ORIGINAL ARTICLE MYCOLOGY

In vitro and *in vivo* role of heat shock protein 90 in Amphotericin B resistance of *Aspergillus terreus*

G. Blum[1]*, B. Kainzner[1]*, K. Grif[1], H. Dietrich[2], B. Zelger[3], T. Sonnweber[4] and C. Lass-Flörl[1]

1) *Division of Hygiene and Medical Microbiology, Innsbruck Medical University,* 2) *Innsbruck Central Animal Experiment Station,* 3) *Department of Pathology, Innsbruck Medical University and* 4) *Department of Internal Medicine I, Innsbruck Medical University, Innsbruck, Austria*

Abstract

Aspergillus terreus (*A. terreus*) is of serious concern because of a high propensity to dissemination and *in vitro* and *in vivo* resistance to Amphotericin B (AmB). The underlying molecular mechanism of AmB is not known yet and here we want to explore whether fungal heat shock protein 90 (HSP90) is involved in polyene resistance in *A. terreus*. AmB-susceptible (ATS) and AmB-resistant (ATR) *A. terreus* and AmB-susceptible *Aspergillus fumigatus* (AFS) were investigated in response to AmB with a special focus on HSP90. HSP90 inhibitors resulted in significant improvement of AmB activity against ATR as minimum inhibitory concentrations (MIC) decreased from 32 to 0.38 mg/L. Gene expression profiling showed a greater basal amount of HSP90 levels in ATR and ATS when compared with AFS. HSP90 blockers in combination with AmB were evaluated in a murine model of disseminated aspergillosis. HSP90 inhibitors were not beneficial for mice infected with ATR, and neither mono- nor combination treatment with AmB yielded clinical improvement. HSP90 inhibition with 17-allylamino-17-demethoxygeldanamycin (17-AAG) was harmful. HSP90 seems to play a vital role in antifungal stress response in all aspergilli tested, whereas HSP90 does not substantiate the origin of AmB resistance in ATR.

Keywords: Amphotericin B, antifungal drug resistance, *Aspergillus terreus*, heat shock protein 90, *in vitro* antifungal susceptibility
Original Submission: 28 November 2011; **Revised Submission:** 22 February 2012; **Accepted Submission:** 14 March 2012
Editor: E. Roilides
Article published online: 21 March 2012
Clin Microbiol Infect 2013; **19:** 50–55
10.1111/j.1469-0691.2012.03848.x

Corresponding author: C. Lass-Flörl, MD, Innsbruck Medical University, Division of Hygiene and Medical Microbiology, Fritz Pregl Str. 3/3, A-6020 Innsbruck, Tirol, Austria
E-mail: cornelia.lass-floerl@i-med.ac.at
*Both authors contributed equally to this work.

Introduction

Invasive fungal infections (IFIs) are an important cause of morbidity and mortality in patients with underlying risk factors (e.g. neutropenia, cancer chemotherapy or AIDS) [1,2]. There are a wide variety of pathogens associated with IFIs, yet the predominant players are *Candida* and *Aspergillus* species [3]. The most frequently isolated mould from clinical specimens is *Aspergillus fumigatus*, but *Aspergillus terreus* appears to be an emerging cause of infection at some institutions (e.g. the Medical University Hospital of Innsbruck, Austria, and the M. D. Anderson Cancer Center in Houston, Texas, USA) [4,5] and an endemic appearance of *A. terreus* is shown [6]. *A. terreus* infections are of serious concern because most isolates exhibit *in vivo* and *in vitro* resistance (MIC ≥2 mg/L) to Amphotericin B (AmB) [7,8]. The reason for this resistance is not known yet and the exact mode of AmB action is still incompletely understood [9–11]. Recent studies identified HSP90 as a regulator for emergence of fungal drug resistance of two leading fungal pathogens, *Candida albicans* and *A. fumigatus* [12,13]. The molecular chaperone HSP90 assists in protein folding, cell signalling, and tumour repression. HSP90 is one of the most represented proteins in the cytosol of eukaryotes as it makes up 1–2% of the complete protein level, also without the influence of stress. Cellular stress can be induced by nutrient deprivation, changed temperature or pH and exposure to various toxins and drugs [14,15]. Another study demonstrated that Mycograb®, a single-chain variable fragment genetically recombinant monoclonal antibody, which acts against the fungal molecular chaperone HSP90, increased AmB efficacy *in vivo* [16]. Biamonte *et al.* [17] showed that pharmacological HSP90 inhibition leads to a degradation of oncogenic proteins and

that HSP90 inhibitors can be used in the treatment of a wide range of diseases [15,17,18], including fungal [12,14,19] and parasitic pathogens [20], both in humans and animals.

