



Biofouling

The Journal of Bioadhesion and Biofilm Research

ISSN: 0892-7014 (Print) 1029-2454 (Online) Journal homepage: www.tandfonline.com/journals/gbif20

The inhibition of *Pseudomonas aeruginosa* biofilm formation by micafungin and the enhancement of antimicrobial agent effectiveness in BALB/c mice

Kohar Annie B. Kissoyan, Wael Bazzi, Usamah Hadi & Ghassan M. Matar

To cite this article: Kohar Annie B. Kissoyan, Wael Bazzi, Usamah Hadi & Ghassan M. Matar (2016) The inhibition of *Pseudomonas aeruginosa* biofilm formation by micafungin and the enhancement of antimicrobial agent effectiveness in BALB/c mice, *Biofouling*, 32:7, 779-786, DOI: [10.1080/08927014.2016.1199021](https://doi.org/10.1080/08927014.2016.1199021)

To link to this article: <https://doi.org/10.1080/08927014.2016.1199021>



Published online: 27 Jun 2016.



Submit your article to this journal [↗](#)



Article views: 491



View related articles [↗](#)



View Crossmark data [↗](#)



Citing articles: 4 View citing articles [↗](#)

The inhibition of *Pseudomonas aeruginosa* biofilm formation by micafungin and the enhancement of antimicrobial agent effectiveness in BALB/c mice

Kohar Annie B. Kissoyan^a, Wael Bazzi^a, Usamah Hadi^b and Ghassan M. Matar^a

^aDepartment of Experimental Pathology, Immunology and Microbiology, American University of Beirut, Beirut, Lebanon; ^bOtolaryngology Head and Neck Surgery, American University of Beirut, Beirut, Lebanon

ABSTRACT

Micafungin inhibits biofilm formation by impeding 1,3- β -D-glucan synthesis in *Candida albicans*. Since *Pseudomonas aeruginosa* also has 1,3- β -D-glucan in its cell wall, this study assessed the effects of antibacterial agents *in vitro* and *in vivo* on micafungin-treated biofilm-forming *P. aeruginosa* isolates. After treatment with micafungin as well as with a panel of four antibacterial agents, biofilm production was significantly reduced as measured by spectrophotometry. The relative mRNA transcription levels for the genes encoding pellicles (*pelC*) and cell wall 1,3- β -D-glucan (*ndvB*), which were measured by quantitative reverse transcription PCR (qRT-PCR), significantly decreased with micafungin treatment. *In vivo*, the survival rates of *P. aeruginosa*-infected BALB/c mice significantly increased after combined treatment with micafungin and each of the antibacterial agents. Of these treatments, the combination of micafungin with levofloxacin had the highest survival rate; this combination was the most effective treatment against *P. aeruginosa*-induced infection.

ARTICLE HISTORY

Received 28 December 2015
Accepted 1 June 2016

KEYWORDS

Micafungin; biofilm;
Pseudomonas aeruginosa;
antibacterial agents; BALB/c
mice

Introduction

Biofilms are sessile microbial populations that are sheathed in self-produced extracellular polymeric substances (EPS) (Mikkelsen et al. 2011; Wei & Ma 2013), which allow microorganisms to withstand environmental assaults and colonize new niches when conditions are favorable. Biofilms are formed by a community of cells attached irreversibly to a surface and are prevalent on various surfaces in nature, in industry and in hospital settings (Mikkelsen et al. 2011; Wei & Ma 2013). Bacteria in biofilms exhibit divergent characteristics compared to their free-living planktonic cell counterparts, such as resistance to the immune system of a host and to antibacterial agents (Wei & Ma 2013).

The opportunistic pathogen *Pseudomonas aeruginosa* is a model organism for biofilm research (Mikkelsen et al. 2011). *P. aeruginosa* is a notorious pathogen in respiratory tract infections, causing diseases that are usually chronic in nature, such as pulmonary infections in intubated patients and patients with cystic fibrosis (CF), bronchiectasis, diffuse panbronchiolitis and chronic obstructive pulmonary disease. The production of biofilms by *P. aeruginosa* hinders the management of such infections because organisms in the biofilm become more resistant

to physical and chemical treatments (Høiby et al. 2001; Zhao & Liu 2010).

Biofilm formation is a highly regulated process that consists of a number of diverse stages, which are controlled by a wide range of factors (Mikkelsen et al. 2011). One survival strategy for environmental bacteria such as *P. aeruginosa* (Høiby et al. 2001) is to grow in a biofilm. Such growth involves the following stages: initial attachment, microcolony formation, maturation, and dispersion or the release of planktonic cells into the environment (Mikkelsen et al. 2011).

