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Antifungal activity of compounds targeting the Hsp90-calcineurin pathway against various mould species

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Objectives: Invasive mould infections are associated with a high mortality rate and the emergence of MDR moulds is of particular concern. Calcineurin and its chaperone, the heat shock protein 90 (Hsp90), represent an important pathway for fungal virulence that can be targeted at different levels. We investigated the antifungal activity of compounds directly or indirectly targeting the Hsp90-calcineurin axis against different mould species.

Methods: The *in vitro* antifungal activity of the anticalcineurin drug FK506 (tacrolimus), the Hsp90 inhibitor geldanamycin, the lysine deacetylase inhibitor trichostatin A and the Hsp70 inhibitor pifithrin- μ was assessed by the standard broth dilution method against 62 clinical isolates of *Aspergillus* spp. and non-*Aspergillus* moulds (*Mucoromycotina*, *Fusarium* spp., *Scedosporium* spp., *Purpureocillium/Paecilomyces* spp. and *Scopulariopsis* spp.)

Results: FK506 had variable antifungal activity against different *Aspergillus* spp. and was particularly active against *Mucor* spp. Geldanamycin had moderate antifungal activity against *Fusarium* spp. and *Paecilomyces variotii*. Importantly, trichostatin A had good activity against the triazole-resistant *Aspergillus ustus* and the amphotericin B-resistant *Aspergillus terreus* as well as the MDR *Scedosporium prolificans*. Moreover, trichostatin A exhibited synergistic interactions with caspofungin against *A. ustus* and with geldanamycin against *Rhizopus* spp. for which none of the other agents showed activity. Pifithrin- μ exhibited little antifungal activity.

Conclusions: Targeting the Hsp90-calcineurin axis at different levels resulted in distinct patterns of susceptibility among different fungal species. Lysine deacetylase inhibition may represent a promising novel antifungal strategy against emerging resistant moulds.

Keywords: tacrolimus, geldanamycin, trichostatin A, pifithrin- μ , heat shock protein 90, heat shock protein 70, histone deacetylase inhibitors

Introduction

Invasive fungal infections (IFIs) are associated with a high mortality rate in patients with haematological malignancies and solid organ transplant recipients.^{1,2} Among filamentous fungi, *Aspergillus fumigatus* is the most frequent cause of IFI, followed by other *Aspergillus* spp. However, as the spectrum of immunosuppressed patients is increasing, other moulds have emerged as opportunistic pathogens.³ *Mucoromycotina* and *Fusarium* spp. account for 5%–10% of IFI cases and are associated with a particularly high mortality rate.^{1,2} *Scedosporium* spp., *Paecilomyces* spp. and *Scopulariopsis* spp. are now well-recognized causes of IFI.^{4–6} Many of these fungal species are less susceptible to conventional antifungal drugs and

some species (e.g. *Scedosporium prolificans* and *Scopulariopsis* spp.) are resistant to multiple current antifungal classes. Among *Aspergillus* spp., *Aspergillus terreus* is intrinsically resistant to amphotericin B, while *Aspergillus ustus* is resistant to triazoles and was associated with breakthrough IFI in patients receiving antifungal prophylaxis.⁷ Alternatives to azole therapy are limited by their toxicity (polyenes) or lack of fungicidal activity (echinocandins). There is an urgent need for novel antifungal drug classes against these recalcitrant moulds.

Heat shock protein 90 (Hsp90) is essential for fungal survival and controls the activity of its client protein calcineurin in stress responses and cell wall repair mechanisms.^{8,9} Another molecular chaperone, Hsp70, is involved in the transfer of client proteins to

Table 1. Antifungal activity of FK506, geldanamycin, trichostatin A and pifithrin- μ against *Aspergillus* and non-*Aspergillus* moulds

Genus/species (n)	MEC ₅₀ (mg/L) (range) ^a			
	FK506	geldanamycin	trichostatin A	pifithrin- μ
<i>A. fumigatus</i> (6) ^b	0.025 (0.025–0.4)	16 (16 to >16)	8 (8–16)	>16
<i>A. flavus</i> (4)	0.1 (0.1–0.2)	>16	>16 (8 to >16)	>16
<i>A. terreus</i> (4)	0.025 (0.025–0.1)	8 (8–16)	2	>16
<i>A. niger</i> (2)	0.006–0.012	16 to >16	2	>16
<i>A. ustus</i> (3)	>0.4	>16	2 (2–4)	>16
<i>A. versicolor</i> (1)	>0.4	>16	2	>16
<i>Rhizopus</i> spp. (5)	>0.4 (0.1 to >0.4)	>16	16 (16 to >16)	>16
<i>Mucor</i> spp. (4)	0.025 (0.012 to >0.4)	16 (4 to >16)	>16	>16
<i>Rhizomucor</i> spp. (2)	0.012–0.025	8 to >16	>16	>16
<i>Lichtheimia</i> spp. (2)	0.2 to >0.4	>16	>16	>16
<i>Cunninghamella</i> spp. (2)	0.05–0.2	16 to >16	8 to >16	>16
<i>Fusarium</i> spp. (6)	>0.4	4 (2–16)	>16	>16
<i>S. apiospermum</i> (4)	0.05 (0.05 to >0.4)	>16	8 (2 to >16)	8 (8–16)
<i>S. prolificans</i> (4)	>0.4	>16	2 (1–8)	16 (16 to >16)
<i>P. lilacinus</i> (5)	>0.4	>16	8 (4–16)	>16
<i>P. variotii</i> (4) ^b	0.025 (0.025 to >0.4)	2 (2 to >16)	2 (0.5–16)	16 (16 to >16)
<i>Scopulariopsis</i> spp. (4)	0.2 (0.05 to >0.4)	>16 (4 to >16)	8 (2 to >16)	>16 (16 to >16)

