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# **Adaptive Resistance Mechanisms of *Aspergillus fumigatus* Biofilms**

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Submitted in fulfilment of the requirements for the  
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School of Medicine

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*“It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change”*

*- Charles Darwin*

## Abstract

Biofilm formation is one of several significant virulence factors associated with life threatening pulmonary infections in immunocompromised individuals caused by *Aspergillus fumigatus*. Previous studies have demonstrated phase dependant antifungal activity against *A. fumigatus* biofilms. Antifungal resistance associated with fungal biofilms is a complex multifactorial phenomenon, and it remains unclear specifically how this manifests itself in *A. fumigatus*. This study therefore aimed to investigate adaptive resistance mechanisms in *A. fumigatus* biofilms.

Different phases of *A. fumigatus* biofilms were grown for 8, 12, 24 and 48h in polystyrene plates in RPMI media. Functional efflux pump activity was subsequently assessed using an Ala-Nap fluorescent uptake assay. Extracellular material was extracted from each phase and the level of extracellular DNA (eDNA) was quantified using a microplate fluorescence assay. The minimum inhibitory concentrations (MIC) of different classes of antifungals were assessed in the presence and absence of different inhibitors using a checkerboard assay, or with a fixed concentration, by the broth microdilution method to assess synergism, antagonism, or otherwise. The presence of eDNA and phenotypic changes in biofilm caused by antifungal agents and inhibitors were assessed by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) techniques. The resultant biofilm biomass for different experiments was evaluated using a crystal violet assay. SYBR green qRT-PCR was used to assess the expression of different genes implicated in biofilm resistance (*AfuMDR 1-4*, *ChiA-E*, *HSP90* and *Fks1*) over the period of multicellular development, using a diffusion chamber in a murine model and a *Galleria mellonella* infection model.

The results from this study demonstrated phase dependant expression of efflux pumps in *A. fumigatus* biofilm populations, which actively contributes to azole resistance. Moreover, voriconazole treatment induced efflux pump expression in both *in vitro* and *in vivo* models. These data suggest that *A. fumigatus* efflux pump proteins, which evolved to become integral to their natural physiological function, have inadvertently induced resistance to azole drugs, albeit in the early phases of biofilm development.

Assessment of *A. fumigatus* biofilm extracellular matrix (ECM), associated with maturing biofilms, showed that eDNA is an important architectural component of the biofilm, helping to maintain its stability. The antifungal sensitivity of different phases of *A. fumigatus* growth decreased significantly in the presence of DNase, indicating that decreased susceptibility to antifungals in the *A. fumigatus* is mediated in part by eDNA. Its release was shown to correlate with chitinase activity, a marker of autolysis, suggestive that autolysis was associated with eDNA release. It was hypothesised that heat shock protein 90 (HSP90) was involved in this autolytic pathway. Therefore, when HSP90 was pharmacologically inhibited this led to a decrease in matrix eDNA level, providing a compelling mechanism through which HSP90 might regulate biofilm antifungal resistance. To test whether these mechanisms of adaptive resistance had any bearing clinically, a *G. mellonella* model was developed. It was shown that each of the key genes were expressed during infection, both in control and antifungal treated larvae. This validates the potential use of this insect model for resistance and virulence studies.

Overall, this study establishes several novel adaptive resistance mechanisms regulating biofilm drug resistance in *A. fumigatus* biofilms. Moreover, it highlights the potential to target these mechanisms as a therapeutic strategy for managing and improving clinical outcomes in these hard-to-treat infections.

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## **Author's declaration**

I declare that the work described in this thesis has been carried out by me unless otherwise acknowledged or cited, under the supervision of Professor Gordon Ramage. I further declare that this thesis has not, in whole or in part, been submitted for any other degree.

Ranjith Rajendran

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## List of publications based on thesis

- **Rajendran R**, Williams C, Lappin DF, Millington O, Martins M, Ramage G.2013. Extracellular DNA release acts as an antifungal resistance mechanism in mature *Aspergillus fumigatus* biofilms. Eukaryot Cell. Epub ahead of print 2013 Jan 11.
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- Fraczek, M, Bromley M, Buied A, Moore C. B, **Rajendran R**, Rautemaa R, Ramage G, Denning D. W. and Bowyer P. 2013. The *cdr1B* efflux transporter is associated with non-*cyp51a*-mediated itraconazole resistance in *Aspergillus fumigatus*. J Antimicrob Chemother.
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- **Rajendran R**, Quinn R, McCulloch E, Murray C, Williams C and Ramage G. 2010. Efflux pumps may play a role in tigecycline resistance in the *Burkholderia* species. International Journal of Antimicrobial Agents. 36, 151-154

## Conference proceedings

- *Aspergillus fumigatus* biofilm extracellular DNA: a novel antifungal resistance mechanism. BSMM. UK. April 2012.
- *Aspergillus fumigatus* biofilms releases extracellular DNA which plays a role in phase dependant antifungal resistance. 5<sup>th</sup>AAA. Turkey. January 2012.
- Phase dependant mechanisms of antifungal resistance in *Aspergillus fumigatus* biofilms. TIMM. Spain. October 2011.
- Multidrug resistance transporter genes play a potential role in *Aspergillus fumigatus* azole resistance. ECCMID. Italy. May 2011.
- Efflux system plays a key role in azole resistance in *Aspergillus fumigatus* biofilms. BSMM. UK. April 2011
- *Pseudomonas aeruginosa* and their small diffusible extracellular molecules inhibit *Aspergillus fumigatus* biofilm formation. ESF meeting. France. January 2011.
- Molecular mechanisms of azole Resistance in *Aspergillus fumigatus*. GMMG meeting, UK. December 2010.
- Azole induced efflux pump expressions in *Aspergillus* biofilms. ESF Fuminomics research meeting. Institute Pasteur, France. October 2010.

## Abbreviations

ABC:	ATP-binding cassette
ABPA:	Allergic bronchopulmonary aspergillosis
AIDS:	Acquired immune deficiency syndrome
Ala-Nap:	Alanine $\beta$ -naphthylamine
ampB:	Amphotericin B
ANOVA:	Analysis of variance
AZL:	Azoles
BAL:	Bronchopulmonary lavage
cDNA:	Complementary deoxyribonucleic acid
CF:	Cystic fibrosis
CFE:	Colony forming equivalent
CLSI:	Clinical and laboratory standards institute
CLSM:	Confocal laser scanning microscopy
COPD:	Chronic obstructive pulmonary disease
CSP:	Caspofungin
CT:	Computed tomography
Ct:	Cycle threshold
CV:	Crystal violet
dH <sub>2</sub> O:	Distilled water
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
ECM:	Extracellular matrix
ECN:	Echinocandins

ECV:	Epidemiologic cut off value
eDNA:	Extracellular DNA
EDTA:	Ethylenediaminetetraacetic acid
EPI:	Efflux pump inhibitor
FUN-1:	2-chloro-4-(2,3-dihydro-3-methyl-1,3-thiazol-2-yl)-methylidene-1-phenylquinolinium iodine
EUCAST:	European committee on antimicrobial susceptibility testing
GdA :	Geldanamycin
GFP:	Green fluorescent protein
GPI:	Glycophosphatidylinositol
HD:	High dose
HSCT:	Haematopoietic stem cell transplant
Hsp:	Heat shock protein
IA:	Invasive aspergillosis
ITZ:	Itraconazole
LB:	Luria-buertani
LD:	Low dose
MAPK:	Mitogen activated protein kinase
MDR:	Multi drug resistance
MFA:	Microplate fluorescence assay
MFS:	Major facilitator superfamily
MIC:	Minimum inhibitory concentration
MOPS:	3-(N-Morpholino)-propanesulfonic acid
OD:	Optical density

PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PEG:	polyethyleneglycol
PI:	Propidium iodide
PM:	Post mortem
PMN:	Polymorphonuclear neutrophil
POL:	Polyenes
PVDF:	Polyvinylidene fluoride
RAPD:	Random amplification of polymorphic DNA
Real time RT-PCR:	Real time reverse transcriptase polymerase chain reaction
RFU:	Relative fluorescence units
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RPMI:	Roswell Park Memorial Institute- 1640 media
SAB:	Sabouraud dextrose agar
SEM:	Scanning electron microscopy
SMIC:	Minimum inhibitory concentration of sessile cells
SOD:	Superoxide dismutase
TB:	Tuberculosis
VRZ:	Voriconazole
WT:	Wild type
XTT:	2,3 bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide

# **Chapter 1**

## **Introduction**

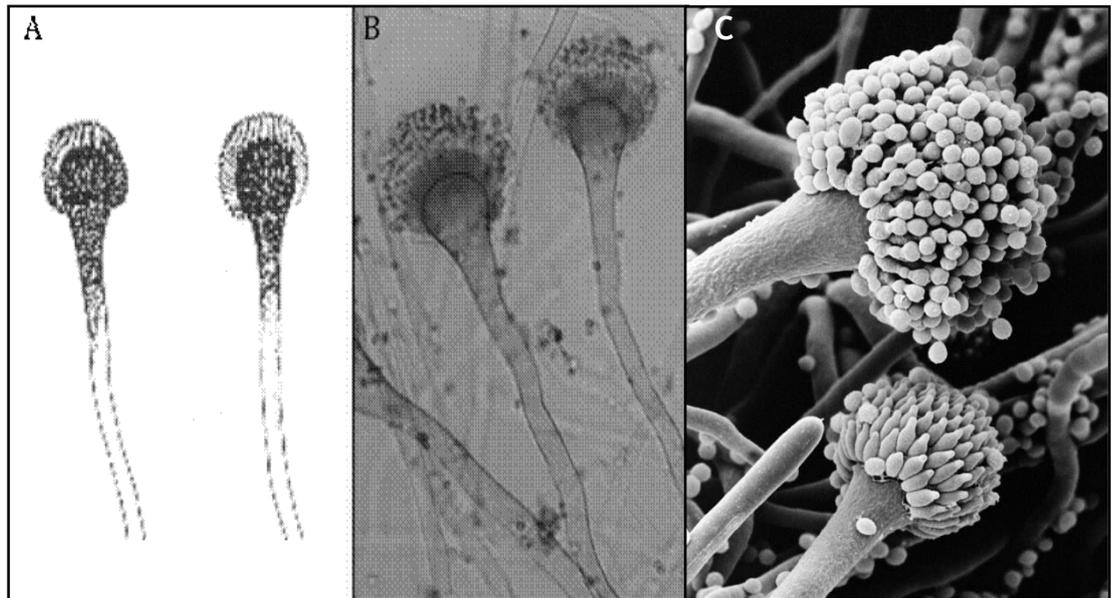
A review on fungal biofilm resistance

## 1.1 *Aspergillus* species

The genus *Aspergillus* is an Eurotiomycetes, which was first classified by Pier Antonio Micheli, an Italian priest and biologist in 1729 (Bennett, 2010). The word '*Aspergillus*' came from its structural resemblance in the shape of an aspergillum (Figure 1.1), used to sprinkle holy water by Roman Catholic priests. *Aspergillus* species are ubiquitous saprophytes in nature, being present in soil, grain and decaying vegetation. The genus consists of more than 200 species and variants, although only ten are pathogenic in humans (Bennett, 2010). *Aspergillus fumigatus* is one of the leading pathogenic species that cause disease in immunocompromised patients (Howard and Arendrup, 2011, Howard et al., 2009). In addition, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus niger* and *Aspergillus terreus* are other pathogenic species that have also been identified as harmful pathogens (Denning, 1998).

### 1.1.1 *Aspergillus fumigatus*

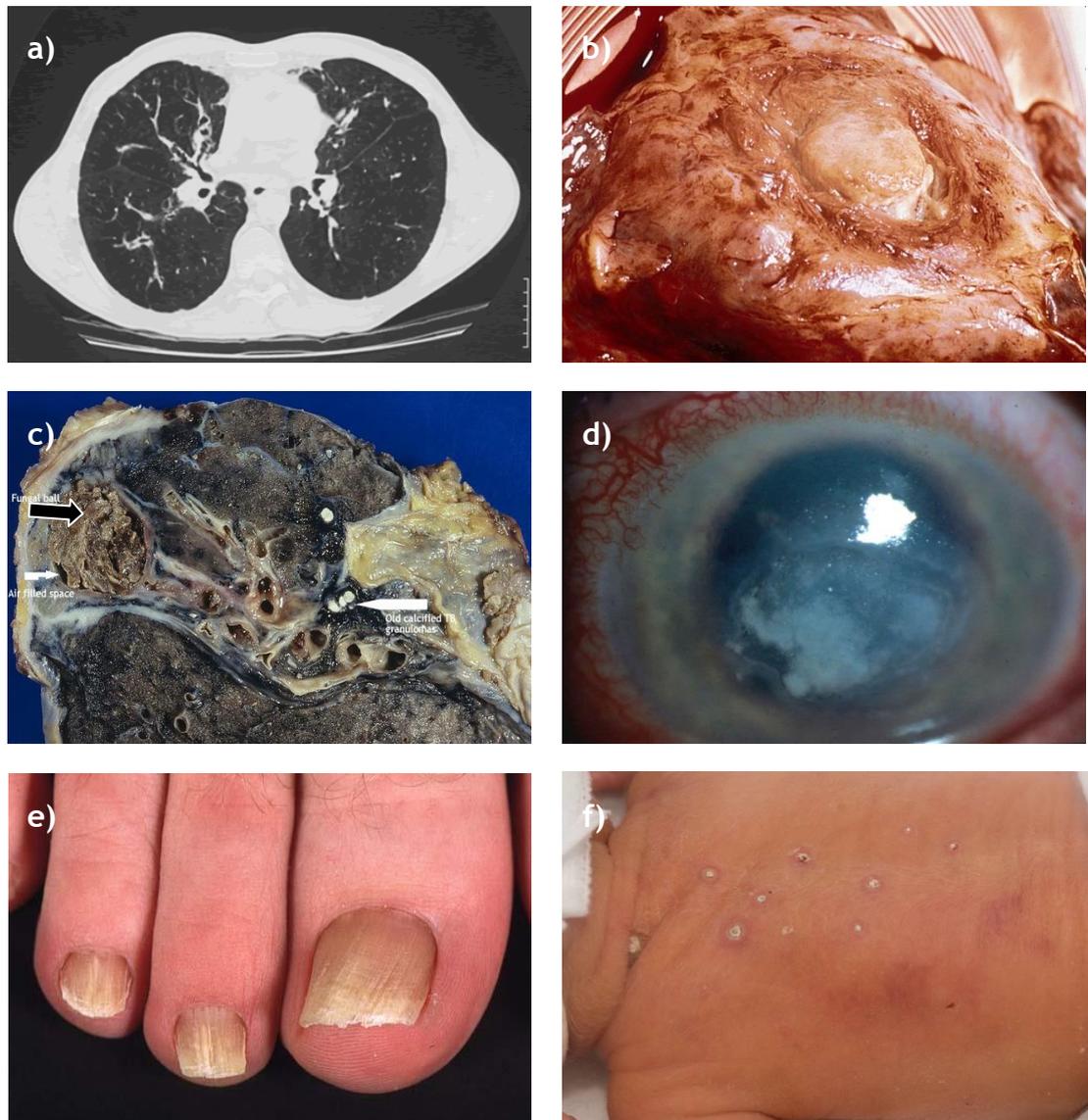
*Aspergillus fumigatus* is an airborne saprotroph, which survives and grows on organic debris. It plays an important role in carbon and nitrogen recycling in the environment (Haines, 1995). The *A. fumigatus* conidiophore sporulates thousands of grey green conidia at a size of 2-3  $\mu\text{m}$  that are readily airborne (Figure 1.1c). As a specie we inhale hundreds of conidia every day from the atmosphere (Dagenais and Keller, 2009). Inhalation by healthy individuals does not normally cause any adverse effects since the innate immune system eliminates the conidia (Latge, 1999). However, a host with a compromised immune system, such as acquired immune deficiency syndrome (AIDS), chronic obstructive pulmonary disease (COPD), chemotherapy and transplant patients, are vulnerable to *A. fumigatus* infections (Palousova et al., 2012, Greenberger, 2012, Bulpa et al., 2007, Mahfouz and Anaissie, 2003, Ruhnke, 2004). Not only does it affect humans, but it also causes serious problems in birds and animals (mallards and other ducks, penguins in zoos and old horses fed with poor-quality hay) (Beernaert et al., 2010, Tell, 2005, Shannon, 1992).