In light of these observations, we investigated the *in vivo* and *in vitro* role of HSP90 in polyene resistance in *A. terreus*.

Materials and Methods

Fungal strains

All *Aspergillus* spp. isolates (species identification via sequencing of ITS3-ITS4, calmodulin, β-tubulin, enolase and cytochrom B) used in this study were derived from clinical specimens. AFS showed AmB MIC of ≤1.0 mg/L, ATS of ≤0.5 mg/L and ATR of ≥32 mg/L.

Susceptibility testing

AmB MIC were determined by Etest® (AB BIODISC, Vienna, Austria) on defined agar plates (RPMI-1640 + MOPS + 2% glucose + 1.5% Agar granulated) performed according to the manufacturer's instructions with an inoculum of $1–4 \times 10^6$ colony forming units (CFU) per mL. HSP90 was blocked with geldanamycin (InVivoGen, Nottingham, UK), 17-AAG (Sigma, Vienna, Austria), tacrolimus FK506 (Sigma) and cyclosporin A (Sandimmun, Novartis, Vienna, Austria). Preparation of each blocking solution was carried out according to the manufacturer's recommendations and added to the chilled RPMI agar. For each substance concentrations of 5, 10 and 20 μM were used.

HSP90 gene expression by northern blotting

To observe the fungal HSP90 biology we determined gene expression of HSP90 in ATR, ATS and AFS. Conidial suspensions (final range of 1×10^5 CFU/mL) were grown in Sabouraud medium (Merck, Vienna, Austria) for 24 h (37°C) with slight shaking and fungi were treated with AmB (Bristol-Myers Squibb, Vienna, Austria) at sub-lethal (S) and lethal (L) concentrations for 15 min. S and L concentrations were 2 mg/L (S) and 5 mg/L (L) and 0.1 mg/L (S) and 1 mg/L (L) for ATR and ATS, and 0.25 mg/L (S) and 1 mg/L (L) for AFS, respectively. Northern blotting was carried out according to a previous protocol [21]. Hybridization probes were generated by PCR using oligonucleotides for the HSP90 gene 5′-TCATGATACGCTCCATGTTG-3′—forward and 5′-GAGGAGCTCAACAAGACCAA-3′—reverse for *A. terreus* and 5′-CAT CTC CTA CCC CAT CTA CC-3′—forward and 5′-CTG TCA AGG AAG GGA GAC TT-3′—reverse for *A. fumigatus*.

HSP90 gene expression by real-time PCR

Isolates (1×10^5 CFU/mL, 24 h at 37°C with slight shaking) were treated with sub-lethal and lethal AmB concentrations for 15 min. RNA was isolated by use of TRI reagent (Sigma) [21]. For cDNA preparation 4 μg of RNA were transcribed using the Superscript-III-RT-Kit (Invitrogen, Vienna, Austria). Real-time PCR experiments were performed on a Light-Cycler 2.0 (Roche) via Light-Cycler Software 4.1. SensiMix™Lite-Kit (Quantace, London, UK) was used for all reactions. Each real-time PCR was carried out in 20-μL glass capillaries (Roche, Vienna, Austria) containing 4 μL SensiMix Lite, 1.5 μL Enzyme Mix, 0.4 μL 50× SYBR Green I solution, 0.8 μL of each corresponding primer (10 μM), 7.5 μL PCR grade water and 5 μL cDNA (80 ng). *A. terreus* primers were designed using HSP90 (GenBank accession no. XM_001216617) and beta-tubulin (*BTU*) (GenBank accession no. NT_165972) genes. For *A. fumigatus* HSP90 (GenBank accession no. XM_742833) and beta-tubulin (*BTU*) (GenBank accession no. AY048754) genes were used. Relative expression was evaluated as previously described [22]. The ratio of the target gene is expressed vs. the control in comparison with the reference gene.