^aThe MEC₅₀ is the concentration achieving $\geq 50\%$ growth inhibition in $\geq 50\%$ of tested isolates.

^bIncluding one ATCC strain.

Hsp90 for their subsequent activation via the Hsp90 chaperone cycle.¹⁰

We have previously shown that targeting the Hsp90-calcineurin pathway may represent a novel antifungal strategy.⁹ FK506 (tacrolimus) is a calcineurin inhibitor commonly used as immunosuppressive therapy. The Hsp90 inhibitor geldanamycin and its derivatives 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) are being studied for anticancer therapy.¹¹ Inhibitors of the histone deacetylases (also referred to as lysine deacetylases), such as trichostatin A, cripple Hsp90 function by inducing Hsp90 acetylation.¹² These compounds display some degree of *in vitro* antifungal activity against *A. fumigatus*,^{12,13} but their activity against other moulds is unknown. Pifithrin- μ (2-phenylethanesulfonamide) inhibits Hsp70 by binding to its C-terminal portion that interacts with Hsp90.¹⁴ The antifungal activity of pifithrin- μ is unknown. In this study, we tested the *in vitro* antifungal activity of these compounds against a wide collection of clinically relevant pathogenic moulds.

Materials and methods

FK506 (Astellas Pharma US, Deerfield, IL, USA), geldanamycin (Selleck Chemicals, Houston, TX, USA), trichostatin A (Selleck Chemicals) and pifithrin- μ (Enzo Life Sciences, Farmingdale, NY, USA) were tested against 2 ATCC strains (*A. fumigatus* MYA 3626 and *Paecilomyces variotii* MYA 3630) and 60 selected clinical isolates collected at Duke University Medical Center (Durham, NC, USA) between 2009 and 2013, including *A. fumigatus* (5), *Aspergillus flavus* (4), *A. terreus* (4), *A. ustus* (3), *Aspergillus niger* (2), *Aspergillus versicolor* (1), *Purpureocillium lilacinus* (formerly *Paecilomyces lilacinus*) (5), *Paecilomyces variotii* (3), *Scopulariopsis* spp. (4), *Rhizopus* spp. (5), *Mucor* spp. (4), *Rhizomucor* spp. (2), *Lichtheimia* spp. (formerly *Absidia* spp.) (2), *Cunninghamella* spp. (2),

Fusarium spp. (6), *Scedosporium apiospermum* (4) and *S. prolificans* (4). The study was approved by the Duke Hospital Institutional Review Board.

Microbroth dilution antifungal susceptibility testing was performed according to the CLSI procedure.¹⁵ Interpretation of results was performed after incubation at 35°C for 24 h (48 h for *S. apiospermum* and *Scopulariopsis* spp.). Based on preliminary studies in *A. fumigatus*,^{12,13} the range of concentrations tested was 0.001–0.4 mg/L for FK506 and 0.015–16 mg/L for geldanamycin, trichostatin A and pifithrin- μ . Because of the fungistatic effect of these compounds, we did not assess the MIC, but the minimal effective concentration (MEC), defined as the concentration at which $\geq 50\%$ growth inhibition was observed by macroscopic and microscopic evaluation.¹⁵ Based on previous results reporting synergism between these inhibitors and standard antifungals,^{12,13,16,17} drug interactions were assessed for some drug combinations on selected strains by chequerboard dilution and evaluated by the fractional inhibitory concentration index (FICI).¹⁸

Results

Among *Aspergillus* spp., susceptibility to FK506 was variable (Table 1). Most *A. fumigatus* and *A. terreus* isolates displayed MECs of 0.025–0.05 mg/L. FK506 was particularly active against *A. niger* (0.006–0.012 mg/L), reaching >90% growth inhibition (Figure S1A, available as Supplementary data at JAC Online). However, *A. flavus* isolates tended to have higher MECs (0.1–0.2 mg/L) and no significant effect of FK506 was observed against *A. ustus* or *A. versicolor*. Trichostatin A had little activity against *A. fumigatus* and *A. flavus* isolates, but had better activity against *A. niger*, *A. terreus*, *A. versicolor* and *A. ustus* (MEC 2 mg/L for 90% of these isolates; Figure S1D). Geldanamycin had little activity and pifithrin- μ was inactive against *Aspergillus* spp.