**Figure 1.1 - *Aspergillus fumigatus*.** (a) First *A. fumigatus* drawing by George W. Fresenius in 1863 showing aspergillum structure (Schmidt and Schmidt, 1999) (b) a micrograph displaying conidial heads of *A. fumigatus*(x920). (c) SEM picture of *A. fumigatus* (Wellcome images, <http://images.wellcome.ac.uk>).

## 1.2 Aspergillosis

*A. fumigatus* causes a spectrum of disease in humans called aspergillosis. The term aspergillosis includes infections and growth of fungus as well as allergic reactions it causes in the host (Latge, 1999). Aspergillosis can occur in variety of organs. It is often started with lungs (pulmonary aspergillosis) and spreads to other sites (invasive aspergillosis [IA]) such as sinuses (Sambatakou et al., 2006b), skin (Carvalho et al., 2008), nails (Mastella et al., 2000), heart (Montoya et al., 2003), kidney (Singh and Husain, 2003), bones (Perfect et al., 2001) and eyes (Vermes et al., 2000) via the bloodstream (Filler and Sheppard, 2006) (Figure 1.2). The percentage incidence of aspergillosis varies with organs (Table 1.1) (Patterson *et al.*, 2000). *A. fumigatus* primarily causes three main syndromes which includes allergic bronchopulmonary aspergillosis (ABPA), aspergilloma and IA.



**Figure 1.2 - Aspergillosis clinical images.** a) ABPA, CT scan image of a patient demonstrating bronchiectasis in the right lung. b) Aspergilloma, PM sample showing fungal ball formation in lung of leukaemia child. c) Aspergilloma, Lung tissue from tuberculosis patient showing cavitation (white arrow) and presence of fungal ball (black arrow). d) *Aspergillus* keratitis, *Aspergillus* growth in eye of a patient with corneal ulcer. e) *Aspergillus* onychomycosis, toenails showing *Aspergillus* infection. f) Primary cutaneous aspergillosis, circular papules with white eschars on the neonate back showing *Aspergillus* infection. Pictures were taken from the *Aspergillus* website ([www.Aspergillus.org.uk](http://www.Aspergillus.org.uk)).

**Table 1.1 - Percentage incidence of aspergillosis with different organs (Patterson et al, 2000).**

<b>Organ</b>	<b>% incidence*</b>
Pulmonary	56
Multiorgan dissemination	19
Skin	5
Paranasal sinuses	5
Central nervous system	6
Tracheobronchitis	1
Other	9

\* n = 595 patients with invasive aspergillosis

**Table 1.2 - Percentage incidence of aspergillosis in relation to underlying patient conditions (Patterson et al, 2000).**

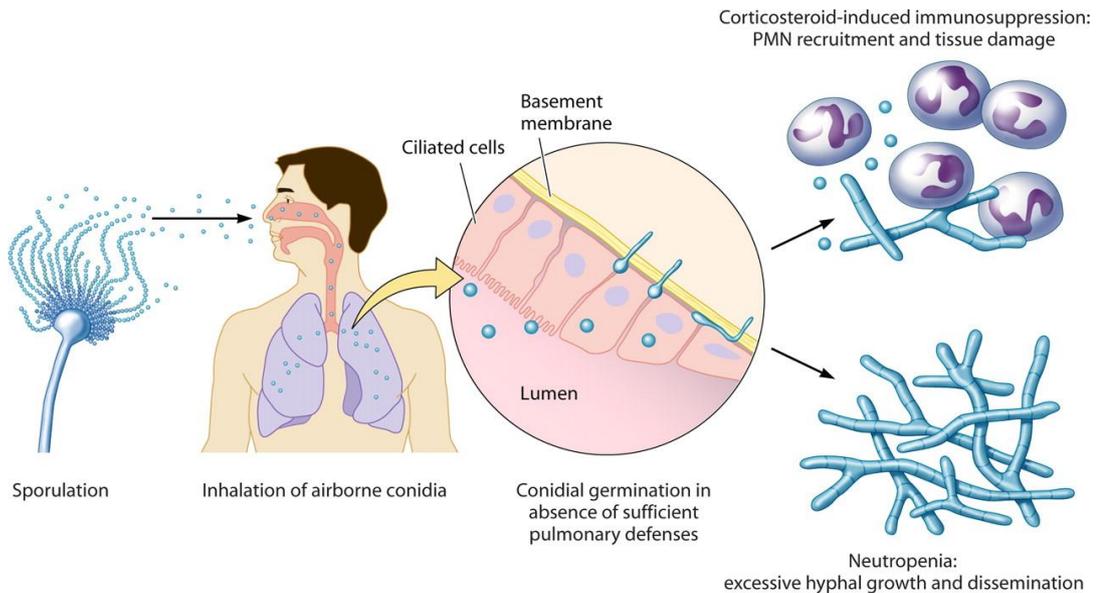
<b>Underlying condition</b>	<b>% incidence*</b>
Allogeneic stem cell transplantation	25
Haematological malignancy	28
Pulmonary disease	9
Solid organ transplantation	9
AIDS	8
Autologous stem cell transplantation	7
Immunosuppressive therapy	6
Other underlying conditions	6
Non-compromised hosts	2

\* n = 595 patients with invasive aspergillosis

### 1.2.1 Allergic bronchopulmonary aspergillosis

ABPA is a condition caused by allergic lung reaction to *A. fumigatus* spores. ABPA primarily affects asthma and cystic fibrosis patients (Greenberger and Patterson, 1988). *A. fumigatus* growth in the trachea and bronchi invokes both type I (formation of IgE) and type III hypersensitivity response (formation of IgG) in the host (Kurup et al., 1994, Kurup, 2000). The type I response to *A. fumigatus* causes mast cell degranulation with broncho constriction and increased capillary permeability. The type III response together with deposits of inflammatory cells within the airway mucous membrane causes necrosis (Zeaske et al., 1988). Some of the common clinical symptoms of ABPA are increased sputum production, wheezing, coughing up mucus with brown, black, or green elements, shortness of breath and fever (Greenberger, 1986).

The standard diagnosis of ABPA are to fulfil the following criteria a) asthma, b) immediate reactivity to *A. fumigatus*, c) high serum IgE level (> 1000ng/mL), d) precipitating antibodies to *A. fumigatus* present in serum, e) eosinophilia and f) central bronchiectasis on chest CT (Greenberger and Patterson, 1988). Treating ABPA is quite difficult and there is no complete cure for ABPA. All treatments are carried out to avoid any lung damage. There are two treatment aspects for ABPA. One is to control inflammation and hypersensitivity reactions by using oral corticosteroids and the other is to control fungal growth by antifungal drugs.



**Figure 1.3 - Infection process of *A. fumigatus*.** *A. fumigatus* spores readily become airborne. Inhalation of airborne conidia by immunocompromised patients causes significant inflammation followed by conidium establishment in the lungs, germination of conidia and PMN recruitment. With neutropenic conditions excessive hyphal growth and dissemination is possible (Dagenais and Keller, 2009).

### 1.2.1.1 Epidemiology

ABPA has a worldwide distribution, there are likely to be significant geographic differences in its prevalence. There are different factors influencing the occurrence of ABPA which includes environmental factors such as atmospheric concentrations of conidia (Esposito et al., 2009, Young et al., 1970, Jayshree et al., 2006), genetic factors such as *CFTR* gene mutation (Miller et al., 1996, Marchand et al., 2001, Eaton et al., 2002), interleukins IL 4,13,15 polymorphisms (Knutsen et al., 2006, Escande et al., 2011), tumour necrosis factor(TNF)- $\alpha$  polymorphisms (Escande et al., 2011), TLR9 polymorphisms (Carvalho et al., 2008), and patients underlying conditions such as asthma. The prevalence of ABPA varies depending on treatment centre and diagnostic criteria used (Mastella et al., 2000). The first ABPA case was reported in UK in 1952 by Hinson(Hinson et al., 1952). In 1967 ABPA cases were reported in USA and Australia, followed by India in 1971(Shah, 1971). The reported incidence of ABPA

in asthma patients varies from 13 to 45% in different parts of the world (Table 1.3). Previous studies have shown that asthma patients exposed to *Aspergillus* had an increased risk of death (Taff et al., 2012). However, none of these studies specifically evaluated ABPA in relation to severity of asthma.

The impaired mucus clearance and airway obstruction in CF patients are other risk factors associated with ABPA. The association of CF and ABPA were reported in 1965 in UK (Loussert et al., 2010). From surveys and epidemiology registries, about 7.8 % of patients in Europe and 7% in United States with CF are estimated to have ABPA. Table 1.4 shows the prevalence of ABPA in Europe (Mastella et al., 2000). As like incidences with asthma, the incidence of ABPA in CF patients also shows geographical variances. In European countries for example the incidence varied from 2.1% in Sweden to 13.6 % in Belgium (Mastella et al., 2000). In USA, the incidence varied from 0.9% in the South West region to 4% in the West region (Geller et al., 1999).

Table 1.3 - Prevalence of ABPA in asthma patients.

Country	Number of Patients with asthma	Prevalence of ABPA (%)	Reference
UK	656	85 (13)	(Ramage et al., 2009b)
USA	100	38 (38)	(Huang and Kao, 2012)
Australia	79	26 (33)	(Martins et al., 2010)
South Africa	500	110 (22)	(Ben-Ami and Kontoyiannis, 2012)
Saudi Arabia	53	12 (23)	(Walker et al., 2010)
India	755	294 (39)	(Nett et al., 2010b)

Table 1.4 - Prevalence of ABPA in CF patients in Europe(Mastella et al., 2000).

Country	Number of patients with CF	Prevalence of ABPA (%)
Austria	169	9 (5.3)
Belgium	44	6 (13.6)
Denmark	320	11 (3.4)
France	2935	217 (7.4)
Germany	3070	353 (11.5)
Ireland	621	68 (11.0)
Netherlands	353	24 (6.8)
Sweden	424	9 (2.1)
UK	4511	271 (6.0)
All	12447	971 (7.8)

### 1.2.2 Invasive aspergillosis

IA is a serious *Aspergillus* infection and involves the initial colonisation and consequential spread to other parts of the body by haematogenous and angioinvasion (Figure 1.4). Some of the common risk factors associated with IA are AIDS, solid organ transplantation, intensive chemotherapy regimens and increased use of immunosuppressive regimens (Baddley, 2011, Herbrecht et al., 2012, Segal and Walsh, 2006, Cornet et al., 2002). Patients with IA have symptoms including fever, cough, chest pain, difficulty breathing and poor response to standard antibiotics (Aleksenko and Gyasi, 2006, Latge, 1999). The earlier the diagnosis the better the treatment but there is no single diagnostic method that is either universally applicable or sensitive or specific enough to establish IA. Radiological evaluation, microscopy and culture from sputum sample, serological test for *Aspergillus* and molecular diagnostic methods are generally used for diagnosis. Antifungal drugs include voriconazole, itraconazole, caspofungin or amphotericin B are used for treatment of IA. The overall success rate with these antifungals varies significantly between different patient groups. Surgical excision and cytokine therapy are other treatment methods in practice for IA (Rajbanshi et al., 2012, Lang et al., 1983, Safdar, 2010, Lehrnbecher et al., 2012).

#### 1.2.2.1 Epidemiology

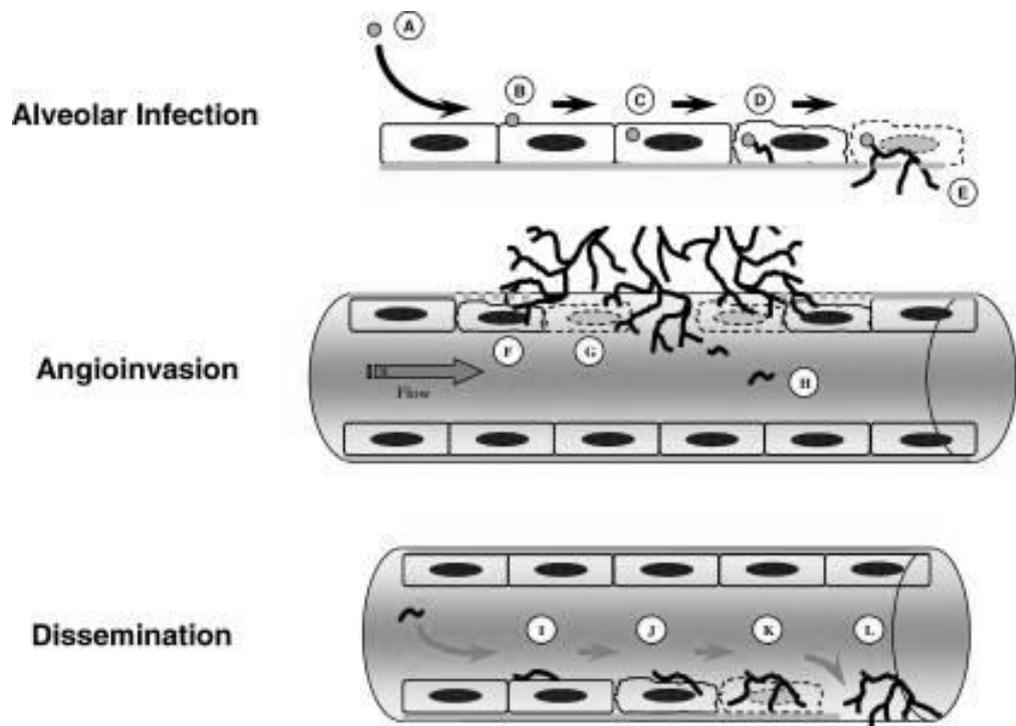
The incidence of IA varies depending on the patients underlying conditions and geographic location (Lang et al., 1983, Walker et al., 2010). A percentage of up to 7% is reported in Europe to have IA (Table 1.5) composed of about 38% in acute myelogenous leukaemia, 50-60% with organ transplant patients and 70-85% in other immunocompromised patients (Rajbanshi et al., 2012, Lang et al., 1983, Walker et al., 2010, Ghannoum et al., 1996, McCulloch et al., 2009). The onset of IA also varies with underlying patient condition. About 72% of heart transplant patients are diagnosed with IA in the first three months of post-surgery (Montoya et al., 2003), with approximately 50% of liver and lung transplant patients diagnosed within 3-5 months (Singh and Husain, 2003). The rate of mortality associated with haematopoietic stem cell transplants ranged from 53.8 to 84.6%

and with solid organ transplants 20 to 66.7% after 3 months. A multicentre study (24 medical centres) from 2001 showed out of 1209 IA cases diagnosed approximately 750 patients died within three months of diagnosis (Perfect et al., 2001).