In vivo combination of AmB and HSP90 blocker

The *in vivo* effect of AmB treatment in combination with HSP90 blockers was investigated with a murine model including 54 female BALB/c-mice (groups A to I; six mice per group; weight 23–28 g; age 14–16 weeks). Mice in groups A-H received intravenous cyclophosphamid (200 mg/kg), mice in group I did not receive immunosuppression. Animals in group A to G were infected intravenously (100 μL) with 2×10^6 CFU of ATR [23]. Mice were treated daily (days 1–5) with the corresponding therapies; group A received 2 mg/kg/day AmB, group B 2 mg/kg/day AmB plus 20 mg/kg/day 17-AAG (less toxic geldanamycin analogue, Invivo Gen), group C 2 mg/kg/day AmB plus 60 mg/kg/day 17-AAG, group D 60 mg/kg/day 17-AAG, group E 2 mg/kg/day AmB plus 50 mg/kg/day cyclosporin A (Sandimmun) and group F 50 mg/kg/day cyclosporin A. Mice in group G, which were immunosuppressed and infected daily received phosphate buffered saline (PBS). Animals in group H (immunosuppressed, not infected) received PBS daily and mice in group I, which were not immunosuppressed and not infected, did not obtain any treatment. High and low level treatment of 17-AAG was investigated [24]. Each substance was prepared according to the manufacturer's recommendations. Mice were observed daily and evaluated by assigning body weight and clinical signs of infection (e.g. scrubby coat, head misalignment, excessive loss of weight and banded back). On day six, surviving animals were sacrificed and several organs were removed for further analyses (histopathological analysis and fungal burden in lungs). Animal studies were in compliance/accordance with the Austrian animal protection law and guidelines for scientific purposes.

Quantitative *Aspergillus* real-time PCR of mouse lungs

ATR burden in lungs was examined by quantitative real-time PCR assay on a Light Cycler 2.0 (Roche) using the real-time quantitative PCR settings on Light Cycler Software Version 4.1. DNA isolation and real-time PCR were carried out as previously described [25,26].

Statistical analyses

All *in vitro* assays were performed on three independent occasions. Statistical analyses were performed using Student's two-tailed *t*-test with values of $p < 0.05$ considered statistically significant.

Results

In vitro combination of HSP90 blockers and AmB

AmB MIC without any HSP90 blocker was ≥32 mg/L for ATR, and ≤1 mg/L for ATS and AFS. In ATR, HSP90 blocking with geldanamycin (5 and 10 μM) decreased MIC to <1 mg/L (Table 1). Cyclosporin A and tacrolimus FK506 also reduced MIC in ATR, but not as strongly as geldanamycin. Similar data were obtained in ATS and AFS. Geldanamycin analog 17-AAG, a less toxic substance used in our mouse model, showed comparable effects to geldanamycin.

Northern blot and real-time PCR analyses of HSP90 after AmB treatment

Gene expression profiling by northern blot (Fig. 1a) and real-time PCR (Fig. 1b) analyses revealed expression of HSP90 in ATR, ATS and AFS with different basal expression levels. Compared with AFS, a higher amount of HSP90 was detected in ATR and ATS. AFS showed a slight HSP90 up-regulation after lethal and sub-lethal AmB treatment; in ATR and ATS, HSP90 gene expression was not up-regulated after lethal AmB treatment.

TABLE 1. ***In vitro*** **effects of various HSP90 blockers on Amphotericin B efficacy**

	Amphotericin B MIC (mg/L) (48 h, Etest)		
HSP90 blockers	**Amphotericin B-resistant *A. terreus***	**Amphotericin B-susceptible *A. terreus***	**Amphotericin B-susceptible *A. fumigatus***
No blocker (control)	>32	0.25	1.0
Geldanamycin (5 μM)	0.75	0.125	0.19
Geldanamycin (10 μM)	0.38	0.003	0.125
Geldanamycin (20 μM)	No growth	No growth	No growth
17-AAG (5 μM)	1.0	0.125	0.19
17-AAG (10 μM)	0.5	0.008	0.19
17-AAG (20 μM)	No growth	No growth	No growth
Cyclosporin A (5 μM)	3.00	0.19	0.19
Cyclosporin A (10 μM)	1.00	0.125	0.19
Cyclosporin A (20 μM)	0.5	0.047	No growth
FK506 (5 μM)	4.00	0.125	0.25
FK506 (10 μM)	1.00	0.125	0.19
FK506 (20 μM)	No growth	No growth	0.094

Tests were performed three times in duplicates and showed similar results.
17-AAG, 17-allylamino-17-demethoxygeldanamycin.