FK506, geldanamycin, trichostatin A and pifithrin- μ displayed distinct and species-specific patterns of antifungal activity against

non-*Aspergillus* moulds (Table 1). All these compounds had no or very little activity against Mucoromycotina, with the exception of FK506, which had good activity against species of *Mucor* and *Rhizomucor* (Figure S1B). FK506 had variable activity against *P. variotii*, *S. apiospermum* and *Scopulariopsis* spp. Geldanamycin was the only compound with significant activity against *Fusarium* spp. (Figure S1A) and was also active against *P. variotii*. Trichostatin A was active against all four *S. prolificans* isolates, with an MEC of ≤ 2 mg/L for three of them (Figure S1A). Trichostatin A also had some activity against other non-*Aspergillus* moulds with the exception of Mucoromycotina and *Fusarium* spp. Pifithrin- μ had little antifungal activity against non-*Aspergillus* moulds.

Drug combinations were tested against selected species that are known to be less susceptible to conventional antifungal drugs. A synergistic effect between trichostatin A and caspofungin was observed against *A. ustus* (FICI=0.5) (Figure S1D). However, caspofungin did not increase the effect of trichostatin A against *S. prolificans*. Neither caspofungin nor amphotericin B was able to potentiate the antifungal activity of geldanamycin against *Fusarium* spp. Various combinations of the new inhibitors were tested against *Rhizopus* spp. for which all single compounds were inactive. A synergistic effect was observed between trichostatin A and geldanamycin (Figure S1B). This combination did not have any positive interaction against other moulds including other Mucoromycotina. While FK506 was highly active against *Mucor* spp., the addition of low levels of posaconazole or voriconazole resulted in a decreased effect of FK506 (Figure S1C).

Discussion

We assessed the *in vitro* antifungal activity of various compounds targeting the Hsp90-calcineurin pathway against a large set of clinically significant *Aspergillus* and non-*Aspergillus* moulds and found distinct species-dependent patterns of antifungal activity.

Most *Mucor* and *Rhizomucor* spp. were highly susceptible to FK506, while *Rhizopus* and *Lichtheimia* spp. were not. This distinct effect of FK506 among Mucoromycotina might be related to some differences in the number of genes encoding calcineurin pathway components.¹⁹ While data with regard to the type of interaction between FK506 and triazoles against *Mucor* spp. remain controversial,^{17,20} we observed that the addition of voriconazole or posaconazole at concentrations corresponding to those achieved in plasma with therapeutic or prophylactic dosing (0.25–1 mg/L) decreased the antifungal activity of FK506. We hypothesize that this negative interaction might be due to the stress response induced by triazoles resulting in increased calcineurin expression by the fungus. This finding would deserve further investigation as mucormycosis is a well-known breakthrough IFI among patients receiving azole prophylaxis. Geldanamycin had significant activity only against *Fusarium* spp. and *P. variotii*. While Hsp90 is an essential gene, its basal level of expression may be much higher than required for growth and survival,⁹ which may explain the limited activity of geldanamycin against most fungal species. Trichostatin A displayed variable antifungal activity and, notably, was particularly active against some resistant mould species, such as the triazole-resistant *A. ustus*, the amphotericin B-resistant *A. terreus* and the MDR *S. prolificans*. As was previously reported for *A. fumigatus*,¹² trichostatin A exhibited synergistic interactions with caspofungin against *A. ustus*. Moreover, the combination of trichostatin A and geldanamycin was synergistic against *Rhizopus* spp.

Because Hsp90 and calcineurin are highly conserved in eukaryotes, fungus-specific inhibition of these targets is difficult to achieve. FK506 has immunosuppressive effects in humans. However, some fungus-specific key regions of calcineurin have been recently identified in *A. fumigatus* and might represent novel antifungal targets.²¹ Hsp90 inhibitors are considered for anticancer therapy,¹¹ but their antifungal effect is reached at concentrations that are above their toxic threshold. Trichostatin A appears to be an interesting candidate as an alternative antifungal therapy because of its good activity at relatively low concentrations (1–2 mg/L) against moulds that are resistant to the first-line antifungal classes such as the azoles and polyenes and because of its potential synergism with echinocandins against *Aspergillus* spp. Trichostatin A was well tolerated in mice, but its very short half-life constitutes a major limitation.²² However, more stable trichostatin A analogues with increased half-lives have been obtained in cancer research (vorinostat and panobinostat). Some of these compounds have been recently approved for anticancer therapy or are under clinical investigation.²³ Thus, lysine deacetylase inhibitors, via indirect inhibition of the Hsp90-calcineurin axis, represent a promising novel antifungal drug class to improve the consistently poor outcomes of IFI due to emerging MDR fungal pathogens.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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