**Table 1.5 - Prevalence of IA in Europe.**

Country	Year	Patient group	Number of patients	Prevalence of IA (%)	Reference
Austria	2000-2003	HSCT*	104	23	(Adams, 2004)
Belgium	2000-2003	Critically ill	1850	6.9	(Walker et al., 2010)
Italy	1999-2003	HSCT	1249	6.3	(Lang et al., 1983)
Spain	1996-2000	HSCT	395	9.4	(Bowman and Free, 2006)
Finland	1990-2001	HSCT	1188	0.8	(Selvaggini et al., 2004)
Poland	1990-2006	liver transplant recipients	277	3.6	(Leach et al., 2012)

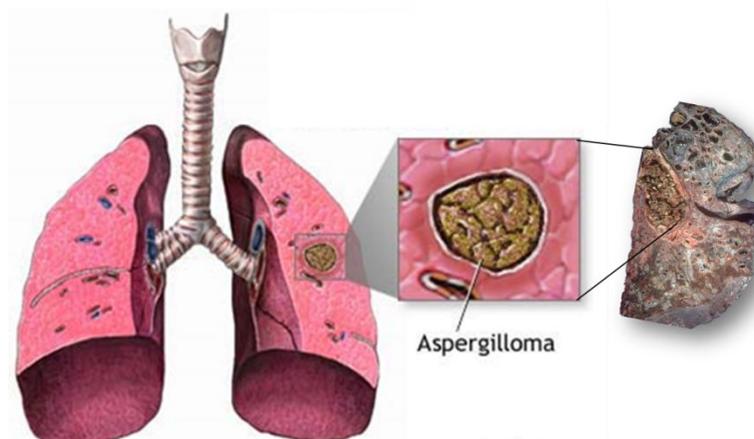
\* HSCT, haematopoietic stem cell transplant



**Figure 1.4 - Model of invasive aspergillosis.** The process of IA involves three steps alveolar infection, angioinvasion and dissemination. (a) Alveolar infection includes adhesion of inhaled conidia (b) with pulmonary epithelial cells, (c) endocytosis within the epithelial cells, (d) germination of conidia and hyphal growth within the epithelial cells, and (e) hyphal extrusion from the epithelial cells. Angioinvasion involves (f) penetration of abluminal surface by emergent hyphae, (g) induced cell damage, and (h) spreading of hyphal fragments via blood stream. Finally in dissemination process (i) the hyphal fragments adhere to the cell surface, (j) cells invasion, (k) cell damage and (l) invasion of other deep organs (Filler and Sheppard, 2006).

### 1.2.3 Aspergilloma

An aspergilloma, also known as fungal ball, is a rounded mass of hyphal material usually combined with mucus and cellular debris, existing within scarred lungs or a pre-existing body cavity (Figure 1.5). The body cavities such as in the lungs caused by cystic fibrosis, tuberculosis, lung cancer, lung abscess, coccidioidomycosis, sarcoidosis and histoplasmosis are most susceptible to an aspergilloma (Chatzimichalis et al., 1998, Kawamura et al., 2000, Massard et al., 1992, Hadjiliadis et al., 2002, Greenberg et al., 2002, Kunst et al., 2006). Typically, patients with aspergilloma are asymptomatic but a small percentage of this *Aspergillus* growth invades into cavity walls and can result in haemoptysis. The size of fungal ball formation can range from 3-7cm in diameter and is commonly diagnosed by sputum culture, chest X-ray, chest CT, bronchoscopy or serum precipitins for *Aspergillus* (Munoz, 2006). Often, no treatment is necessary for aspergilloma but patients with haemoptysis need medical attention (Chen et al., 1997). Angiography can be used to detect the location of bleeding and surgery may be necessary to remove the fungal ball formation and to prevent further bleeding. Antifungal drugs such as azoles are also used for aspergilloma treatment but none has been shown to eradicate *Aspergillus* growth.



**Figure 1.5 - Model of aspergilloma.** Aspergilloma, a rounded mass of hyphal material exists within scarred lungs. (Adam images, 3434\_72EWJ, www.adamimages.com)

### 1.2.3.1 Epidemiology

Currently, information about the incidence of aspergillomas is limited. Unlike other types of aspergillosis the incidence of aspergilloma was reported in patients with normal immunity but structurally abnormal lungs, with pre-existing cavities. One of the common underlying pulmonary conditions reported to be associated with development of aspergilloma was TB (Karas et al., 1976, Massard et al., 1992, Regnard et al., 2000, Kawamura et al., 2000). About 25 - 80% of TB cases were reported to have aspergilloma (Chen et al., 1997). A study from France reported the incidence of aspergilloma from 1974-1991, where 57% of patients were reported to be associated with TB (Massard et al., 1992). Later in 1992-1997 the association of TB was reported to be only 17% (Chatzimichalis et al., 1998). Similar pattern of reduction in percentage of TB associated aspergilloma of 82% in 1977-1987 and 60% in 1987-1997 reported by another study from France indicates the epidemiologic shifts in TB associated aspergilloma (Regnard et al., 2000). Pulmonary sarcoidosis, bronchiectasis from any cause and other pulmonary cavities are the other conditions shown to be associated with incidence of aspergilloma (Chen et al., 1997, Hadjiliadis et al., 2002, Kawamura et al., 2000, Lee et al., 2004)

### 1.3 Treatment of Aspergillosis

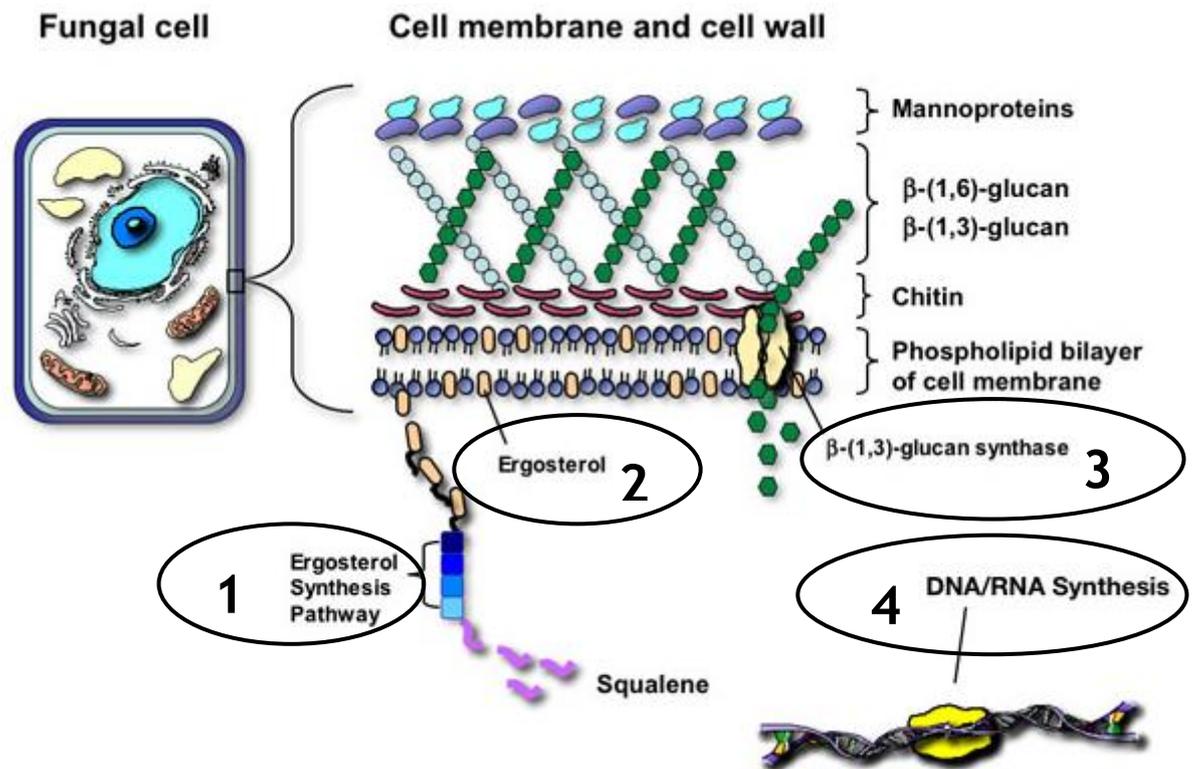
Treatment options for aspergillosis are limited. Due to severity of aspergillosis in immunocompromised patient prophylaxis treatment is quite common. The use of pharmacological agents to control or treat aspergillosis is the first line of treatment option by clinicians. According to a study from USA about 47% of IA patients undergo monotherapy and 29% received combination therapy (Karas et al., 1976). The monotherapy includes azole (60-70%), polyene (13-15%) and echinocandin treatment (10%) (Karas et al., 1976, Kawamura et al., 2000). For patients with an aspergilloma, surgery is the only effective option (Regnard et al., 2000). The different treatment options and complications are now discussed in the following sections.

### 1.3.1 Prophylaxis

Aspergillosis morbidity and mortality is associated with the neutropenia resulting from intensive cancer chemotherapy, stem cell transplantation, lung and liver transplant recipients. Given the high risk associated with aspergillosis, it is not surprising that prophylaxis against *A. fumigatus* is apparently a sensible therapeutic approach. One of the common prophylaxis methods for aspergillosis is by minimising the patient exposure to airborne spores by providing a filtered air environment (Levenson et al., 1991). It is achieved by two means, laminar air-flow and high efficient particulate air (HEPA) filter (Buckner et al., 1978, Navari et al., 1984, Petersen et al., 1988, Berthelot et al., 2006). Control of *Aspergillus* growth by pharmacologic agents is the next most common prophylaxis approach by clinicians (Drew et al., 2004, Conneally et al., 1990). Initially, the polyene drug amphotericin B was used to prevent aspergillosis. Later, due to drug toxicities, amphotericin B was discontinued to use for prophylaxis. The triazole drugs are the next improvement in prophylactic agents. In late 1980s, fluconazole was introduced and has changed the epidemiology of aspergillosis following stem cell transplantation. In the 1990s, itraconazole was tested for prophylaxis (Grant and Clissold, 1990). Later another improved triazole drug posaconazole was licensed for prophylaxis treatment with neutropenic patients (Petraitiene et al., 2001). According to the recent reports an echinocandin drug caspofungin may be an attractive option for prophylaxis treatment due to comparatively less adverse effects than others (Massard et al., 1992, Hadjiliadis et al., 2002). There are different pharmacological agents tested for controlling fungal infections and the prophylaxis regimen is still not clear and standardised.

### 1.3.2 Antifungal drugs

Antifungal therapy is a method of eradicating fungi from host by using pharmacological agents exploiting differences between human and fungal cells(Dixon and Walsh, 1996). The formulation of antifungal agents are mainly administered orally (54.2%) or by intravenous methods (Kawamura et al., 2000) (Table 1.6). Unlike antibacterial drugs, to date relatively few antifungal targets were exploited because fungal cells are closely related to humans. There are only three antifungal targets, cell wall components, membrane sterols and DNA synthesis that have been found with varying degrees of success(Kathiravan et al., 2012, Dixon and Walsh, 1996) (Figure 1.6). Fungal membrane sterols are different enough from humans so antifungal agents targeting this can kill fungi but not mammalian cells(Ghannoum and Rice, 1999). Fungal cells synthesise  $\beta$ -glucans, an important cell wall component, it is absent in human cells so drugs targeting glucan biosynthesis display low side effects(Kathiravan et al., 2012). Some of the main points of consideration with antifungal therapy is drug specificity, broad spectrum of antifungal activity, fungicidal rather than fungistatic, drug interactions, and low cost. Currently, there are three principal classes of antifungals azoles, polyenes and echinocandins used in antifungal therapy(Dixon and Walsh, 1996).



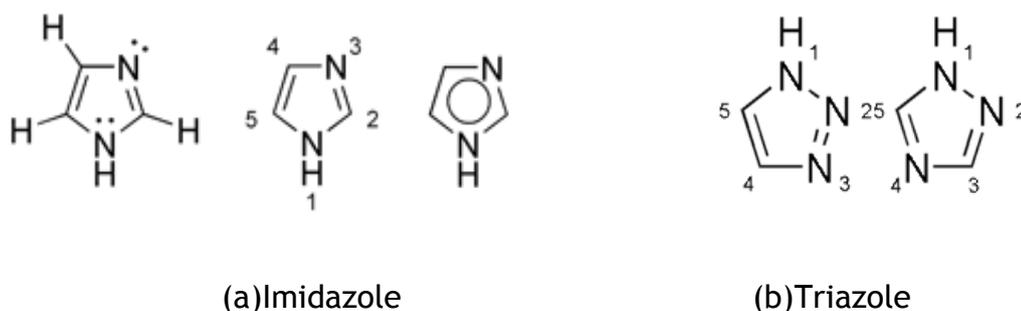
**Figure 1.6 - Antifungal drug targets.** 1) Azoles inhibit *Erg11*, affects ergosterol biosynthesis, which cause fungal membrane stress. 2) Polyenes bind with membrane sterol, thereby forming trans membrane pores, which cause cell leakage 3) Echinocandins inhibit  $\beta$ -(1, 3) - glucan synthase, thus disrupt cell-wall integrity 4) Flucytosine inhibits DNA and RNA synthesis, disturbs the translation of certain crucial proteins (adapted from Doctor Fungus [[http://www.doctorfungus.org/thedrugs/antif\\_pharm.htm](http://www.doctorfungus.org/thedrugs/antif_pharm.htm)]).