Biofilm formation by *P. aeruginosa* requires the expression of exopolysaccharides such as Pel and Psl, the regulation of which is post-transcriptionally repressed by RsmA, which is in turn controlled by a complex regulatory system involving sensor kinases and accessory components (Mikkelsen et al. 2011). These mechanisms allow bacteria to respond suitably to a given environmental stimulus; therefore, an improved understanding of these mechanisms and signals is crucial in designing novel therapeutic approaches (Mikkelsen et al. 2011) and may aid in controlling these infections and developing alternative treatments (Wei & Ma 2013). At the molecular level, the *pelC* gene encodes

for a glucosyltransferase involved in the migration of polysaccharides (Vasseur et al. 2007).

Micafungin is a semi-synthetic lipopeptide that belongs to the echinocandin family. This compound inhibits the enzyme 1,3- β -D-glucan synthase at the FKS catalytic subunit in a noncompetitive, concentration-dependent manner, thereby inhibiting the synthesis of 1,3- β -D-glucan. 1,3- β -D-glucan is a major component of the cell wall of most fungal cells that is also present in *P. aeruginosa*. Studies have revealed that the *ndvB* gene encodes for the glucosyltransferase enzyme, which is necessary for the development of 1,3- β -D-glucan in *P. aeruginosa* (Mennink-Kersten et al. 2008; Coulon et al. 2010; Beaudoin et al. 2012). Previously, Bazzi et al. (2013) showed that micafungin can disrupt the biofilm structure of *P. aeruginosa*, thus possibly exposing the core cells to treatment. Therefore, this study was undertaken to assess the effect of micafungin in combination with each of the four antibacterial agents *in vitro* and *in vivo*. Spectrophotometry was used to determine the relative gene transcription levels of the biofilm-encoding genes *pelC* and *ndvB* in samples treated *in vitro*, and the effects of these treatments were further evaluated *in vivo* using male BALB/c mice.

Materials and methods

Bacterial isolate

A biofilm-forming *P. aeruginosa* clinical isolate, PAN 14, was obtained from a patient with nosocomial infections (Zahreddine et al. 2012). The isolate was confirmed using Gram staining, colonial morphology, pigment production, and API20 NE kits (bioMérieux, SA 69820, Marcy l'Etoile, France). Antibacterial susceptibility testing was performed using the Kirby-Bauer disk diffusion method, and the minimum inhibitory concentration (MIC) was determined by broth dilution, both in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Cockerill et al. 2011) using the antibiotics ceftazidime (GlaxoSmithKline, Verona, Italy), levofloxacin (Sigma-Aldrich, Inc., St Louis, MO, USA), ciprofloxacin (Sigma-Aldrich, Inc.), and aztreonam (Bristol-Myers Squibb, Anangi, Italy).

Assessment of biofilm formation with micafungin in combination with antibacterial agents using microtiter plates

P. aeruginosa isolate PAN 14 was used in a microtiter plate assay to assess the effect of micafungin on biofilm formation in combination with the MIC doses of four different antibacterial agents: ceftazidime, aztreonam, ciprofloxacin and levofloxacin.

The development of biofilms of *P. aeruginosa* after the addition of micafungin (10 mg ml⁻¹) and MIC doses of the four antibacterial agents was monitored for four days using the protocol described by Bazzi et al. (2013) with minor modifications. Briefly, 100 μ l of the PAN 14 isolate suspension were transferred in triplicate into a 96-well polystyrene microtiter plate (Costar 3788, Corning Incorporated, Corning, NY, USA). These wells were considered the untreated controls. The protocol for treating the bacterial cells with 100 μ l of micafungin sodium (10 mg ml⁻¹) and/or 100 μ l of MICs of each of the four antibacterial agents is provided in Table 1. Following incubation, planktonic bacteria were discarded, and after rinsing, a 150- μ l aliquot from each well was transferred into a new polystyrene microtiter plate; the absorbance was then measured at 630 nm using an automated microplate reader at 630 nm (ELx800; BIO-TEK, Winooski, VT, USA). Standard deviations (SDs) were calculated, and Student's *t*-test was performed. The results were considered significant when *p*-values were < 0.05.

RNA extraction

Extraction of total RNA was performed on 10 samples of untreated PAN 14, isolates treated with and without micafungin, and with combinations of micafungin and each of the four antibacterial agents. The extraction was performed using a duplicate of the microtiter plate described above. A 1.5-ml aliquot of the biofilm suspension in LB broth was transferred to a 2-ml collection tube and then subjected to RNA extraction using the Power Biofilm RNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's procedure.

Table 1. Microtiter plate assay (plates #1, #2 & #3): experimental design.