In vivo combination of HSP90 blockers and AmB

Overall, survival was significantly better in all groups with no 17-AAG, as seen in Fig. 2 ($p < 0.05$). Survival was 100% in group A (AmB), group E (AmB and cyclosporin A) and group F (cyclosporin A), as well as in control group G (ATR and PBS), H (no infection and PBS) and I (control). Survival in group B (AmB and 20 mg/kg 17-AAG), group C (AmB and 60 mg/kg 17-AAG) and group D (60 mg/kg 17-AAG) was 83.3%, 66.7% and 16.7%, respectively. Mice infected with ATR showed clinical abnormalities such as scrubby coat, as well as excessive loss of weight and head misalignment. Quantitative PCR was applied to measure *A. terreus* load in lungs (Fig. 3). Groups B and F showed the lowest fungal CFU, whereas all other groups had comparable CFU amounts. Fungal dissemination was identified in all ATR infected groups via histopathological analyses (data not shown).

Discussion

Our *in vitro* data showed that harnessing HSP90 function increased AmB activity in *A. terreus*. Cowen *et al.* [12,13] demonstrated that HSP90 plays an important role in antifungal drug resistance. Compromising HSP90 function in *C. albicans* reduces resistance to azoles and blocks de novo emergence of resistance. The same effects were also noticed in *A. fumigatus* for echinocandin resistance. Cowen *et al.* [13,27] also showed that blocking the HSP90 client protein calcineurin with cyclosporin A and FK506 reduced fungal drug resistance. The immunosuppressant geldanamycin is now in clinical development as an anticancer agent and it binds directly to the ATP-binding pocket of HSP90, thus altering its function [18]. In our study, geldanamycin decreased AMB MIC of ATR from >32 mg/L to <1 mg/L. Similar, yet slightly reduced, effects were observed for cyclosporin A and FK506 in ATR, ATS and AFS. Northern blotting demonstrated an approximately equal basal amount of HSP90 gene expression in ATR and ATS. No significant up-regulation in response to AmB in comparison to untreated controls was detected in both isolates. This higher basal HSP90 equipment in *A. terreus* makes this species probably more stable against different environmental and other exogenous stress exposures. Thus, northern blotting and real-time PCR showed marginal up-regulation of equal genes in AFS,

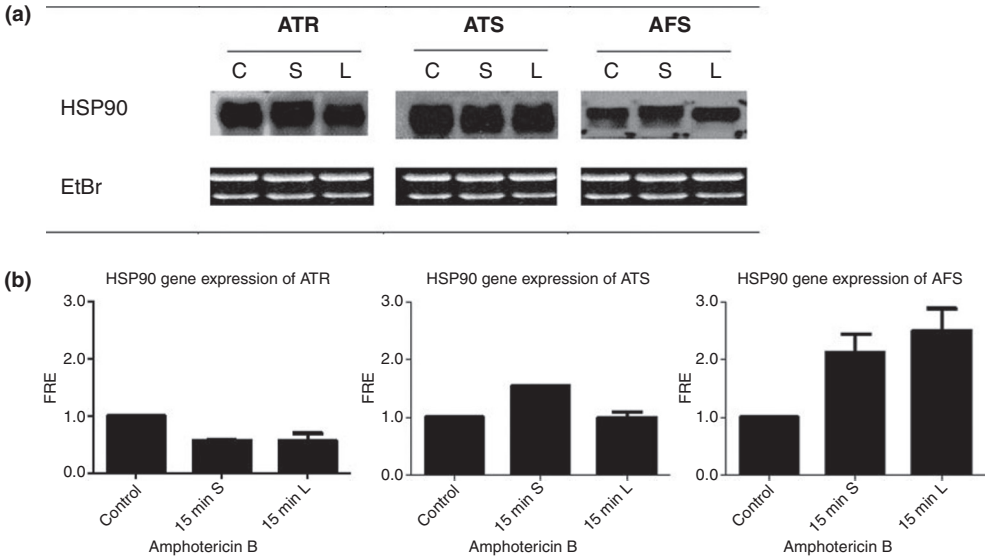

FIG. 1. Northern blot (a) and RT-PCR analysis (b) of HSP90 in *Aspergillus* spp. Incubation time was 15 min with sub-lethal (S) and lethal (L) concentrations of AmB. Tests were performed three times in duplicates and showed similar results. Gene expression levels of HSP90 (a) of Amphotericin B-resistant (ATR) and Amphotericin B-susceptible *A. terreus* (ATS) were compared with susceptible *A. fumigatus* (AFS). Ethidiumbromide (EtBr) staining of 26S and 18S rRNA was used as a loading control (black box). Regulation of target genes (b) in response to AmB and untreated control was determined using crossing points (CP) in comparison to the corresponding reference gene beta-tubulin. The$2^{-\Delta\Delta C_t}$ relative expression ratio of the target gene HSP90 determined with the$2^{-\Delta\Delta C_t}$ method and fold relative expression (FRE) are shown.