**Table 1.6 - List of antifungal drugs and classes, formulation and year introduced.**

<b>Class</b>	<b>Generic name</b>	<b>Formulation</b>	<b>Year</b>
<b>Azole</b>	Ketoconazole	oral, topical	1981
	Fluconazole	oral, topical, intravenous	1990
	Itraconazole	oral, intravenous	1992
	Voriconazole	oral, intravenous	2002
	Posaconazole	oral, intravenous	2006
<b>Echinocandin</b>	Caspofungin	intravenous	2002
	Micafungin	intravenous	2005
	Anidulafungin	intravenous	2006
<b>Polyene</b>	Amphotericin B	oral, intravenous	1960
	Amphotericin B lipid complex	intravenous	1995
	Amphotericin B Liposomal	intravenous	1997
<b>Other</b>	Flucytosine	oral	1964

### 1.3.2.1 Azoles

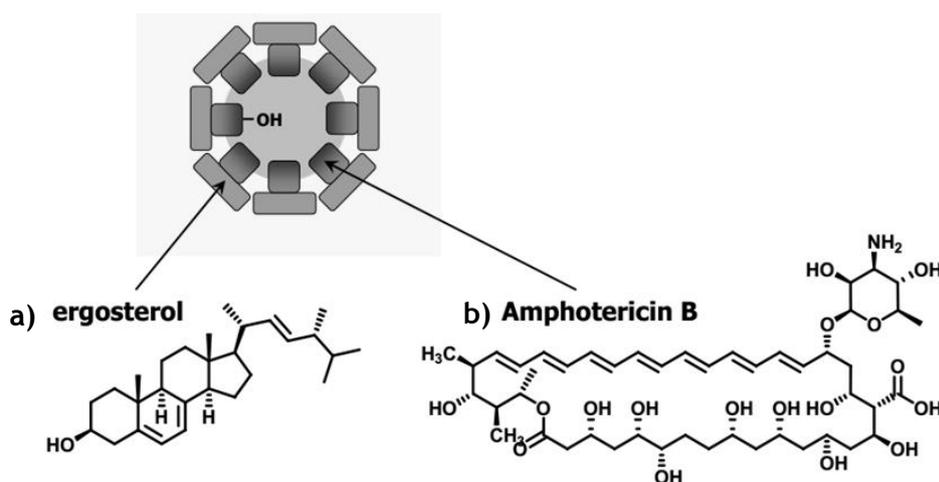
Azole drugs are the mainstay of treatment for aspergillosis. They inhibit the fungal cytochrome P450 dependent enzyme called lanosterol 14 $\alpha$ -demethylase. The inhibition of this enzyme depletes the ergosterol synthesis from lanosterol, which affects the fungal membrane leading to fungal growth inhibition. Azole drugs can be categorised into two main classes, imidazoles and triazoles. Both classes share the same mechanism of action but are slightly different in structure. Imidazole is a five membered synthetic ring (Figure 1.7 a) that confers antifungal activity. It includes ketoconazole, miconazole and tioconazole. The poor antifungal response and toxicity associated with imidazoles led to the development of new azoles, namely triazoles (Maertens, 2004). Triazoles are developed by a minor modification in imidazole ring (Figure 1.7 b), which gives improved activity and less adverse effects. Triazoles include fluconazole, itaconazole, voriconazole and posaconazole. According to a recent survey fluconazole (60.5%) was one of the most used antifungal agents in European hospitals and voriconazole (70%) in USA and Canada (Karas et al., 1976, Kawamura et al., 2000).



**Figure 1.7 - Azoles.** Structure of two different classes of azoles, (a) imidazole and (b) triazole.

### 1.3.2.2 Polyenes

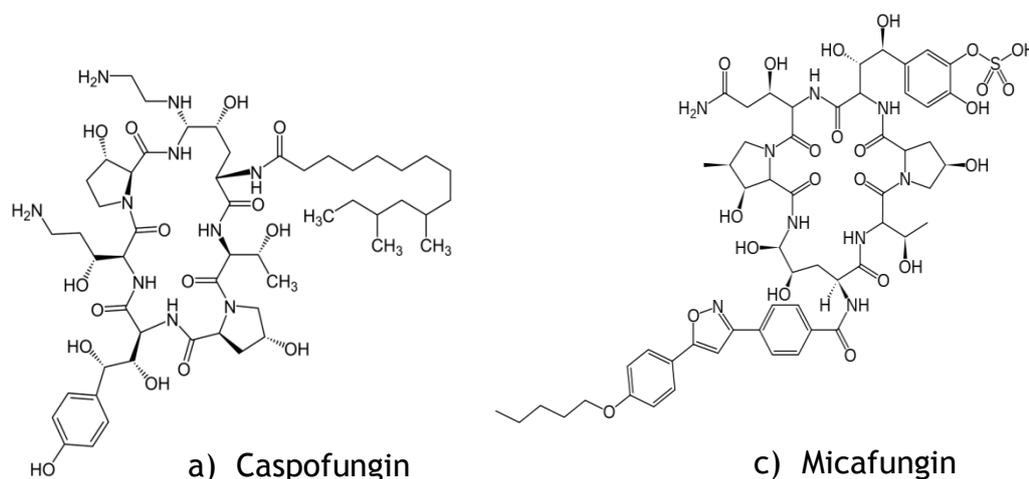
The polyene class of antifungals remain an important antifungal option for life threatening invasive aspergillosis. Polyenes are naturally-occurring poly-unsaturated compounds consist of seven conjugated carbon-carbon double bonds, a carboxyl group, an ester and a primary amino group along the side chain (Figure 1.8 b). Amphotericin B is one of the organic polyene compounds first isolated in the mid-1950s from *Streptomyces nodosus* (Dixon and Walsh, 1996). Amphotericin B presents a broad spectrum of antifungal activity. It binds with membrane sterols, forming a transmembrane channel causing cell leakage, leads to cell death (Figure 1.8) (Ghannoum and Rice, 1999, Milhaud et al., 2002). One of the problems with polyenes is their drug target, sterols. Both mammalian (cholesterol) and fungal (ergosterol [Figure 1.8a]) cell membranes contain sterols, they are similar in structure so polyenes can be cytotoxic to host cells by forming pores within the cell membrane (Baginski and Czub, 2009, Ghannoum and Rice, 1999). Later studies mixed amphotericin B with lipid emulsions and demonstrated reduced toxicity, while having similar efficacy. Then a different lipid formulation of Amphotericin B was developed and in 1990s three distinct formulations were approved, ABCD, Amphotericin B lipid complex and unilamellar vesicle liposomal formulation.



**Figure 1.8 - Polyenes.** Amphotericin B binds with membrane sterol and alters permeability of cell membrane. Chemical structure of (a) ergosterol and (b) amphotericin B.

### 1.3.2.3 Echinocandins

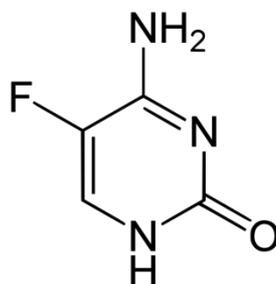
Previously mentioned antifungal classes, azoles and polyenes are associated with drug toxicities, problem with drug interactions and nonlinear pharmacokinetics (Sheehan et al., 1999, Worth et al., 2008, Lewis, 2011, Albengres et al., 1998). The lipid formulations of amphotericin B were demonstrated to be less toxic but their acquisition cost is high. Given these drawbacks, scientists were forced to look for new drug targets. In 1970s, researchers found a new class of antifungals to treat fungal infections called echinocandins. Echinocandins are non-competitive inhibitors of 1, 3 -  $\beta$  glucan synthase, an enzyme necessary for the synthesis of fungal cell wall glucan (Dixon and Walsh, 1996). Inhibition of glucan synthesis disrupts the cell wall integrity leads to fungal growth inhibition or cell death. The echinocandins display broad spectrum of antifungal activity. They are fungicidal against some species including *Candida* and fungistatic to others such as *Aspergillus* (Canton et al., 2010, Chen et al., 2011a). Caspofungin was the first licensed echinocandins drug in the market then micafungin (Figure 1.9) and anidulafungin were licensed and available for antifungal treatment.



**Figure 1.9 - Echinocandins.** Chemical structure of (a) caspofungin and (b) micafungin.

#### 1.3.2.4 Flucytosine

Flucytosine is a pyrimidine chemical compound (Figure 1.10). It is an antimetabolite type of antifungal drug that inhibits translation of mRNA in fungal cells by replacing uracil with 5-fluorouracil in RNA. It also inhibits DNA synthesis by obstructing thymidylate synthetase via 5-fluorodeoxy-uridine monophosphate (Vermees et al., 2000). It is active against some strains of *Candida*, *Aspergillus*, *Cryptococcus* and *Cladosporium* spp both in *in vitro* as well as *in vivo* conditions. Resistance to flucytosine is quite common with its use alone in antifungal treatment. Mutations in cytosine permease or cytosine deaminase enzymes are shown to be associated with flucytosine resistance (Polak and Scholer, 1975). To avoid resistance flucytosine is always administered with polyenes or azoles or with both antifungals as combination therapy (Esposito et al., 2009). Flucytosine is commercially available as oral capsules since 1972 however its intravenous formulation is no longer available.



**Figure 1.10 - Flucytosine.** Chemical structure of flucytosine.

## 1.4 Emergence of antifungal resistance

Resistance to antimicrobial drugs are well documented and remains a topic of discussion by both the scientific and non-scientific community (Neu, 1992, Okeke et al., 2005). Over the past decade antifungal resistance has emerged in pathogenic fungi such as *C. albicans* and *A. fumigatus* (Helmerhorst et al., 1999, Hsueh et al., 2005, Pfaller et al., 2011, Messer et al., 2006). Antifungal resistance has been associated with treatment failure in patients with life threatening fungal infections such as candidiasis and aspergillosis. The term ‘antifungal resistance’ can be described as a relative decrease in susceptibility of fungus to an antifungal agent when compared to other isolates. Standardised test protocols from the Clinical and Laboratory Standards Institute (CLSI, USA) or the European committee on antimicrobial susceptibility testing (EUCAST, Europe) is often used for *in vitro* susceptibility testing to detect resistance (Pfaller et al., 2010, Arendrup et al., 2010, Arendrup et al., 2011, 2008, Arendrup et al., 2012, Canton et al., 2009). Results from these *in vitro* tests are useful in calculating clinical breakpoints and epidemiological cut off values (ECV) (Table 1.7). The cut off values are valuable data helpful to identify the acquired resistance in fungi (Meletiadiis et al., 2012).

**Table 1.7 - MIC of azole drugs and ECVs for *A. fumigatus* (Lewis, 2007b).**

Antifungal agent	No. of isolates tested	MIC ( $\mu\text{g/ml}$ )		
		Range	Mode	ECV (%) <sup>a</sup>
Itraconazole	637	0.015-2	0.25	1 (99.8)
Posaconazole	637	0.06-1	0.03	0.25 (99.8)
Voriconazole	637	0.12-4	0.25	1 (99.2)

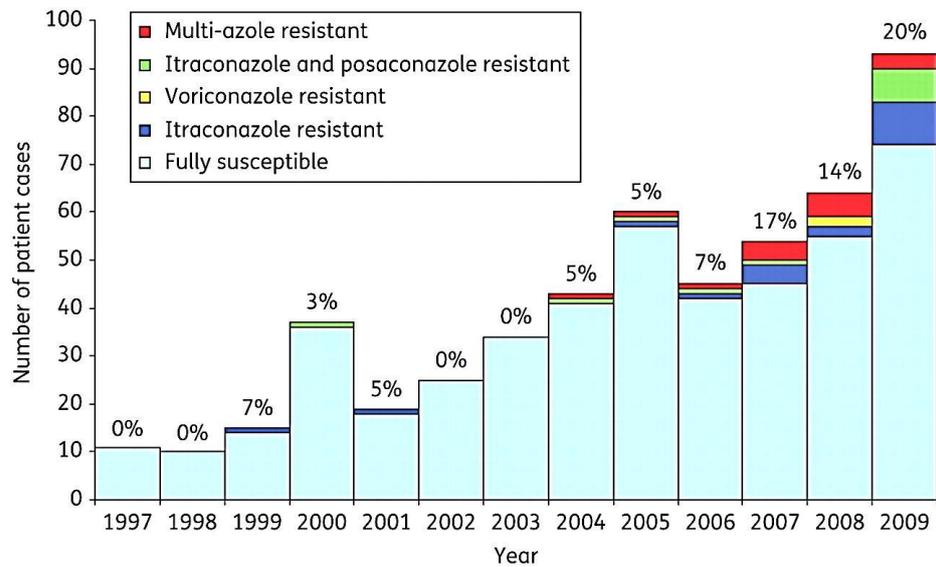
<sup>a</sup> Values in bracket indicate the percentage of MICs  $\leq$  ECV.

Azole drugs are one of the recommended antifungal agents for both clinical settings and in agriculture. In clinical settings azoles are the first line treatment choice for aspergillosis, with echinocandins and polyenes as second line. Recent surveys report the association of long term treatment and prophylaxis in relation to antifungal resistance (Auberger et al., 2012, Escribano et al., 2012). In the last ten years, azole resistance has predominantly been described for *A. fumigatus* (Figure 1.11a)(Howard and Arendrup, 2011). Azoles drugs are the only oral formulated agent available for antifungal treatment that is often used over long period of time, for example the CF patients exposed to itraconazole for along time were associated with higher prevalence of azole resistance (Burgel et al., 2012). In the Netherlands, the prevalence of azole resistance was reported to vary between 0.9 to 9.4%, and the use of azoles for protecting agriculture crops and material preservation were described to be a risk factor (Verweij et al., 2012).