Microtiter plate #1	Microtiter plate #2	Microtiter plate #3
NC	NC	NC
PC (PAN 14)	PC (PAN 14)	PC (PAN 14)
PAN 14 + Micafungin	PAN 14 + Micafungin	PAN 14 + Micafungin
PAN 14 + Micafungin + ceftazidime	PAN 14 + Micafungin + ceftazidime*	PAN 14 + Micafungin + ceftazidime \diamond
PAN 14 + Micafungin + aztreonam	PAN 14 + Micafungin + aztreonam*	PAN 14 + Micafungin + aztreonam \diamond
PAN 14 + Micafungin + ciprofloxacin	PAN 14 + Micafungin + ciprofloxacin*	PAN 14 + Micafungin + ciprofloxacin \diamond
PAN 14 + Micafungin + levofloxacin	PAN 14 + Micafungin + levofloxacin*	PAN 14 + Micafungin + levofloxacin \diamond

*: the antimicrobial agent was added after incubation of the bacterium with micafungin for 2 h.

\diamond : the antimicrobial agent was added after incubation of the bacterium with micafungin for 4 h.

Reverse transcription PCR (RT-PCR) and quantitative reverse transcription PCR (qRT-PCR)

RT-PCR was performed on the RNA extracted from untreated, micafungin-treated and micafungin-antibacterial combination-treated isolates of the *in vitro* samples using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Werk Penzberg, Germany). Quantitative RT-PCR was performed on the cDNA synthesized from the *P. aeruginosa* isolates to determine the transcription levels of the *ndvB* and *pelC* genes. The *rpoD* gene, encoding the principal sigma factor [σ (70)] in *P. aeruginosa*, was used as a reference housekeeping gene. The qRT-PCR assay was performed with the CFX-96 Real-Time Apparatus (BioRad, Munich, Germany) following the MIQE Guidelines (Minimum Information for Publication of Quantitative Reverse Transcription PCR Experiments).

In vivo experiments

Male BALB/c mice, six to eight weeks old, were obtained from the Animal Care Facility at the American University of Beirut. All animal experiments were approved by and conducted in accordance with the guidelines of the American University of Beirut Institutional Animal Care and Use Committee (IACUC).

The lethal dose (LD_{50}) of PAN 14 in BALB/c mice was identified using the method described by Nowotny (1979). To determine the LD_{50} for the PAN 14 isolate, 12 male BALB/c mice six to eight weeks old were divided into four groups, each containing three mice. Groups 1–4 each received a different bacterial dose, 10^8 CFU per injection, 10^7 CFU per injection, 10^6 CFU per injection and 10^5 CFU per injection, respectively. All the injections were performed intraperitoneally; saline was used as a diluent, and each preparation had a volume of 0.2 ml.

The therapeutically relevant *in vivo* MIC equivalent dose of the antibacterial agents used (levofloxacin and ceftazidime) was extrapolated from their *in vitro* MIC according to the following formula:

Antibacterial agent *in vivo* MIC dose (μg) = [Antibacterial agent *in vitro* MIC ($\mu\text{g } \mu\text{l}^{-1}$) \times *in vitro* MIC broth volume (μl) \times *P. aeruginosa* administered *in vivo*]/*P. aeruginosa in vitro* MIC reaction.

The injections were prepared so that the maximum total volume to be administered per mouse per day would not exceed 0.5 ml. The mice were monitored for weight loss and survival for 10 days after receiving the bacterial challenge. Dead mice were dissected and their tissues were homogenized and cultured on MacConkey agar to confirm the cause of death (Nowotny 1979).

To assess the efficacy of micafungin in combination with antibacterial agents in treating biofilm-forming strains *in vivo*, 35 mice were divided into seven groups of five mice each. The experimental design was as described in Table 2. All injections were performed intraperitoneally. The infected mice were injected with twice the LD_{50} of PAN 14. MIC doses of the antibacterial agents ceftazidime (2.4 mg ml^{-1}) and levofloxacin (2.4 mg ml^{-1}) were used. The micafungin dose was $2 \text{ mg kg}^{-1} \text{ day}^{-1}$ (0.6 mg ml^{-1}). The mice were treated and monitored over a period of 10 days to determine the survival rate. Dead mice were dissected, and their tissues were homogenized and cultured on MacConkey agar to confirm the cause of death.

Statistical analysis

Statistical analysis of the microtiter plate assay results and comparisons of the transcription levels of genes using unpaired *t*-tests were performed with the Graph-Pad *t*-test calculator. Additionally, Kaplan–Meier curves were used to estimate the survival rates for each group, and the log rank (Mantel–Cox) test was used to compare the rates. *p*-values < 0.05 were considered to be statistically significant.

Results

Minimum inhibitory concentration and antibacterial susceptibility

The MICs of the antibiotics against PAN 14 were as follows: ceftazidime, $2 \mu\text{g ml}^{-1}$; levofloxacin, $2 \mu\text{g ml}^{-1}$; ciprofloxacin, $0.125 \mu\text{g ml}^{-1}$; and aztreonam $4 \mu\text{g ml}^{-1}$. According to CLSI standards, the isolate PAN 14 was susceptible to the entire panel of tested antibiotics.