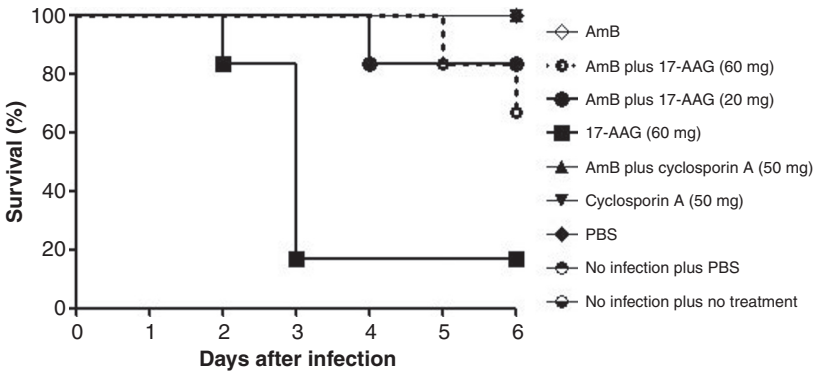

FIG. 2. Kaplan–Meier survival curve of mice infected with 2×10^6 CFU of ATR/mouse. Mice were treated from days 1 to 5 and sacrificed on day 6 post-challenge. All regimes were given once daily.

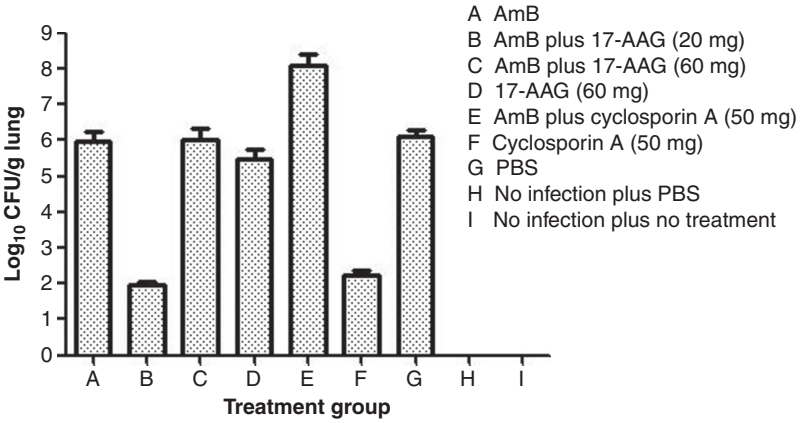

FIG. 3. Quantitative *A. terreus* real-time PCR of mouse lungs. BALB/c-mice received immunosuppression to assure fungal dissemination and were infected intravenously with 2×10^6 conidia of ATR.

illustrating stress interaction emanating from the exposure to AmB. A well-established murine model of disseminated aspergillosis gave detailed *in vivo* insight into 6-day survival and fungal tissue burden by comparing combination therapies with AmB and HSP90 inhibitors.

Our *in vitro* data were not confirmed *in vivo* as antifungal therapy of AmB in combination with HSP90 inhibitors did not reduce fungal burden significantly and showed no improvement in survival. Pathological and PCR analysis revealed fungal growth in all infected groups and did not indicate a reduction of fungal dissemination. Mice receiving AmB plus 17-AAG (20 mg) showed reduction in fungal load, yet the survival was lower. A significant lower survival appeared in those groups receiving HSP90 blockers alone or in combination with AmB, indicating that this agent might negatively influence fungal host defence. Regarding fungal tissue burden and mortality, HSP90 blockers alone or in combination with AmB may be too toxic for *in vivo* therapy for invasive aspergillosis due to ATR.