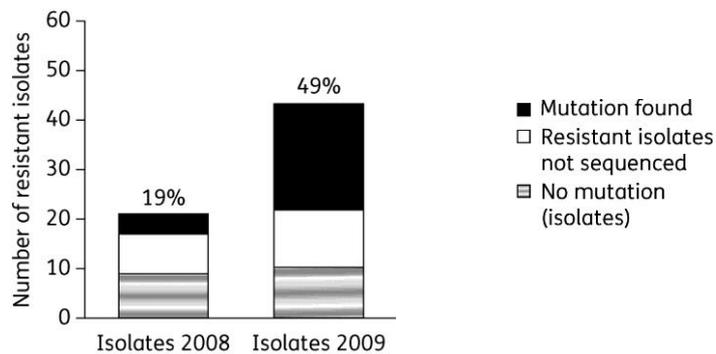
Currently, *A. fumigatus* resistance to azoles has been reported all over the world, including Belgium, Denmark, France, Norway, Spain, Sweden, Netherland and UK in Europe, India, China and Japan in Asia and USA and Canada in North America(Lockhart et al., 2011, Snelders et al., 2008, Morio et al., 2012, Chowdhary et al., 2012, Bueid et al., 2010, Mortensen et al., 2010, Vermeulen et al., 2012, Bowyer et al., 2011). Recently a study from University hospital of South Manchester published azole susceptibility data of 749 *A. fumigatus* isolates collected from 1997-2009. This study reports a significant rise in the number of azole resistant isolates from 7% in 1999 to 20% in 2009 (Figure 1.11a). Among 64 resistant isolates from 2008-09, 97% are resistant to itraconazole, 3% are voriconazole only resistant and 78% are multi azole resistant isolates (Howard et al., 2009, Bueid et al., 2010). One of the commonly reported mechanisms of azole resistance in *A. fumigatus* isolates is mutation within the *cyp51A* gene responsible for ergosterol biosynthesis. However, in Manchester the number of resistant isolates from 2008-09 with *cyp51A* mutations is less compared to other areas, about 43% of isolates were non- *cyp51A* mutants (Figure 1.11b) (Bueid et al., 2010). This indicates some other novel mechanism of resistance must be responsible. Currently, resistance to other class of antifungals are less known. *A.*

*fumigatus* resistance to caspofungin has been reported by several studies in relation to mutation or overexpression of *Fks1* gene (Lewis *et al.*, 2011, Walker *et al.*, 2010, Gardiner *et al.*, 2005, Arendrup *et al.*, 2008). *Fks1* is the  $\beta$ -glucan synthase gene that produces glucan, which is deposited in the extracellular surface forming the biofilm matrix (Beauvais *et al.*, 2001). The extracellular glucan was shown to be associated with fungal biofilm resistance by impeding drug diffusion (Nett *et al.*, 2010b). Antifungal resistance is often associated with multicellular development and biofilm formation by fungal cells (d'Enfert, 2006). Fungal biofilm-associated infections are frequently recalcitrant to conventional therapy because of resistance to antifungal agents. The following sections in this chapter discuss in detail about fungal biofilm formation and its associated mechanisms of antifungal resistance.

a)



b)



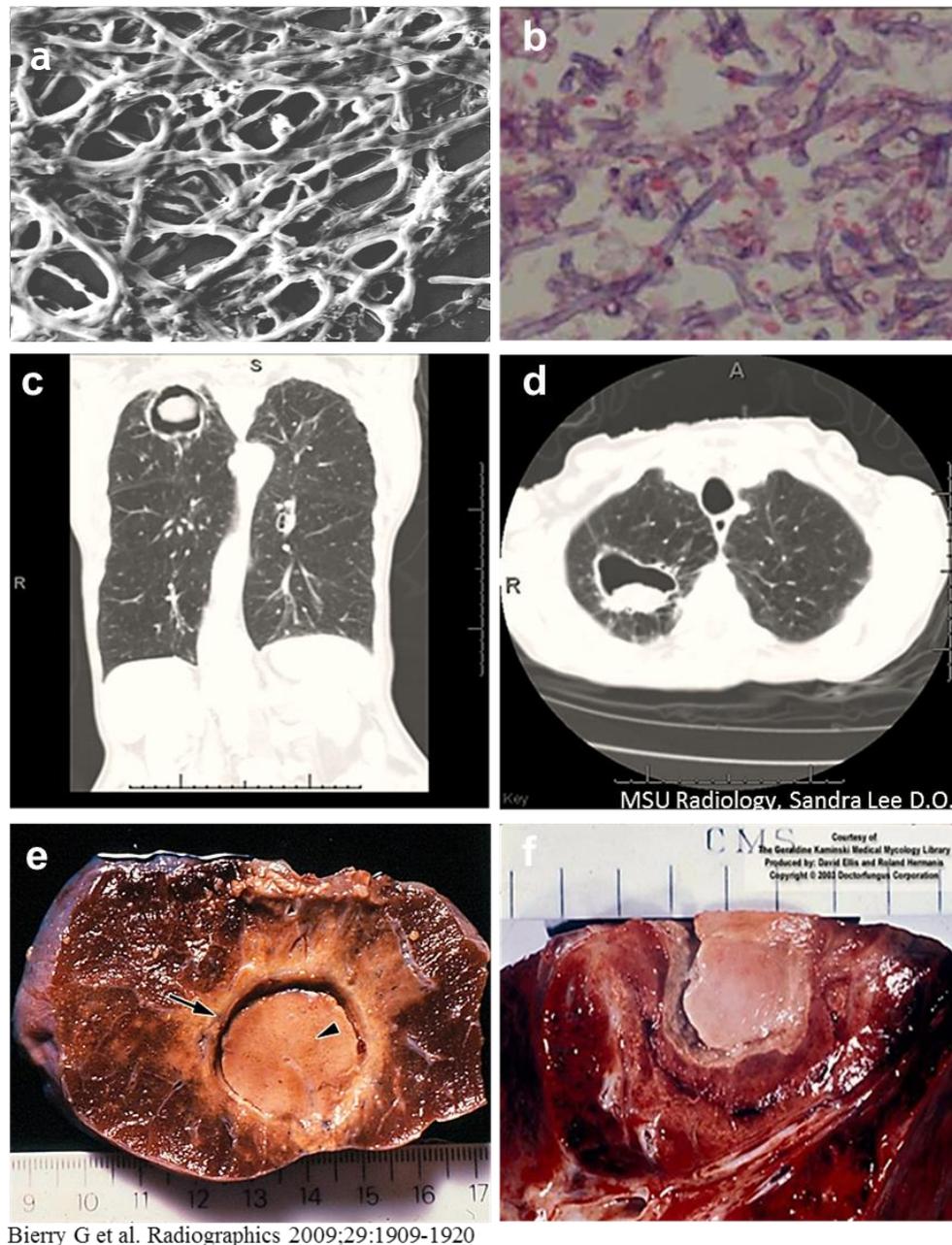
**Figure 1.11 - Prevalence of azole resistance in *Aspergillus fumigatus* 1997-2009.**

a) Bar graph showing the percentage of overall azole resistant isolates for each year above each column and colour code indicates the proportion of resistant isolates to individual azole drug or multiazole drugs. b) Graph showing the percentage of azole resistance isolates in 2008 and 2009 with *cyp51A* mutations above each column. (Bueid *et al.*, 2010).

## 1.5 Clinical significance of *Aspergillus* multicellular development

*A. fumigatus* can cause a wide spectrum of clinical disease and its growth as aspergilloma is relatively difficult to treat in comparison to other aspergillosis. Aspergillomas can develop in immune competent hosts, but usually require a pre-existing cavity such as those resulting from prior tuberculosis. The aspergilloma, a localised infection consisting of a spherical mass of hyphae has clear biofilm characteristics (Figure 1.12). Another form of aspergillosis infection, aspergillary bronchitis, is characterized by bronchial casts containing mucus and mycelia, which are associated with pathological damage (Young *et al.*, 1970). Compact masses are formed which may be expectorated. Moreover, bronchopulmonary lavage (BAL) in some patients with aspergillosis reveals the presence of numerous hyphae in the form of a complex multicellular mycetoma structure when examined histologically (Jayshree *et al.*, 2006). In contrast, IA disease is more diffuse with multiple points of angioinvasion within the pulmonary tissue. Nevertheless, filamentous intertwined hyphae are important to this process, as in other forms of aspergillosis (Mowat *et al.*, 2007b). Notably, antifungal treatment is often ineffectual, which may relate to the *Aspergillus* growth phenotype.

Clearer evidence of *Aspergillus* biofilms is demonstrated in infections affecting other sites. Aspergilli can enter the host through alternative routes causing other serious biomaterial related biofilm infections, including catheters, joint replacements, cardiac pace makers, heart valves and breast augmentation implants (Langer *et al.*, 2003, Rosenblatt and Pollock, 1997, Jeloka *et al.*, 2011, Escande *et al.*, 2011). *Aspergillus* is also frequently associated with complex sinus infections, which in canines have been described as superficial mucosal fungal plaque (Day, 2009, Laury and Delgaudio, 2010, Sato *et al.*, 2010, Grosjean and Weber, 2007). The urinary tract, whilst less frequently associated with *A. fumigatus*, has been reported to support an aspergilloma (Lee, 2010, Muller *et al.*, 2011).



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**Figure 1.12 - Filamentous growth of *Aspergillus fumigatus*.** (a) Scanning electron micrograph of *A. fumigatus* biofilm (Mowat et al., 2009), (b) Histologic section of aspergilloma shows branching hyphae of *Aspergillus* mixed with few red cells and neutrophils (Challa et al., 2011). (c-d) Chest radiograph shows a thin walled cavitory lesion within the right upper lobe measuring 4.8 x 5.2 x 5.4 cm. and CT of the thorax demonstrates soft tissue mass within the cavitory lesion this makes the diagnosis of aspergilloma most likely (Sandra Lee D.O., MSU Radiology). (e-f) a gross specimen slice shows a centrally located aspergilloma (arrowhead) delimited by a fibrous wall (arrow) (Bierry et al., 2009).

## 1.6 *Aspergillus* biofilms

Microbiologists have historically studied planktonic (free floating and homogeneous cells) in pure-culture. However, there has been a paradigm shift as the link between sessile (surface attached and heterogeneous cells) and microbial pathogenesis and human infection is now widely accepted (Costerton et al., 1995). There has been much debate within the mycology community of what specifically constitutes a biofilm. The ability of fungi to attach to a surface and/or to one another and to be enclosed within an extracellular matrix (ECM) is sufficient to fit the basic criteria of a microbial biofilm. From the available literature it is increasingly clear that different *Aspergillus* species do have this overall capacity, which is hardly surprising given that 80% of all microorganisms are proposed to exist within multi-cellular communities. Moreover, 65% of human infection is biofilm associated, which is related to the increasing number of immunocompromised patients and the escalating use of biomaterials in medicine (Ramage et al., 2009a, Donlan, 2002, Blankenship and Mitchell, 2006, Polak and Scholer, 1975).

Biofilms are defined as highly structured communities of microorganisms that are either surface-associated or attached to one another, and are enclosed within a self-produced protective extracellular matrix (ECM) (Ramage et al., 2009a). The advantages to an organism of forming a biofilm include protection from the environment, resistance to physical and chemical stress, metabolic cooperation and a community based regulation of gene expression. Fungi growing within biofilms exhibit unique phenotypic characteristics compared to their planktonic counterpart cells, particularly increased resistance to antimicrobial agents (Ramage et al., 2009a). In addition to providing safe sanctuary for microorganisms biofilms may also act as reservoirs for persistent sources of infection in a patient and as such adversely effect the health of an increasing number of individuals, including patients with HIV-infection, cancer, transplants, patients requiring surgery or intensive care and newborn infants (Calderone, 2002, Ramage et al., 2006).

The adhesion and colonization of complex fungal populations onto biological and innate surfaces, such as the oral mucosa or denture acrylic substrates, is commonplace for clinically relevant fungi (Ellepola and Samaranayake, 1998, Holmes et al., 2002, Radford et al., 1999, Williams et al., 2011). A wide variety of environmental factors contribute to the initial surface attachment, including the flow of the surrounding medium (urine, blood, saliva, mucus), pH, temperature, osmolarity, bacteria, presence of antimicrobial agents and host immune factors (Hawser et al., 1998, Baillie and Douglas, 2000, Richard et al., 2005, Ramage et al., 2002c, Ramage et al., 2008, Chandra et al., 2001b). Fungal biofilms have defined phases of development that have been described through the use of defined model systems (Al-Fattani and Douglas, 2004, Baillie and Douglas, 1998a, Chandra et al., 2001a, Garcia-Sanchez et al., 2004, Nikawa et al., 2003, Ramage et al., 2001a, Ramage et al., 2008). These key phases include arriving at an appropriate substratum, adhesion, colonisation, extracellular matrix (ECM) production, biofilm maturation and dispersal (Blankenship and Mitchell, 2006, Chandra et al., 2001a, Ramage et al., 2001b). Understanding this entire process has enabled us to begin to unravel some of the mechanisms involved in resistance.

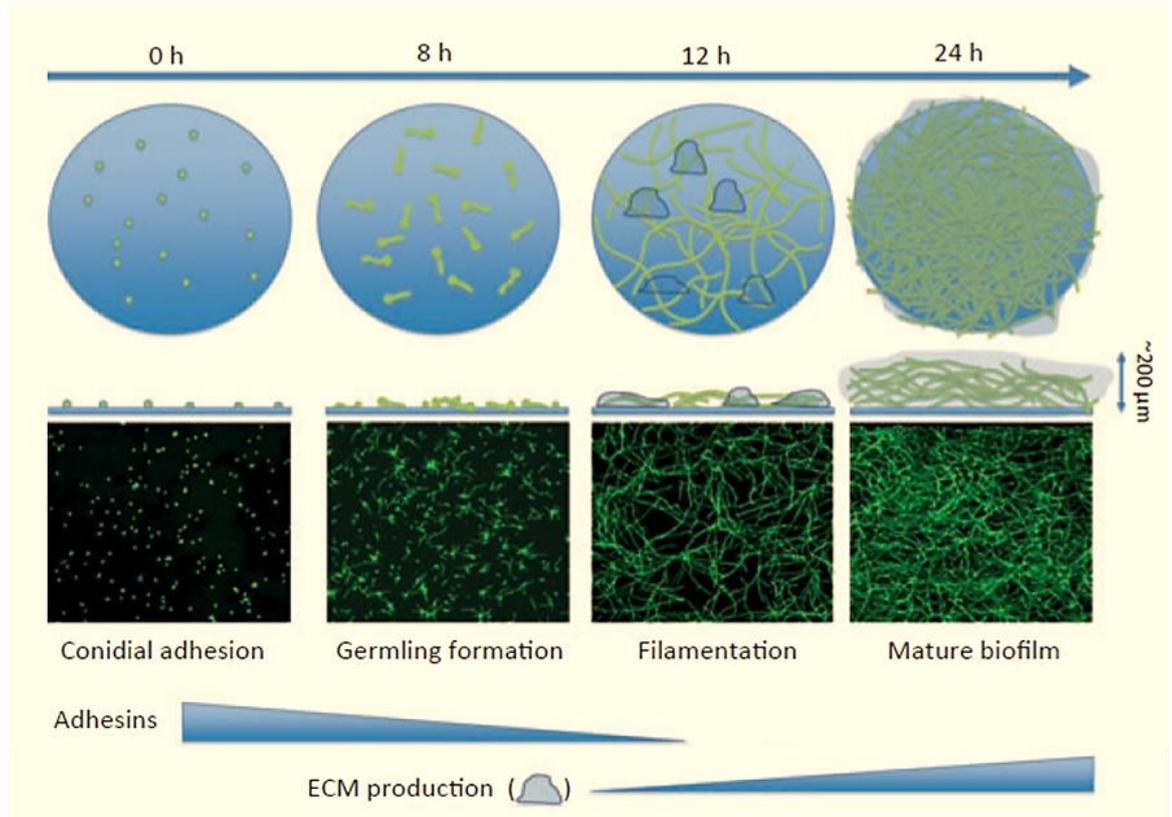
### 1.6.1 Biofilm development

The initial non-clinical based studies explored whether an *A. fumigatus* multicellular structure (or mycelial mass) fits the criteria for a biofilm, and represents a source of continuing debate (Chandrasekar and Manavathu, 2008, Mowat et al., 2008c). A key factor as observed early in these investigations was the critical importance of conidial seeding density, a phenomenon also described in studies of *A. niger* biofilms (Villena and Gutierrez-Correa, 2006). Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) demonstrated an optimal conidial seeding density of  $1 \times 10^5$  conidia/mL of liquid medium. Therefore, structural morphology and integrity was dependant upon the concentration of conidia (Mowat et al., 2007b). This biofilm system was then amenable to high throughput testing required for the screening of clinical isolates and defined mutants, or for testing the susceptibility of antifungal

agents (Mowat et al., 2008a). This concentration differs from those described elsewhere, where it was reported that an optimized conidial density of  $1 \times 10^6$  conidia/mL was used, a concentration also reported within an epithelial-biofilm co-culture system (Seidler et al., 2008, Villena and Gutierrez-Correa, 2006). However, this log difference was due to subtleties of biofilm model systems, which both utilise different substrates and media.