Table 2. The experimental design for the *in vivo* experiment, with and without antibacterial agent, in *P. aeruginosa* infected male BALB/c mice.

Days	Group I (PC)	Group II (NC1)	Group III	Group IV	Group V (NC2)	Group VI	Group VII
Day 1 (t=0)	PAN 14*	Micafungin	PAN 14	PAN 14 & micafungin	Micafungin	PAN 14	PAN 14 & micafungin
Day 1 (t=2 h)	–	Ceftazidime	Ceftazidime	Ceftazidime	Levofloxacin	Levofloxacin	Levofloxacin
Days 2–10	–	Micafungin & ceftazidime	Ceftazidime	Micafungin & ceftazidime	Micafungin & levofloxacin	Levofloxacin	Micafungin & levofloxacin

*PAN 14: strong biofilm forming strain of *P. aeruginosa* (dose used: $2LD_{50}$).

Table 3a. Results of the microtiter plate assay: Plate 1.

Samples	PLATE #1 Average rounded	SD	Statistical analysis Student's <i>t</i> -test (<i>p</i> value)	Significance
NC	1.396	0.146978		
PC (PAN 14)	>3	0	0.0001	Significant
PAN 14 + micafungin	2.782	0	0.0001	Significant
PAN 14 + mica+ ceftazidime	1.952	0	0.0028	Significant
PAN 14 + mica + aztreonam	>3	0	0.0001	Significant
PAN 14 + mica + ciprofloxacin	2.523	0.58709	0.0321	Significant
PAN 14 + mica + levofloxacin	2.335	0	0.0004	Significant

Table 3b. Results of the microtiter plate assay: Plate 2.

Samples	PLATE #2 Average rounded	SD	Statistical analysis Student's <i>t</i> -test (<i>p</i> value)	Significance
PC (PAN 14)	>3	0	0.0001	Significant
PAN 14 + micafungin	>3	0	0.0001	Significant
PAN 14 + mica+ ceftazidime	2.689	1.12618	0.0001	Significant
PAN 14 + mica + aztreonam	2.509	0.35753	0.0076	Significant
PAN 14 + mica + ciprofloxacin	2.846	0.08415	0.0012	Significant
PAN 14 + mica + levofloxacin	2.665	0.24422	0.0015	Significant

Table 3c. Results of the microtiter plate assay: Plate 3.

Samples	PLATE #3 Average rounded	SD	Statistical analysis Student's <i>t</i> -test (<i>p</i> value)	Significance
PC (PAN 14)	>3	0	0.0001	Significant
PAN 14 + micafungin	2.746	7.07E-05	0.0001	Significant
PAN 14 + mica+ ceftazidime	2.399	1.350348	0.0003	Significant
PAN 14 + mica + aztreonam	2.532	0.25244	0.0071	Significant
PAN 14 + mica + ciprofloxacin	2.55	0.47057	0.0154	Significant
PAN 14 + mica + levofloxacin	2.509	0.51336	0.0317	Significant

Assessment of biofilm formation with micafungin in combination with antibiotics using a microtiter plate assay

The *in vitro* results indicated significant inhibition of biofilm formation by *P. aeruginosa* (average OD, *p*-value < 0.05) when treated with micafungin compared to the untreated isolate (PC) (Table 3a, b, and c). Furthermore, a combination of micafungin and either ceftazidime or levofloxacin, which were added either simultaneously (Table 3a) or after incubation for 4 h (Table 3c), appeared to have the greatest effect in decreasing biofilm formation, as measured by decreasing OD values. However, a combination of micafungin and either aztreonam or levofloxacin applied after a 2-h incubation period was more effective than the other combinations of micafungin (Table 3b).

qRT-PCR

The relative gene transcription level of *ndvB* decreased when the PAN 14 isolate was treated with micafungin and decreased further when treated with a combination of micafungin and the other antibacterial agents. There was a 1,000-fold decrease in the relative transcription level of *ndvB* when the isolate was treated with aztreonam alone and a 500-fold decrease in the transcription level of *ndvB*

when it was treated with a combination of micafungin and ceftazidime (Table 4). Treated isolates had lower *pelC* gene transcription levels than the positive control. Although treatment with micafungin alone led to a 1,281-fold decrease in the expression of *pelC*, treatment with a combination of micafungin and the other antibacterial agents led to decreases of 100-fold, 500-fold, 333.334-fold and 125-fold with the addition of aztreonam, ceftazidime, levofloxacin and ciprofloxacin, respectively. The transcription level of the *rpoD* gene remained constant in all the tested samples, indicating that the observed decreases in the transcription levels of the target genes are not due to differences in the levels of cDNA. Furthermore, the melting curves analyzed using the Bio-Rad CFX Manager software (BioRad) revealed one peak for each assay performed, signifying that the results obtained are reliable.