In clinical practice these drugs have several side-effects such as hepatotoxicity of geldanamycin or nephrotoxicity of calcineurin inhibitors and in fact, these immunosuppressants render patients to be highly susceptible to fungal infections [28], probably reduced immune response. These major drawbacks of direct HSP90 blocking substances explain the worse outcome in our animal setting, although 17-AAG, a less toxic blocker, was used. Pachl *et al.* [29] demonstrated that a recombinant antibody of *C. albicans* HSP90 in combination with AmB improved clinical outcome. Mycograb®, a human recombinant monoclonal antibody, affects fungal pathogens specifically and does not influence host immune response, and in general acts in a different way to pharmacological inhibitors [30] tested herein. Mycograb® plus lipid-associated AmB produced significant clinical and culture-confirmed improvement in outcome for patients with invasive candidiasis. Also, in larvae of the greater wax moth *Galleria mellonella* and in a murine model of disseminated candidiasis the combination therapy with HSP90 inhibitors and azole rescued larvae from lethal *C. albicans* infections and enhanced the therapeutic efficacy of azoles *in vivo* [16]. In comparison to our data, the reasons for such differences in outcome are not clear in detail as HSP90 inhibitors target fungal lifeblood directly and thus deregulate fungal stress management and increase vulnerability against drugs decrease fungal vitality.

Our data showed that fungi treated with the highest dosage of HSP90 inhibitors *in vitro* failed to grow anymore. However, such fungicidal *in vitro* effects may be overlapped *in vivo*. It is known that HSPs have a dual function depending on their intracellular or extracellular location: intracellular HSPs have a protective function and extracellular HSPs mediate immunological functions. In our case it is imaginable that application of cyclophosphamid, selection of pathogenic fungi (*C. albicans* vs. *A. terreus*), laboratory animals (BALB/c mice vs. CD1 mice) and choice of drugs (azoles vs. AmB) influence fungal progression and drug–drug interactions followed by complexities of immune responses. Also, combination of AmB plus 17-AAG might increase toxicity issues in our study. It is not known yet how an extension of treatment and further reduction of 17-AAG dosage would have positively influenced the outcome in our test animals.

In conclusion, we observed that blocking HSP90 *in vitro* enhances antifungal activity of AmB against ATR, ATS and AFS, and the basal equipment of HSP90 is higher in ATR and ATS compared with AFS; this renders *A. terreus* to be probably more 'environmentally resistant' than AFS. At higher dosages HSP90 inhibitors were fungicidal *per se* for all tested isolates. *In vivo*, application of HSP90 inhibitors does not improve outcome. Fungal HSP90 seems to play a central role in stress handling, and seems not to be the specific origin of AmB resistance in *A. terreus*.

Funding

This work was supported by MFF (119) and TWF (2010-1-1069).

Transparency Declaration

The authors have declared that no competing interests exist.

References

1. Wingard J, Ribaud P, Schlamm H, Herbrecht R. Changes in causes of death over time after treatment for invasive aspergillosis. *Cancer* 2008; 112: 2309–2312.
2. Segal B. Aspergillosis. *N Engl J Med* 2009; 360: 1870–1884.
3. Richardson M, Lass-Florl C. Changing epidemiology of systemic fungal infections. *Clin Microbiol Infect* 2008; 14 (suppl 4): 5–24.
4. Lass-Flörl C, Rath P, Niederwieser D *et al. Aspergillus terreus* infections in haematological malignancies: molecular epidemiology suggests association with in-hospital plants. *J Hosp Infect* 2000; 46: 31–35.
5. Lass-Flörl C, Mayr A. Diagnosing invasive fungal diseases – limitations of microbiological diagnostic methods. *Expert Opin Med Diagn* 2009; 3: 1–10.
6. Blum G, Perkhofer S, Grif K *et al.* A 1-year *Aspergillus terreus* surveillance study at the University Hospital of Innsbruck: molecular typing of environmental and clinical isolates. *Clin Microbiol Infect* 2008; 14: 1146–1151.
7. Lass-Flörl C, Perkhofer S, Mayr A. Invitro susceptibility tesing in fungi: a global perspective on a variety of methods. *Mycoses* 2010; 53: 1–11.