Fungal biofilms, like bacterial biofilms, have defined developmental phases that include arrival at an appropriate substrate, adhesion, colonisation, polysaccharide production and biofilm maturation, and dispersal (Chandra et al., 2001a, Donlan, 2002, Blankenship and Mitchell, 2006). Following initial conidial seeding there is a lag phase (conidial adhesion), germination (6 to 8 h), filamentation and formation of a monolayer (12 h), followed by increased structural complexity, ECM production and maturation (24 h). During this time the depth of the biofilm increases from 10 to 200  $\mu\text{m}$  (Mowat et al., 2007b). These phase dependant growth characteristics play a key role in the outcome of antifungal treatment. The development of *A. fumigatus* biofilms are illustrated in Figure 1.13.

The process of *A. niger* biofilm formation can also be divided into distinct phases: (i) adhesion, which is strongly increased by *A. niger* spore hydrophobicity; (ii) an initial growth and development phase from spore germination to surface colonization; (iii) a maturation phase, in which biomass density is highly increased with development of an internal channel organization (Gutierrez-Correa and Villena, 2003). These channels appear to allow fluids to pass through, favouring a better mass transfer (Villena et al., 2010, Villena and Gutierrez-Correa, 2006, Villena and Gutierrez-Correa, 2007a). There is also different spatial growth coordination when fungus adheres to the surface. This coordination responds to steric interactions between hypha and tips in contact with surfaces. At short distances, binary interactions (tip-hyphae) involve a local spatial rearrangement, resulting in a slowing down of the tip extension rate and consequently in a control of maximum biomass surface density (Villena *et al.*, 2010).



**Figure 1.13 - *Aspergillus fumigatus* biofilm development.** The different phases of biofilm development are represented schematically, from initial adhesion of conidia, germling formation (8 h), a monolayer of intertwined hyphae (12 h) and mature 3D filamentous biomass (c. 200  $\mu\text{m}$ ) encased within ECM (24 h). These are also illustrated in the adjacent confocal laser scanning micrographs stained using FUN1 (Molecular Probes) (Ramage et al., 2011).

### 1.6.2 Adhesion

Filamentous growth is a fundamental feature of fungal biofilms and is an important morphological characteristic of *A. fumigatus* required during the development of an aspergilloma (Ramage et al., 2009a). The initial establishment of these chronic infections involves the germination of conidia and subsequent hyphal invasion of the lung tissues (Filler and Sheppard, 2006). Fungal spores adhere to compatible surfaces through several mechanisms, which include complex interactions of physical and biological processes. Physical properties of support like hydrophobicity, electrostatic charge and surface roughness are important at the initial adhesion step of bacteria, as well as yeasts and filamentous fungi (Bigerelle et al., 2002, Cunliffe et al., 1999, Dufrene, 2000, Webb et al., 1999).

A small class of amphipathic proteins called hydrophobins principally mediate adhesion in filamentous fungi, and have recently been shown to play a role in fungal biofilm development (Armenante et al., 2010, Bruns et al., 2010, Perez et al., 2011). Hydrophobins stabilize the adhesion of spores to both natural and artificial hydrophobic surfaces possibly generating morphogenetic signals (Linder et al., 2005, Wosten, 2001, Scholtmeijer et al., 2001). Hydrophobins, a family of small-secreted proteins with a characteristic pattern of eight cysteine residues, have been reported in *A. fumigatus* to be responsible for the strong adhesion forces of  $2858 \pm 1010$  pN during spore adhesion to surfaces (Dupres et al., 2010, Dague et al., 2008). It seems that conidium contact per attachment is required to trigger germination (Shaw et al., 2006).

It has been shown that when *A. niger* biofilms are under stress caused by low water activity ( $a_w$ ) then high amounts of extracellular material are secreted (Villena and Gutierrez-Correa, 2007c). In some plant pathogenic fungi like *Bipolaris sorokiniana* the production of extracellular material appears to be important for adhering conidia and germlings to the host surface (Apoga et al., 2001). For the development of *A. niger* biofilms the spore rough surface is important for its first physical attachment to the support surface and this

process is also helped by the production of adhesive substances forming a pad beneath spores; this has been found when different supports were used, indicating that the adhesive substances are part of the adsorption process (Villena and Gutierrez-Correa, 2007b, Gamarra et al., 2010, Lord and Read, 2011).

Further studies of the genetic basis of biofilm formation have revealed a role for *medA*, which has recently been characterized with respect to conidiation, host cell interactions and virulence (Gravelat et al., 2010). Here it was reported that in addition to its role in conidiophore morphology, it was shown that its mutant phenotype was impaired in biofilm production in addition to adherence to plastic, pulmonary epithelial cells, endothelial cells and fibronectin *in vitro*. Moreover, this strain had reduced capacity to damage pulmonary epithelial cells and stimulate pro-inflammatory cytokines, which was reflected by reduced virulence in both an invertebrate (*Galleria mellonella*) and a mammalian model (mouse) of invasive aspergillosis (Gravelat et al., 2010).

It was also shown that deletion of *cspA* (cell surface protein A), which encodes a repeat-rich glycosylphosphatidylinositol (GPI)-anchored cell wall protein, causes weakening of the conidial cell wall (Levdansky et al., 2010). Analysis of double mutants indicated that *CSPA* interacts with the cell wall protein-encoding genes *ECM33* and *GEL2*, deletion of which results in strongly reduced conidial adhesion, increased disorganization of the conidial cell wall, and exposure of the underlying layers of chitin and beta-glucan. Given the number of genes integral to adhesion and cell wall structure, it is likely that many play a pivotal role in the different phases of biofilm formation, and as biofilm becomes an accepted term within the *Aspergillus* research community many future studies will elucidate the molecular pathways for its development.

### 1.6.3 Molecular Mechanisms

Very few reports on the molecular biology and functional genomics of *Aspergillus* biofilms have been published, yet a recent study reported global transcriptional and proteomic biofilm specific changes in *A. fumigatus* (Bruns et al., 2010). Planktonic- and biofilm-grown mycelium at 24 and 48 h growth were analysed using microarrays and 2-D gel electrophoresis. Both biofilm- and time-dependent regulation of many proteins and genes associated with primary metabolism was demonstrated, indicating an energy dependant developmental stage of young biofilms. Biofilm maturation showed a reduction of metabolic activity and an upregulation of hydrophobins, and proteins involved in the biosynthesis of secondary metabolites, such as gliotoxin (Bruns et al., 2010). Specifically, it was shown that 36 protein spots changed in biofilm mycelium of *A. fumigatus* in comparison to planktonic mycelium, and 78 protein spots changed significantly during biofilm maturation. Based on FunCat categorisation these included proteins involved in 'metabolism', 'protein with binding function or cofactor requirement' and 'cellular transport, transport facilitation and transport routes'. Transcriptional profiling demonstrated that 740 genes were differentially regulated (179 up- and 561 down-regulated) with respect to 24 h biofilm versus planktonic cells. The up-regulated genes were mainly involved in protein synthesis, metabolism, energy conservation, and encoded for proteins with binding function or cofactor requirement. Many down-regulated genes were involved in signal transduction, cell type differentiation, interaction with the environment, biogenesis of cellular components, regulation of metabolism and protein function, as well as cell and protein fate (Bruns et al., 2010). Recent work has used RNA-Seq to compare the transcriptomes of biofilm and liquid planktonic growth, where sequencing identified 3,728 differentially regulated genes in the two conditions (Gibbons et al., 2011). In addition to many genes that are likely to reflect the different growth demands, these investigations identified many up-regulated genes involved in transport, secondary metabolism, and cell wall and surface functions. Mapping of these genes showed significant spatial structure across the genome. 1,164 genes were down-regulated, which were involved in primary metabolic functions, including carbon

and amino acid metabolism. Interestingly, these were not spatially structured across the genome. This work has provided some initial insight into the genetics of biofilm formation.

## 1.7 Mechanisms of fungal biofilm resistance

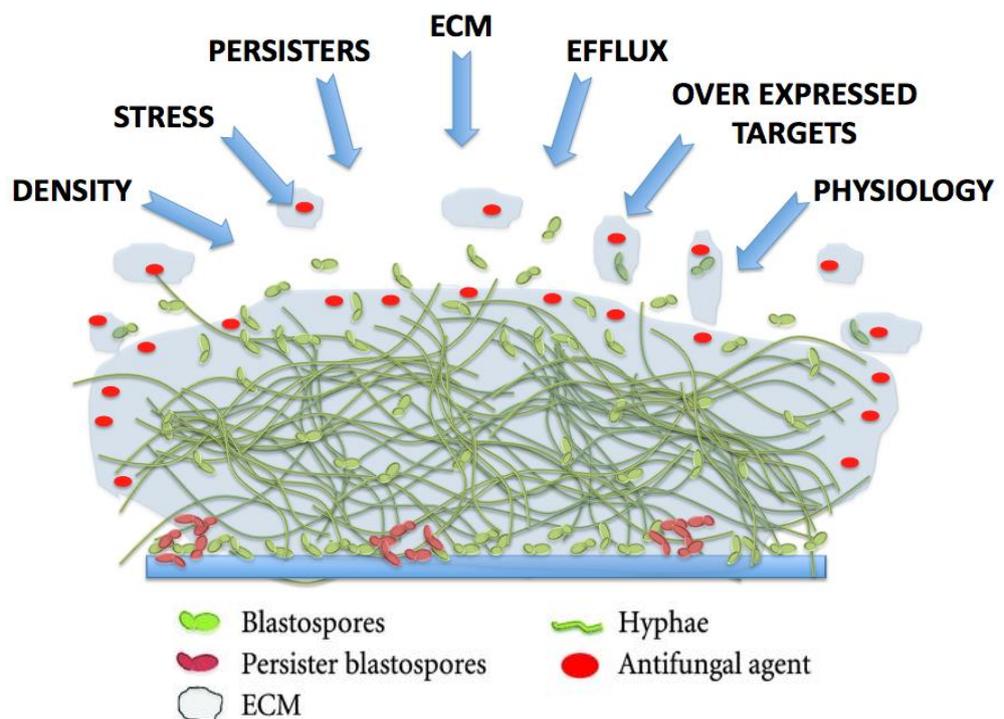
One of the defining characteristics of biofilms is their increased resistance to antimicrobial agents. Fungi have been reported to be up to 1000-fold more resistant to antifungal agents than planktonic free floating cells, yet this recalcitrance to antimicrobial therapy has yet to be fully elucidated (Di Bonaventura et al., 2006, Ramage et al., 2001a, Tre-Hardy et al., 2008). Despite some antifungal agents being efficacious against fungal biofilms, particularly the echinocandins and liposomal amphotericin B formulations, the intrinsic resistance exhibited by these complex structures has promoted detailed investigation (Kuhn et al., 2002, Ramage et al., 2002b, Bachmann et al., 2003, Bachmann et al., 2002, Mowat et al., 2008a).

Antifungal resistance is both complex and multi-factorial (Figure 1.14). It can be inducible in response to a compound, or an irreversible genetic change resulting from prolonged exposure. Specifically, these include alterations or over-expression of target molecules, active extrusion through efflux pumps, limited diffusion, tolerance and cell density, which are all characterised mechanisms utilised by fungi to combat the effects of antifungal treatment (Niimi et al., 2010). Planktonic cells generally rely on irreversible genetic changes to maintain a resistant phenotype, whereas biofilms are able to persist due to their physical presence and the density of the population, which provides a resistant phenotype irrespective of defined genetic alterations. The biofilm formation and its underlying resistance mechanisms in *Candida* species are well documented compared to other fungal species. *C. albicans* is one of the top four fungal pathogens causing nosocomial infections in humans. The morphology of *A. fumigatus* biofilms is somewhat analogous to a *Candida* species (Mowat et al., 2008a, Mowat et al., 2008c, Chandra et al., 2001a). The following section will

now describe some of the pivotal factors that play a role in fungal biofilm resistance with an emphasis on the paradigm, i.e. *Candida* species.

### 1.7.1 Physiological state

The general physiological state of cells in sessile populations has also been implicated to influence the susceptibility profiles of biofilms. Metabolic dyes assays (e.g. XTT-based assays) confirm that cells within biofilms are undergoing mitochondrial respiration during development (Chandra et al., 2001a, Hawser, 1996, Ramage et al., 2001a, Kuhn et al., 2003, Mowat et al., 2008a). Other factors including the effect of growth rate on *C. albicans* biofilm resistance has also been studied, where varying the rates were shown to play no role in resistance to amphotericin B (Baillie and Douglas, 1998a). Similarly, biofilms of *C. albicans* grown under glucose and iron limited conditions were shown to both be highly resistant to amphotericin B (Baillie and Douglas, 1998b). Furthermore, studies of biofilms grown under anaerobic conditions demonstrated that *C. albicans* biofilms were resistant to high levels of amphotericin B and different azole antifungals (Dumitru et al., 2004). Nevertheless, factors including pH, temperature, oxygen availability and other environmental stresses will alter the biofilm architecture and possibly antifungal sensitivity (Kucharikova et al., 2011, Pettit et al., 2010). Therefore, whilst the physiological state of the cell may have a minor role in resistance (e.g. dormancy), it is more likely that more complex factors are involved.