In vivo experiments

The LD₅₀ of PAN 14 in the six- to eight-week-old male BALB/c mice was determined to be 3×10^7 CFU ml⁻¹.

The *in vivo* experiments indicated that the survival of mice infected with twice the LD₅₀ of *P. aeruginosa* (PAN 14) was significantly increased (*p*-value = 0.001) when the mice were treated with micafungin in combination with levofloxacin. After treatment and a monitoring period of 10 days, the mice in Group I (positive control)

Table 4. Relative gene expression for *ndvB* and *pelC* genes from *in vitro* treated and untreated samples and the fold decrease compared to the positive control.

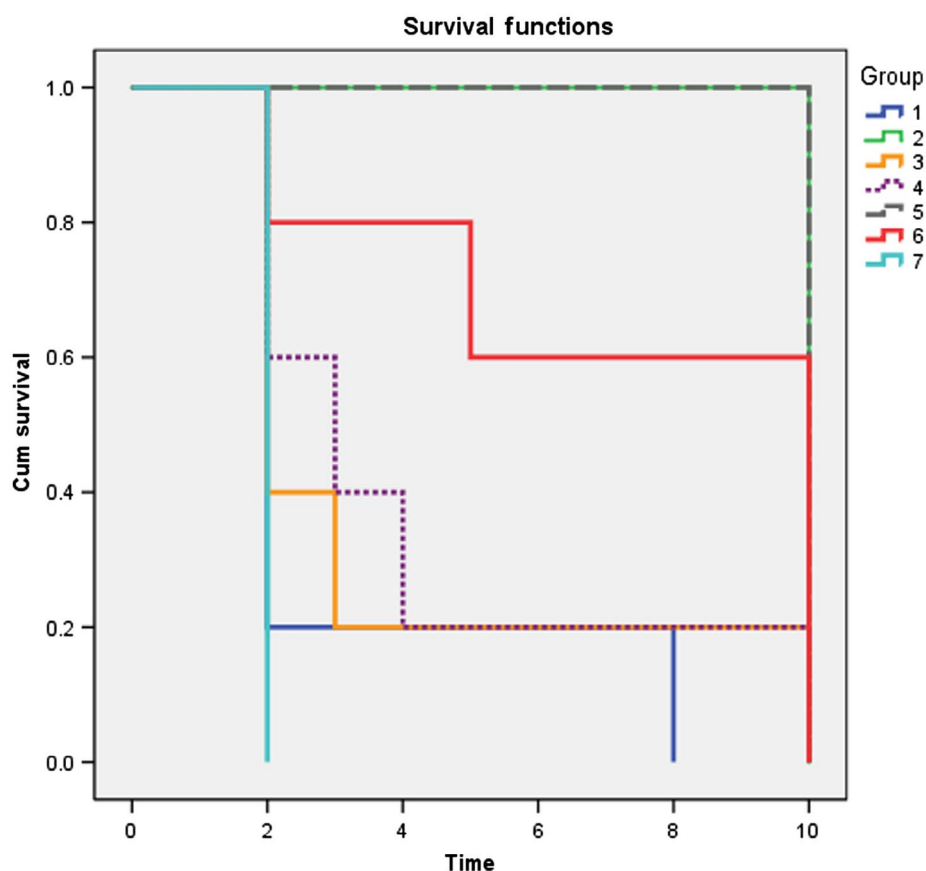
	Relative gene expression ratio			
	<i>ndvB</i>	Fold decrease: i/f	<i>pelC</i>	Fold decrease: i/f
Bacteria (B)	1	–	1	–
B + Micafungin	0.555	1.802	0.781	1.281
B + Levofloxacin	0.481	2.079	0.015	66.667
B + Ceftazidime	0	–	0.034	29.412
B + Ciprofloxacin	0.033	30.303	0.021	47.619
B + Aztreonam	0.001	1,000	0.001	1,000
B + Micafungin + levofloxacin	0.112	8.929	0.003	333.334
B + Micafungin + ceftazidime	0.002	500	0.002	500
B + Micafungin + ciprofloxacin	0.119	8.403	0.008	125
B + Micafungin + aztreonam	0.099	10.101	0.001	1,000

exhibited 0% survival, while the mice in groups II and V (negative controls for the combination of micafungin with either ceftazidime or levofloxacin, respectively) had 100% survival rates. The mice treated with ceftazidime alone (Group III) and a combination of micafungin and ceftazidime (Group IV) both had a survival rate of 20%, while those treated with levofloxacin alone (Group VI) exhibited 0% survival vs 60% survival when treated with a combination of micafungin and levofloxacin (Group VII). Upon dissection of the dead mice and culture of the homogenized tissues, *P. aeruginosa* was grown in culture,

indicating that all the deaths were due to the experimental infection. The results were statistically significant according to the log rank (Mantel–Cox) test (Figure 1).