8. Walsh T, Petraitis V, Petraitiene R *et al.* Experimental pulmonary aspergillosis due to *Aspergillus terreus*: pathogenesis and treatment of an emerging fungal pathogen resistant to Amphotericin B. *J Infect Dis* 2003; 188: 305–319.
9. Lass-Flörl C, Cuenca-Estrella M, Denning D, Rodriguez-Tudela J. Antifungal susceptibility testing in *Aspergillus* spp. according to EUCAST methodology. *Med Mycol* 2006; 44 (suppl 1): 319–325.
10. Sokol-Anderson ML, Brajtburg J, Medoff G. Amphotericin B-induced oxidative damage and killing of *Candida albicans*. *J Infect Dis* 1986; 154: 76–83.
11. Pierce AM, Pierce HD Jr, Unrau AM, Oehlschlager AC. Lipid composition and polyene antibiotic resistance of *Candida albicans* mutants. *Can J Biochem* 1978; 56: 135–142.
12. Cowen LE, Lindquist S. Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. *Science* 2005; 309: 2185–2189.
13. Cowen L, Singh S, Köhler J *et al.* Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. *Proc Natl Acad Sci USA* 2009; 106: 2818–2823.
14. Pearl LH, Prodromou C, Workman P. The Hsp90 molecular chaperone: an open and shut case for treatment. *Biochem J* 2008; 410: 439–453.
15. Soti C, Nagy E, Giricz Z, Vigh L, Csermely P, Ferdinandy P. Heat shock proteins as emerging therapeutic targets. *Br J Pharmacol* 2005; 146: 769–780.
16. Matthews RC, Rigg G, Hodgetts S *et al.* Preclinical assessment of the efficacy of mycograb, a human recombinant antibody against fungal HSP90. *Antimicrob Agents Chemother* 2003; 47: 2208–2216.
17. Biamonte MA, Van de Water R, Arndt JW, Scannevin RH, Perret D, Lee WC. Heat shock protein 90: inhibitors in clinical trials. *J Med Chem* 2010; 53: 3–17.
18. Roe SM, Prodromou C, O'Brien R, Ladbury JE, Piper PW, Pearl LH. Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J Med Chem* 1999; 42: 260–266.
19. LaFayette SL, Collins C, Zaas AK *et al.* PKC signaling regulates drug resistance of the fungal pathogen *Candida albicans* via circuitry comprised of Mkc1, calcineurin, and Hsp90. *PLoS Pathog* 2010; 6: e1001069.
20. Pallavi R, Roy N, Nageshan R *et al.* Heat shock protein 90 as a drug target against protozoan infections: biochemical characterization of HSP90 from *Plasmodium falciparum* and *Trypanosoma evansi* and evaluation of its inhibitor as a candidate drug. *J Biol Chem* 2010; 285: 37964–37975.
21. Arenderup M, Perkhofer S, Howard SJ *et al.* Establishing *in vitro-in vivo* correlations for *Aspergillus fumigatus*: the challenge of azoles versus echinocandins. *Antimicrob Agents Chemother* 2008; 52: 3504–3511.
22. Pfaffl M. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Adics Res* 2001; 29: e45.
23. Lass-Flörl C, Gunsilius E, Gastl G *et al.* Diagnosing invasive aspergillosis during antifungal therapy by PCR analysis of blood samples. *J Clin Microbiol* 2004; 42: 4154–4157.
24. Xie Q, Thompson R, Hardy K *et al.* A highly invasive human glioblastoma pre-clinical model for testing therapeutics. *J Transl Med* 2008; 6: 77.
25. van Burik JA, Schreckhise RW, White TC, Bowden RA, Myerson D. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. *Med Mycol* 1998; 36: 299–303.
26. Bowman J, Abruzzo G, Anderson J *et al.* Quantitative PCR assay to measure *Aspergillus fumigatus* burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate. *Antimicrob Agents Chemother* 2001; 45: 3474–3481.
27. Cowen LE. Hsp90 orchestrates stress response signaling governing fungal drug resistance. *PLoS Pathog* 2009; 5: e1000471.
28. Steinbach WJ, Reedy JL, Cramer RA Jr, Perfect JR, Heitman J. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. *Nat Rev Microbiol* 2007; 5: 418–430.
29. Pachl J, Svoboda P, Jacobs F *et al.* A randomized, blinded, multicenter trial of lipid-associated Amphotericin B alone versus in combination with an antibody-based inhibitor of heat shock protein 90 in patients with invasive candidiasis. *Clin Infect Dis* 2006; 42: 1404–1413.
30. Stewart GR, Young DB. Heat-shock proteins and the host–pathogen interaction during bacterial infection. *Curr Opin Immunol* 2004; 16: 506–510.