**Figure 1.14 - Schematic overview of fungal biofilm resistance mechanisms.** Generic overview of key biofilm resistance mechanisms associated with *C. albicans*, but which are likely to be common to other fungi. This figure illustrates the density and complexity of the *C. albicans* biofilm, with different morphotypes present surrounded by ECM. The arrows represent the different factors that drive antifungal resistance within the biofilm, including density, stress, persisters, ECM, efflux, over-expressed targets and the general physiology of the biofilm. These have been placed according to their contribution to resistance, with those that have a greater effect closer to the middle and those with less impact at the edges (Ramage et al., 2012).

### 1.7.2 Cell density

The architecture of biofilms is highly ordered to enable the perfusion of nutrients and expulsion of waste products. Mature biofilms, whilst densely populated, exhibit spatial heterogeneity with microcolonies and water channels being present, features are common to both bacterial and fungal biofilms (Chandra et al., 2001a, Lawrence et al., 1991, de Beer et al., 1994). Cell density is therefore an important resistance factor within complex biofilm populations of yeast and filamentous fungal biofilms. It was demonstrated that both planktonic and resuspended biofilm cells exhibited azole sensitivity at low cell numbers ( $10^3$  cells/ml), which became increasingly resistant as the density of the cells increased tenfold (Perumal et al., 2007), a phenomenon also been demonstrated in *A. fumigatus* (Lass-Flörl et al., 2003). A phase-dependant increased antifungal resistance has been shown in *A. fumigatus* and *C. albicans*, respectively (Mowat et al., 2008a, Mukherjee et al., 2003), which support the idea that the physical density of the biofilm produces recalcitrance to antifungal agents.

Within dense biofilms there is cooperation between individual cells through quorum sensing processes, which provide the ability of microorganisms to communicate and coordinate their behaviour via the secretion of signalling molecules in a population dependent manner (Miller and Bassler, 2001). In fungi this was first described in *C. albicans* when Hornby and colleagues identified farnesol *trans*, *trans* 3,7,11-trimethyl-2,6,10-dodecatrien-1-ol (Hornby et al., 2001). Exposing *C. albicans* to exogenous farnesol results in genome wide responses, including genes involved in drug resistance (*CaFCR1* and *CaPDR16*) (Cao et al., 2005, Enjalbert and Whiteway, 2005). It has now been shown that quorum sensing in *C. albicans* is likely driven by the two component regulatory system of Chk1p (Kruppa et al., 2004). However, when deleted the  $\Delta$ *chk1* strain shows a similar azole resistance profile to that of wild type (Perumal et al., 2007), indicating that regulatory circuit that controls biofilm resistance may be yet to be discovered, or cell density is not a defined biofilm resistance factor. However, given that echinocandins are highly effective against biofilms suggests cell density has a limited effect (Bachmann et al., 2002). In addition, previous

work has shown that disrupted biofilms that are resuspended and tested using the CLSI methodology in comparison to planktonic cells retain a resistant phenotype (Ramage et al., 2002a), indicating alternative mechanisms of resistance. The process of quorum sensing in *A. fumigatus* biofilms is unclear. The quorum sensing molecule farnesol was shown to be associated with cell wall integrity pathway in *A. fumigatus* (Dichtl et al., 2010), but its role in controlling cell density or biofilm resistance is not known.

### 1.7.3 Alterations of drug targets

The azoles are generally fungistatic against yeasts, including *Candida* species, and fungicidal against moulds, such as *Aspergillus* species. The fungistatic nature of the azoles towards *C. albicans* induces a strong directional selection on the surviving population to evolve drug resistance (Anderson, 2005, Cowen, 2008). In fact, high levels of azole resistance in *C. albicans* clinical isolates often accumulate through multiple mechanisms including the alteration of *ERG11* (Anderson, 2005). Azoles actively target the 14  $\alpha$ -demethylase enzyme, blocking ergosterol biosynthesis and leads to depletion of the ergosterol content of membranes and results in the accumulation of toxic sterol pathway intermediates, such as 14 $\alpha$ -methylergosta-8,24(28)-dien-3 $\beta$ ,6 $\alpha$ -diol, which inhibits growth (Akins, 2005, Cannon et al., 2007). The principle drug target, Erg11p, can develop point mutations or be overexpressed (Akins, 2005, Cannon et al., 2007, White, 1997). Common mutations in the Erg11p that confer moderate azole resistance are S405F, Y132H, R467K and G464S (Marichal et al., 1999, Perea et al., 2001, Sanglard et al., 1998, Blosser and Cramer, 2012). In *A. fumigatus*, point mutations in *ERG11* such as N22D, M220 (I, V, K, T), G54 (R, E, K, V), Y491H, T440A, M236 (V, K, T) and S35S or overexpression of *ERG11* gene were shown to be associated with azole resistance (Blosser and Cramer, 2012, Diaz-Guerra et al., 2003, Edlind et al., 2001, Mellado et al., 2001, Chen et al., 2005)

Given the importance of ergosterol as a target of azoles and the high level resistance exhibited by these structures, then the sterol composition of *C. albicans* biofilms has been investigated. Sterol analyses showed that ergosterol levels were significantly decreased in intermediate (12 h) and mature phases (48 h), compared to those in early-phase biofilms (6 h) (Mukherjee et al., 2003). In contrast, in one of the first *C. albicans* biofilm studies to use microarray analysis over-expression of *ERG25* and *ERG11* was reported (Garcia-Sanchez et al., 2004). Enrichment or redistribution of ergosterols in biofilm membranes may explain their resistance to both azole- and polyene derived antifungal agents. For example, *C. albicans* biofilms cultured in a flow cell for 36 h were compared to planktonic cells, where it was shown that a subpopulation of blastospores from the biofilm were 10 times more resistant to amphotericin B than planktonic populations (Khot et al., 2006). Transcriptional analysis of planktonic and the biofilm subpopulation for ergosterol genes and beta-1, 6-glucan pathways indicated a possible association between the high level of resistance and differential regulation of *CaERG1*, *CaERG25*, *CaSKN1*, and *CaKRE1*. Therefore, changes in both the cell membrane and the cell wall may be important determinants of resistance in the biofilm. Subsequent work has shown that transcriptional responses in young and mature biofilms after exposure to high doses of fluconazole or amphotericin B demonstrated differential antifungal drug responses (Nailis et al., 2010). Exposure of both young and mature biofilms to fluconazole induced up-regulation of genes encoding enzymes involved in ergosterol biosynthesis (*CaERG1*, *CaERG3*, *CaERG11* and *CaERG25*), particularly biofilms exposed for longer periods (22 h). Whereas, treatment of both young and mature biofilms with amphotericin B resulted in an overexpression of predominantly *CaSKN1*, with a modest upregulation of *CaKRE1*. Removal of the antifungal in this study depleted further transcriptional changes, except for *CaSKN1*, which was impacted by prior fluconazole exposure. It was speculated that this related to biofilm regrowth. Increased ergosterol gene expression has also been reported *in vivo* in a *C. albicans* central venous catheter biofilm model, demonstrating the importance of the molecule within the biofilm (Nett et al., 2009). Induction of ergosterol genes has also been described in *C.*

*dublinsi*, where incubation with fluconazole and formation of biofilm was coupled with up-regulation of the *CdERG3* and *CdERG25* (Borecka-Melkusova et al., 2009). Moreover, up-regulation of genes involved with ergosterol biosynthesis has been described in *C. parapsilosis* biofilms (Rossignol et al., 2009), which are also resistant to azole antifungal therapy (Katragkou et al., 2008). Overall these data highlight the importance of ergosterol in biofilm resistance. Recent studies have shown that simvastatin, which impairs cholesterol metabolism in humans, is capable of inhibiting *C. albicans* biofilms, thus providing a potential novel strategy of combating these tenacious infections (Liu et al., 2009).

#### 1.7.4 Efflux pump mediated resistance

The primary molecular mechanism leading to high level azole resistance in *C. albicans* is increased efflux of drug, mediated mostly by the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters (Albertson et al., 1996, Lopez-Ribot et al., 1999, Sanglard et al., 1997). The ABC transporters in *C. albicans* constitute a multi-gene family, which includes several *CDR* genes (*CDR1-4*) (Prasad et al., 1995, Walsh et al., 1997). The ABC transporters include a membrane pore composed of transmembrane segments and two ABCs on the cytosolic side of the membrane, which provide the energy source for the pump (Higgins, 1992, Balzi and Goffeau, 1995). Importantly, multiple antifungal agents can be substrates for these transporters and thus their over-expression can lead to cross-resistance among different drugs, particularly azoles. Among members of the MFS, which are secondary transporters and use proton-motive force across the plasma membrane, the *MDR1* gene encodes a major facilitator that has been implicated in *C. albicans* azole resistance, and its over-expression leads to exclusive fluconazole resistance (White, 1997, Williams et al., 2011). Echinocandin sensitivity is unaffected by efflux pumps (Niimi et al., 2006).

Genes encoding for drug efflux pumps have been reported in biofilms to be differentially regulated during development and upon exposure to antimicrobial agents include *CaCDR1*, *CaCDR2* and *CaMDR1* (Lepak et al., 2006, Mateus et al.,

2004, Mukherjee et al., 2003, Ramage et al., 2002a). In the first study to investigate the role of efflux pumps, it was demonstrated that expression of genes encoding both types of efflux pump was upregulated during the course of biofilm formation and development. Both *CaCDR1* and *CaCDR2* were upregulated in 24 and 48 h biofilms, whereas *CaMDR1* was transiently upregulated at 24 h (Ramage et al., 2002a). However, their contribution to resistance in the biofilm phenotype was placed in doubt when a set of *C. albicans* isogenic strains deficient in efflux pumps carrying single- and double-deletion mutations ( $\Delta cdr1$ ,  $\Delta cdr2$ ,  $\Delta MDR1$ ,  $\Delta cdr1/\Delta cdr2$ , and  $\Delta MDR1/\Delta cdr1$ ) that rendered the cells hypersusceptible to fluconazole when planktonic retained the resistant phenotype during biofilm growth. In a subsequent investigation, *C. albicans* biofilms were formed through three distinct developmental phases that were associated with high fluconazole resistance. Again, the same set of isogenic *C. albicans* strains were utilised where it was shown that 6h old biofilms formed by double and triple mutants were >4 to 16-fold more susceptible to fluconazole than the wild-type strain (Mukherjee et al., 2003). At 12 and 48h, all the strains became highly resistant to this azole, indicating lack of involvement of efflux pumps in resistance at late stages of biofilm formation.

Cell density studies of the efflux pump isogenic strains showed these remain hypersensitive at low cell concentrations yet resistant at high cell concentrations and in biofilm, indicating a contributory resistance role of cell density (Perumal et al., 2007). Nevertheless, similar to the study by Ramage and co-workers (Ramage et al., 2002a), *C. albicans* biofilms were shown to express *CaCDR* and *CaMDR1* genes in all three phases (6, 12, and 48h), whilst planktonic cells expressed these genes transiently. In fact, GFP promoter studies have shown induction of efflux pumps after 15 min adherence to provide a tolerant biofilm phenotype (Mateus et al., 2004). Animal studies have also shown that biofilms formed on implanted catheters express efflux pumps (Andes et al., 2004, Nett et al., 2009). Transcript upregulation of *CaCDR2* at 12h (1.5-fold) and *CaMDR1* at both 12h (2.1-fold) and 24 h (1.9-fold) was demonstrated (Nett et al., 2009). In *C. glabrata*, similar results are reported, where expression of *CgCDR1* and *CgCDR2* was investigated during the early (6h), intermediate (15h),

and mature (48h) phases of biofilm development. At 6 h and 15h, the biofilms exhibited approximately 1.5- and 3.3-fold upregulation of *CgCDR1* and 0.5- and 3.1-fold upregulation of *CgCDR2*, respectively, in comparison to planktonic cells (Won Song et al., 2008).

Sequence analysis suggest that *A. fumigatus* has 278 different MFS and 49 ABC transporters (Nierman et al., 2005). *A. fumigatus* MDR pumps have been described in several studies, and have been shown to be associated with increased resistance to itraconazole (da Silva Ferreira et al., 2004, Nascimento et al., 2003). Currently, however, there is little evidence to suggest that these play an active role in clinical biofilm resistance (Cannon et al., 2009). This will be explored within this thesis.

### 1.7.5 Extracellular matrix

ECM is a defining characteristic of fungal biofilms, providing the cells protection from hostile factors such as host immunity and antifungal agents (Ramage et al., 2009a). In some of the pioneering work by the Douglas group *C. albicans* ECM was shown to increase when biofilms are grown under dynamic flow conditions (Al-Fattani and Douglas, 2004, Baillie and Douglas, 2000, Hawser et al., 1998). However, subsequent work has shown that while diffusion is hampered by ECM, penetration of antifungal drugs is not thought to play a key role in biofilm resistance (Al-Fattani and Douglas, 2004). Recent studies have provided new insights that suggest the chemical composition of ECM and its regulation may play a central role in resistance.

The composition of the ECM of these biofilms in *C. albicans* and *C. tropicalis* is complex, comprising of protein, hexosamine, phosphorus, uronic acid, and carbohydrates (Al-Fattani and Douglas, 2006). Recently it has been shown that extracellular DNA is another important component of the ECM (Martins et al., 2010). One of the principle carbohydrate components is beta-1,3 glucans, as treatment of *C. albicans* biofilms with beta-1,3 glucanase helps detach biofilms from a substrate (Al-Fattani and Douglas, 2006). Its contribution is confirmed in

a series of investigations by the Andes group where it was shown to increase in *C. albicans* biofilm cell walls compared to planktonic organisms, and was also detected in the surrounding biofilm milieu and as part of the ECM (Nett et al., 2007). Beta-1,3 glucans has also been shown to increase in investigations of three specific phases of biofilm development grown on both denture acrylic and catheter substrates (Yeater et al., 2007). Its contribution to resistance was realized when it was also shown that biofilm cells walls bound 4-5 fold more azole than equivalent planktonic cells, and culture supernatant bound a quantifiable amount of this antifungal agent. Moreover, beta-1,3 glucanase markedly improved the activity of both fluconazole and amphotericin B. Addition of exogenous biofilmECM and commercial beta-1,3 glucan also reduced the activity of fluconazole against planktonic *C. albicans in vitro*(Nett et al., 2007). The group have recently shown that the ECM  $\beta$ -1,3 glucan is synthesised from *Fks1p* using a defined knockout and over-expressing strain (Nett et al., 2010b). This study demonstrated that beta-1,3, glucan is responsible for sequestering azoles, acting as a 'drug sponge' and conferring resistance on *C. albicans* biofilms (Nett et al., 2010b). Further studies have shown that they are also responsible for sequestering echinocandins, pyrimidines, and polyenes (Nett et al., 2010a). This has been confirmed independently where *ampB* was shown to physically bind *C. albicans* biofilms and beta-glucans (Vediyappan et al., 2010). Subsequent studies have identified a role for the *CaSMI1* in biofilm ECM production and development of a drug resistant phenotype, which appears to act through transcription factor *CaRImp* and glucan synthase *Fks1p* (Nett et al., 2011a).