Discussion

The destruction of a biofilm facilitates the infiltration of antibacterial agents through the compromised biofilm structure and allows immune defense components to more easily access the infectious bacteria. Therefore, anti-biofilm compounds may be a promising adjuvant

**Figure 1.** Kaplan–Meier survival graph (p -value = 0.001) of the *in vivo* experiment. The group numbers correspond to those in Table 2.

with antibacterial agents for therapeutic and/or prophylactic use (Rasamiravaka et al. 2015). Micafungin, by inhibiting the synthesis of 1,3- β -D-glucan, which is present in fungal cell walls and also in *P. aeruginosa*, may be one of these anti-biofilm agents. Previously, Bazzi et al. (2013) showed that micafungin can disrupt the biofilm structure of *P. aeruginosa*, thereby exposing the core cells to treatment.

In this study, the *in vitro* results of combining micafungin with antibacterial agents, especially the combination of micafungin and either ceftazidime or levofloxacin, indicated significant inhibition of biofilm formation in *P. aeruginosa* compared to the untreated isolates. The consistency in the results of the *in vitro* experiments with these two antibiotics prompted further study of their effects *in vivo*.

The qRT-PCR assay results for the aztreonam-treated samples showed the highest fold decreases in the transcription levels of *ndvB* and *pelC*. Similarly, Walton et al. (1997) have shown that aztreonam is more effective than aminoglycosides *in vitro*; however, clinical trials indicated that the use of aztreonam entails serious adverse effects. Therefore, this antibiotic was not used in the present *in vivo* experiments.

The array of traditional antibacterial agents with antipseudomonal activity includes the aminoglycosides, ceftazidime, cefepime, aztreonam, the carbapenems (except for ertapenem), ciprofloxacin and levofloxacin (El Solh & Alhajhusain 2009). This observation agrees with the present qRT-PCR data, which showed that the antibacterial agents alone induced an inhibitory effect on biofilm formation as detected by a decrease in the transcription levels of the *ndvB* and *pelC* genes.

However, the combination of the antibacterial agents with micafungin further decreased the transcription level of *pelC*, whereas with the *ndvB* gene, a decrease was only observed when levofloxacin was used in combination with micafungin. Similarly, Bonfiglio (2001) also identified levofloxacin as a good option for the treatment of *P. aeruginosa* infections following their study in which the *in vitro* activity of levofloxacin against 300 *P. aeruginosa* strains was compared to that of a panel of antibacterial agents and those results were confirmed by time-kill studies (Bonfiglio 2001). Likewise, Tennenberg et al. (2006) suggested that levofloxacin may be included in the treatment of pneumonia caused by *P. aeruginosa*, based on microbiological data, pharmacokinetic and pharmacodynamic aspects and clinical trials (Bonfiglio 2001; Tennenberg et al. 2006).

By comparing the effects of levofloxacin and ciprofloxacin on *P. aeruginosa*, it has been shown that the MIC values against *P. aeruginosa* are lower for ciprofloxacin than for levofloxacin, which is in agreement with the results of

this study (Tennenberg et al. 2006; Lister & Wolter 2008). However, the widespread tissue dissemination of levofloxacin leads to better absorption of the drug, which may compensate for the differences in the intrinsic activities as measured by MICs (Tennenberg et al. 2006; Lister & Wolter 2008). Despite the lower intrinsic strength of levofloxacin compared to ciprofloxacin, the superior pharmacokinetics of levofloxacin results in the achievement of nearly identical pharmacodynamics (Lister & Wolter 2008). This phenomenon might explain the better *in vivo* survival of infected mice treated with a combination of micafungin and levofloxacin. Furthermore, the pharmacokinetic parameters of levofloxacin indicate that it is absorbed rapidly after an intraperitoneal injection (Griffith et al. 2006), the route of infection used in the *in vivo* experiments.

Upon comparing the *in vitro* and *in vivo* results for levofloxacin and ceftazidime, the elevated MIC levels due to efflux pumps *in vitro* may or may not correlate with the *in vivo* experimental effects of the drug (Griffith et al. 2006). The inconsistencies between the *in vitro* and *in vivo* results may be explained by the notion that different conditions can trigger different mechanisms in bacteria (Davies et al. 1988). Furthermore, the host immune system may account for some of these discrepancies (Chen et al. 2011; Polak 2013).

Levofloxacin was more effective *in vivo* than *in vitro*. This difference can be explained by the fact that the overexpression of efflux pumps such as MexEF-OprN increases the MIC of levofloxacin *in vitro*, while these pumps are not overexpressed *in vivo* according to Griffith et al. (2006), thus suggesting that the MexEF-OprN system is less efficient than the MexAB-OprM or the MexCD-OprJ efflux pumps at expelling levofloxacin *in vivo*.

