels and its precursors. Additionally, their ability to block the synthesis of intermediate products in the mevalonate pathway critically influences cellular functions, thus being regarded as apoptosis-inducing agents.[30,41] Yeast mitochondrial dysfunction and respiratory deficit have been also associated to statins.[40] On the other hand, the toxicity of azoles against fungi results from the inhibition of the cytochrome *P*450-dependent C-14 lanosterol α-demethylase.[18]

An advantage of the synergistic interaction between these two kinds of drugs would be the low hydrophobicity and toxicity of statins for humans, as compared with the azole-family drugs. In this study, the *in vitro* activity of five azoles (clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole) and five statins (atorvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin), either alone or in combinations, was tested against the low-virulent opportunistic pathogen yeasts *S. cerevisiae* and *C. utilis* by means of an agar-well diffusion bioassay in order to detect possible synergistic effects and to identify the most promising combinations.

Materials and methods

Antifungal agents

Clotrimazole, fluconazole, ketoconazole and miconazole nitrate were purchased from Parafarm Laboratories (Argentina), and itraconazole was purchased from Sigma Chemical Co. (St Louis, MO, USA). Drugs were obtained as powders and stock solutions were prepared at a concentration dependent on the potency of each tested drug. Commercial statins (10 mg per tablet) of: Liparex® (atorvastatin), Mevlor® (lovastatin), Pravacol® (pravastatin), Crestor® (rosuvastatin) and Tanavat® (simvastatin) were used to prepare standard-stock solutions. In order to obtain the active β-hydroxyacid form of statins, commercially provided in the lactone inactive form, a preliminary conversion was carried out as previously described.[8,31] Subsequently, purified statins in the β-hydroxyacid form were extracted with HPLC-grade ethyl acetate and quantification by reversed-phase HPLC (RP-HPLC) was performed as previously described,[8] in order to confirm final concentration.

Yeast strains

The strains *S. cerevisiae* ATCC 32051 (American Type Culture Collection), and *Candida utilis* Pr_{1-2} (PROIMI-MIRCEN Culture Collection, Tucumán, Argentina), were systematically used for bioassays.