The overexpression of multidrug efflux pumps and mutational changes in target enzymes can limit the efficacy of levofloxacin (Lister & Wolter 2008); therefore, combination therapy with this drug might be a better option. Moreover, global surveillance programs estimate that 20–30% of *P. aeruginosa* isolates are resistant to levofloxacin and ciprofloxacin, and even higher resistance rates in certain regions have been reported (Lister & Wolter 2008).

The use of a combination of antibacterial and antifungal agents has been reported as a successful approach in addition to surgical treatment for the management of a patient who presented with *P. aeruginosa* and *C. parapsilosis* endocarditis (Daas et al. 2009). In addition, a combination of micafungin and cefepime has been reported to be a clinically successful approach for the treatment of a patient infected with *Candida albicans* and *P. aeruginosa* (Pomahac et al. 2012). Additionally, in a Japanese study by Ishikawa et al. (2006) micafungin was suggested as a safe

and effective antifungal agent to treat patients with febrile neutropenia refractory to antibacterial agents, thereby suggesting the broader use of micafungin.

Combination therapy using micafungin with antibacterial agents is a potential treatment regime for reducing unsuitable antibacterial use and mitigating resistance. Similar results have been reported by Bazzi et al. (2013) and additional research in this area may be helpful in further exploring this treatment option.

In conclusion, it appears that micafungin enhances therapeutic outcomes by disrupting biofilm structure and exposing the bacteria to antibacterial agents. While micafungin in combination with levofloxacin appears to have the best outcomes both *in vitro* and *in vivo* in *P. aeruginosa*-induced infections, studies are underway to determine the effects of micafungin and/or other antibacterial agents on translational and post-translational modifications using confocal laser microscopy to establish a cause-and-effect relationship between the observed decrease in transcription levels of biofilm-encoding genes, their corresponding proteins and the final biofilm product.

Acknowledgements

The authors would like to thank Hikma Pharmaceuticals PLC and Astellas Pharma Inc. for providing micafungin.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

- Bazzi W, Sabra A, Zahreddine L, Khairallah MT, Baroud M, Hadi U, Matar GM. 2013. The inhibitory effect of micafungin on biofilm formation by *Pseudomonas aeruginosa*. *Biofouling*. 29:909–915. doi:<http://dx.doi.org/10.1080/08927014.2013.816299>
- Beaudoin T, Zhang L, Hinz AJ, Parr CJ, Mah T. 2012. The biofilm-specific antibiotic resistance gene *ndvB* is important for expression of ethanol oxidation genes in *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* 194:3128–3136. doi:<http://dx.doi.org/10.1128/JB.06178-11>
- Bonfiglio G. 2001. Is levofloxacin as active as ciprofloxacin against *Pseudomonas aeruginosa*? *Chemotherapy*. 47:239–242.
- Chen TL, Fung CP, Lee SD. 2011. Spontaneous eradication of a NDM-1 positive *Klebsiella pneumoniae* that colonized the intestine of an asymptomatic carrier. *J. Chin. Med. Assoc.* 74:104. doi:<http://dx.doi.org/10.1016/j.jcma.2011.01.022>
- Cockerill FA, Wikler MA, Bush K, Dudley MN, Eliopoulos GM, Hardy DJ, Hecht DW, Hindler JA, Patel JB, Powell M, et al. 2011. Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement. *Clin. Lab. Stand. Inst.* 31:1–165.
- Coulon C, Vinogradov E, Filloux A, Sadovskaya I. 2010. Chemical analysis of cellular and extracellular carbohydrates of a biofilm-forming strain *Pseudomonas aeruginosa* PA14. *PLoS ONE*. 5:1–10. doi:<http://dx.doi.org/10.1371/journal.pone.0014220>
- Daas H, Abuhmaid F, Zervos M. 2009. Successful treatment of *Candida parapsilosis* and *Pseudomonas aeruginosa* infection using medical and surgical management in an injecting drug user with mitral and aortic valve endocarditis: a case report. *J. Med. Case Rep.* 3:6598. doi:<http://dx.doi.org/10.1186/1752-1947-3-6598>
- Davies SN, Martin D, Millar JD, Aram JA, Church J, Lodge D. 1988. Differences in results from *in vivo* and *in vitro* studies on the use-dependency of N-methyl aspartate antagonism by MK-801 and other phencyclidine receptor ligands. *Eur. J. Pharmacol.* 145:141–151. doi:[http://dx.doi.org/10.1016/0014-2999\(88\)90225-7](http://dx.doi.org/10.1016/0014-2999(88)90225-7)
- El Solh AA, Alhajhusain A. 2009. Update on the treatment of *Pseudomonas aeruginosa* pneumonia. *J. Antimicrob. Chemother.* 64:229–238. doi:<http://dx.doi.org/10.1093/jac/dkp201>
- Griffith DC, Corcoran E, Lofland D, Lee A, Cho D, Lomovskaya O, Dudley MN. 2006. Pharmacodynamics of levofloxacin against *Pseudomonas aeruginosa* with reduced susceptibility due to different efflux pumps: do elevated mics always predict reduced *in vivo* efficacy? *Antimicrob. Agents. Chemother.* 50:1628–1632. doi:<http://dx.doi.org/10.1128/AAC.50.5.1628-1632.2006>
- Høiby N, Krogh Johansen H, Moser C, Song Z, Ciofu O, Kharazmi A. 2001. *Pseudomonas aeruginosa* and the *in vitro* and *in vivo* biofilm mode of growth. *Microbes Infect.* 3:23–35.
- Ishikawa T, Takata T, Tomoyose T, Masuda M, Nakachi S, Koga S, Tsukada J, Matsuura A, Utsunomiya A, Saburi Y, Tamura K. 2006. The efficacy of micafungin as an empiric therapy for febrile neutropenic patient's refractory to antibacterial agents. *Jpn. J. Chemother.* 54:125–128. doi:<http://dx.doi.org/10.1125/chemotherapy1995.54.125>
- Lister PD, Wolter DJ. 2008. Resistance challenges threatening the treatment of *Pseudomonas aeruginosa* infections with levofloxacin: the role of a levofloxacin-imipenem combination for prevention of resistance. [cited 2015 Oct]. Available from: http://www.infectweb.com/only/artsrv2008_3.pdf
- Mennink-Kersten MASH, Ruegebrink D, Verweij PE. 2008. *Pseudomonas aeruginosa* as a cause of 1,3-β-D-glucan assay reactivity. *Clin. Infect. Dis.* 46:1930–1931. doi:<http://dx.doi.org/10.1086/588563>
- Mikkelsen H, Sivaneson M, Filloux A. 2011. Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. *Environ. Microbiol.* 13:1666–1681. doi:<http://dx.doi.org/10.1111/j.1462-2920.2011.02495.x>
- Nowotny A. 1979. Determination of toxicity. In: *Basic exercises in immunochemistry*. New York: Springer; p. 303–305.
- Polak S. 2013. *In vitro* to human *in vivo* translation – pharmacokinetics and pharmacodynamics of quinidine. *ALTEX*. 30:309–318.
- Pomahac B, Pribaz J, Eriksson E, Bueno EM, Diaz-Siso J, Rybicki FJ, Annino DJ, Orgill D, Caterson EJ, Caterson SA, et al. 2012. Three patients with full facial transplantation. *New England J. Med.* 366:715–722. doi:<http://dx.doi.org/10.1056/NEJMoal111432>

- Rasamiravaka T, Labtani Q, Duez P, El Jaziri M 2015. The formation of biofilms by *Pseudomonas aeruginosa*: a review of the natural and synthetic compounds interfering with control mechanisms. *BioMed Res. Int.* 2015:1–17. doi:<http://dx.doi.org/10.1155/2015/759348>
- Tennenberg AM, Davis NB, Wu S-, Kahn J. 2006. Pneumonia due to *Pseudomonas aeruginosa*: the levofloxacin clinical trials experience. *Curr. Med. Res. Opin.* 22:843–850. doi:<http://dx.doi.org/10.1185/030079906X100195>
- Vasseur P, Soscia C, Voulhoux R, Filloux A. 2007. PelC is a *Pseudomonas aeruginosa* outer membrane lipoprotein of the OMA family of proteins involved in exopolysaccharide transport. *Biochimie.* 89:903–915. doi:<http://dx.doi.org/10.1016/j.biochi.2007.04.002>
- Walton MA, Villarreal C, Herndon DN, Hegggers JP. 1997. The use of aztreonam as an alternate therapy for multi-resistant *Pseudomonas aeruginosa*. *Burns.* 23:225–227.
- Wei Q, Ma LZ. 2013. Biofilm matrix and its regulation in *Pseudomonas aeruginosa*. *Int. J. Mol. Sci.* 14:20983–21005. doi:<http://dx.doi.org/10.3390/ijms141020983>
- Zahreddine LT, Khairallah MT, Sabra AH, Baroud M, Hadi U, Matar GM. 2012. Expression levels of virulence factors with up-regulation of hemolytic phospholipase C in biofilm-forming *Pseudomonas aeruginosa* at a tertiary care center. *J. Appl. Res.* 12:87–97.
- Zhao T, Liu Y. 2010. N-acetylcysteine inhibit biofilms produced by *Pseudomonas aeruginosa*. *BMC Microbiol.* 10:140. doi:<http://dx.doi.org/10.1186/1471-2180-10-140>