In addition to *CaFKS1*, a zinc-response transcription factor *CaZAP1* has been shown to be a negative regulator of ECM soluble beta-1,3 glucan in both *in vitro* and *in vivo C. Albicans* biofilm models through expression profiling and full genome chromatin immunoprecipitation (Nobile et al., 2009). Conversely, two glucoamylases, *CaGCA1* and *CaGCA2*, are thought to have positive roles in matrix production. A group of alcohol dehydrogenases *CaADH5*, *CaCSH1*, and *CaLFD6* also have roles in matrix production, with *CaADH5* acting positively, and *CaCSH1* and *CaLFD6* acting negatively (Nobile et al., 2009). It is thought that these

alcohol dehydrogenases generate quorum-sensing aryl and acyl alcohols, which co-ordinate biofilm maturation. Collectively, it appears that *C. albicans* ECM production is highly regulated and is a key resistance factor. It is also present on a number of other *Candida spp.*, including *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. dubliniensis* (Silva et al., 2009, Silva et al., 2011).

Significantly less is known regarding the role of *A. fumigatus* biofilm ECM in antifungal resistance. In an aerial static model, the presence of extracellular hydrophobic ECM is composed of galactomannan, alpha-1,3 glucans, monosaccharides, polyols, melanin, and proteins, including major antigens and hydrophobins (Beauvais et al., 2007b). This study demonstrated that hydrophobic matrix cohesively bound hyphae together, and that the matrix increased with maturity of the developing structure. Further studies report that a new galactosaminogalactan and the galactomannan were the major polysaccharides of the *in vivo* *A. fumigatus* ECM (Loussert et al., 2010). Extensive ECM production was also reported as the maturity of the biofilm increases (Mowat et al., 2009). For *A. niger*, after germination upon a support, the new hyphae also produce an ECM (Villena and Gutierrez-Correa, 2007b). The production of ECM has also been reported elsewhere, where it has been shown to be produced on both polystyrene and cystic fibrosis (CF) bronchial epithelial cells by *A. fumigatus*, that were resistant to antifungal agents (Seidler et al., 2008).

Martinez and Casadevall (2006) reported that *C. neoformans* also have the ability to form biofilm structures *in vitro*, and produces ECM (Martinez and Casadevall, 2006). In *Pneumocystis spp.* confocal microscopy has revealed organisms enmeshed in ECM. Intense monoclonal antibody staining to the major surface glycoproteins and an increase in (1,3)- $\beta$ -D-glucan content was also evident, suggesting that these components contributed to resistance (Cushion et al., 2009). *Blastoschizomyces capitatus*, *Malassezia pachydermatis*, *Sacchromyces cerevisiae*, *Rhizopus oryzae*, *Lichtheimia corymbifera*, *Rhizomucor pusillus* and *Apophysomyces elegans* are all reported to produce ECM in their biofilms (Beauvais et al., 2009, Cannizzo et al., 2007, D'Antonio et al., 2004,

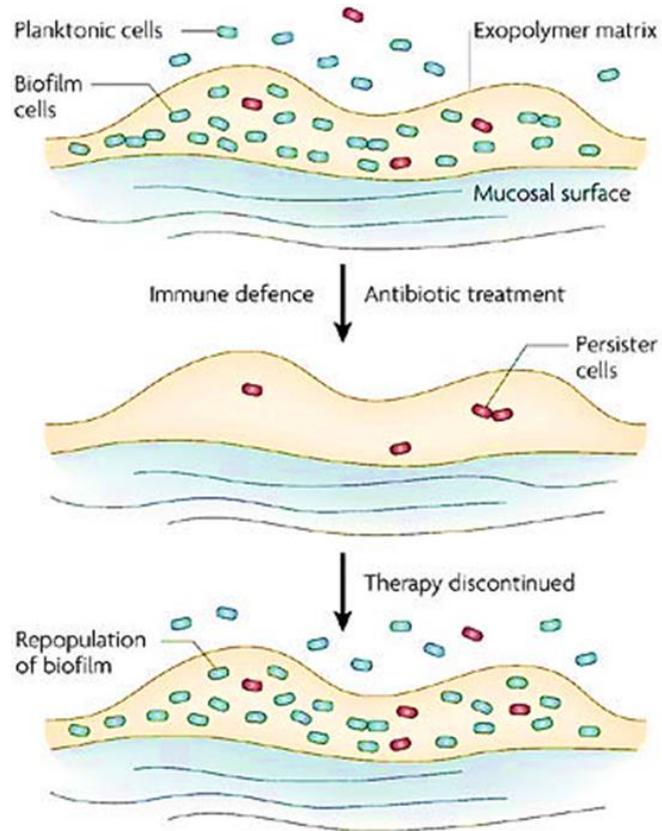
Singh et al., 2011). Therefore, ECM clearly plays a critical role in biofilm resistance for a wide number of clinically important fungi, and is one of the most significant and regulated resistance mechanisms utilized in the biofilm phenotype. Significantly less is known regarding *A. fumigatus* biofilm ECM and its role in antifungal resistance.

### 1.7.6 Persisters

Persister cells are an important mechanism of resistance in chronic infections (Fauvart et al., 2011), and a mechanism of resistance that has gathered some attention recently in fungal biofilms (Bink et al., 2011, LaFleur et al., 2006, Lewis, 2008). Persister cells are “dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics” (Lewis, 2010)(figure 4). In *C. albicans* biofilms a small subset of yeast cells have been described that are highly resistant to amphotericin B following adhesion, which is independent to up-regulation of efflux pumps and cell membrane composition (Khot et al., 2006, LaFleur et al., 2006). The first study to describe persister cells in fungi, described as a subpopulation of highly tolerant cells. In this study *C. albicans* persisters were detected only in biofilms and not in different planktonic populations (LaFleur et al., 2006). Reinoculation of cells that survived killing of the biofilm by amphotericin B produced a new biofilm with a new subpopulation of persisters, suggesting that these were not mutants but phenotypic variants of the wild type, and that attachment to a substratum initiated dormancy. The presence of persisters in *C. albicans*, *C. krusei* and *C. parapsilosis* biofilms treated with amphotericin B was also described (Al-Dhaheri and Douglas, 2008). It was further hypothesized that the periodic application of antimicrobial agents may select for strains with increased levels of persister cells, so 150 isolates of *C. albicans* and *C. glabrata* were obtained from cancer patients who were at high risk for the development of oral candidiasis and who had been treated with topical chlorhexidine once a day. It was shown that the persister levels of the isolates varied from 0.2 to 9%, and strains isolated from patients with long-term carriage had high levels of persisters, whereas those from transient carriage did not (Lafleur et al., 2010). Therefore, in this clinically

relevant scenario prolonged and ineffectual antifungal treatment may be beneficial to the biofilm population, which may be responsible for antimicrobial drug failure and relapsing infections.

The role of reactive oxygen species (ROS) in sessile *C. albicans* cells was investigated as they are known to be induced by high concentrations of miconazole, allowing 1-2% of miconazole-tolerant cells to persist (Vandenbosch et al., 2010). Superoxide dismutases (Sods) were found to be differentially expressed by miconazole treated sessile *C. albicans* cells compared to untreated cells. Inhibition of superoxide dismutase resulted in a 18-fold reduction of the miconazole-tolerant persister cells and increased endogenous ROS levels in these cells (Bink et al., 2011). In biofilms from strains lacking  $\Delta sod4/\Delta sod5$  at least 3-fold less miconazole-tolerant persisters were observed and ROS levels were increased compared to the isogenic wild type. Therefore, miconazole-tolerant persisters are linked to the ROS-detoxifying activity of Sods. Whether this is the definitive molecular basis for *C. albicans* persister cells or a tolerance mechanism still remains to be determined, but these subpopulations are clearly another important fungal biofilm resistance mechanism.



**Figure 1.15 - Persister cells in biofilm.** Antibiotic treatment kills normal cells (coloured green) in both planktonic and biofilm populations. The persister cells in planktonic population (pink) are killed by host immune system, but in the biofilm structure the ECM protects the persister cells (pink) from host defences. The survived persister cells then repopulate the biofilm and infect the host (Lewis, 2007a).

### 1.7.7 Tolerance

Stress responses have become more fully realized as defined mechanisms of antifungal resistance. Pathogenic fungi encounter a range of physiological stresses from different environments, including temperature changes, ionic stress, changes in osmolarity, and oxidative stress, such as that experienced in the phagosomes of neutrophils (Cannon et al., 2007). These stresses are sensed through various receptors, which elicit responses through conserved signaling pathways. One of the most important is the mitogen-activated protein kinase (MAPK) signal transduction network, and the many others are subject to review (Cannon et al., 2007). It was first shown that the mitogen-activated protein kinase (MAPK) MCK1p, which is activated by contact stress, is involved in biofilm development. Moreover, the null mutant (*MCK1Δ*) biofilms were azole sensitive, in contrast to the sessile wild type and both planktonic strains. This indicates that Mck1p is involved in biofilm resistance through a stress pathway (Kumamoto, 2005).

Calcineurin is a  $\text{Ca}^{2+}$ -calmodulin-activated serine/threonine-specific protein phosphatase that plays many critical stress roles in the fungal cell, including amongst other things antifungal drug responses (Steinbach et al., 2007). In planktonic cells, calcineurin is critical for *C. albicans* survival during azole treatment (Sanglard et al., 2003). Inhibiting calcineurin pharmacologically or impairing calcineurin function genetically, has synergistic activity with fluconazole and renders the azoles fungicidal against *C. albicans* (Onyewu et al., 2003). Calcineurin has also been implicated in mediating resistance to the azoles in both *in vitro* and *in vivo* models of biofilm formation (Uppuluri et al., 2008). *C. albicans* cells in biofilms are up to 1,000-fold more resistant to fluconazole than planktonic cells, indicating that inhibitors could be used in combinations as novel therapeutic interventions to treat or prevent biofilms, whereas *C. dubliniensis* were unable to form biofilms in the presence of calcineurin inhibitors (Chen et al., 2011b). Similar studies have evaluated the efficacy of a voriconazole-micafungin combination against *C. albicans* biofilms. Voriconazole significantly antagonized the fungicidal effect of micafungin against biofilms. To

investigate the mechanism of antagonism, an inhibitor of calcineurin was evaluated, which reversed the voriconazole-induced resistance to micafungin (Kaneko *et al.*, 2010). This study also suggested that heat shock protein 90 (HSP90) molecular chaperone played a role in this antagonism. HSP90 regulates complex cellular circuitry in eukaryotes and potentiates the emergence and maintenance of resistance to azoles and echinocandins in *C. albicans*, at least in part via calcineurin (Cowen and Lindquist, 2005). It physically interacts with the catalytic subunit of calcineurin, keeping it stable and poised for activation (Singh *et al.*, 2009). It is likely that HSP90 plays a significant role in fungal biofilm resistance, but further work is required to demonstrate this. For example, a recent investigation of the *C. glabrata* biofilm proteome demonstrated upregulation of a heat shock protein (Hsp12p) and other stress proteins (Trx1p, Pep4p) (Seneviratne *et al.*, 2010).

## 1.8 Aims and hypotheses

It is evident from the current literature over the past decade that *A. fumigatus* has become increasingly associated with undefined resistance mechanisms. Our increased knowledge and understanding of fungal infections has revealed that the biofilm phenotype may explain some of this antifungal resistance. It was the general hypothesis of this study that *A. fumigatus* resistance was related to adaptation to its environment during morphological transition within the maturing biofilm, which endowed it with ability to withstand antifungal challenge.

The aims of this study were therefore to 1) investigate adaptive resistance mechanisms in *A. fumigatus* biofilms by determining how they adapt and respond to antifungal challenge during the different phases of growth and 2) develop an insect model to investigate these characteristics *in vivo*.

## Chapter 2

# Efflux mediated azole resistance in *Aspergillus fumigatus* biofilms

## 2.1 Introduction

As discussed in the previous chapter, therapy for fungal disease is limited to a small number of drug classes including azoles, echinocandins and polyenes. The azole class, which includes fluconazole, itraconazole, voriconazole and posaconazole, have been shown to be effective in the treatment of invasive aspergillosis (IA) and are the mainstay treatment of this disease (Oren et al., 2006, Raad et al., 2008, Sambatakou et al., 2006a, Denning et al., 1989). Although the triazoles have proven efficacy with good safety profiles, they have been shown to be associated with resistance through their continuous use (Howard et al., 2009, Meneau and Sanglard, 2005, Mosquera and Denning, 2002). The mortality of patients with multi-azole resistant invasive aspergillosis is was 88% compared with 30-50% who were infected with azole sensitive strains (Howard et al., 2009). Mutations within the *cyp51A* gene involved in the ergosterol biosynthesis pathway have been reported to cause azole cross-resistance (Howard et al., 2009, Mellado et al., 2007, Snelders et al., 2010, Snelders et al., 2008). However, a recent study reported that 43% of azole resistant isolates did not carry the *cyp51A* mutation, indicating that other mechanisms of resistance were responsible.

Another commonly described azole resistance mechanism is mediated by multi-drug resistance pumps, which are involved in the active extrusion of antimicrobial molecules, which includes azoles (Cannon et al., 2009). The major facilitator superfamily (MFS) represents key transmembrane proteins that have been shown to be clinically important in different pathogenic fungi (Morschhauser, 2010, Cannon et al., 2009). Among members of the ABC transporters the *MDR* genes have been widely implicated in *Candida albicans* azole resistance (Wakiec et al., 2007, White, 1997). These have also been shown to be important in *C. albicans* biofilms, where they have been reported to be transiently expressed and are thought to have a role in detoxification (Andes et al., 2004, Ramage et al., 2002a, Mukherjee et al., 2003).

It has been shown that *A. fumigatus* also have the capacity to form biofilms encased in polymeric matrix, which is the most likely growth modality with a fungus ball (Mowat et al., 2008c, Loussert et al., 2010). As these biofilms grow they are differentially sensitive to antifungal drugs (Mowat et al., 2008b). Sequence analysis suggest that *A. fumigatus* has 278 different MFS and 49 ABC transporters (Nierman et al., 2005). *A. fumigatus* MDR pumps have been described in several studies, and have been shown to be associated with increased resistance to itraconazole (da Silva Ferreira et al., 2004, Nascimento et al., 2003). Currently, however, there is little evidence to suggest that these play an active role in clinical resistance (Cannon et al., 2009). Nevertheless, it could be hypothesised that these pumps are activated as the biofilm develops to regulate homeostasis, and following exposure to an antifungal compound. This activity may explain phase related resistance profiles previously reported (Mowat et al., 2008b).

The aim of this chapter was to investigate the biological activity of efflux pumps in *A. fumigatus* and its differential expression with respect to morphological development and azole treatment.

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## 2.2 Material and methods

### 2.2.1 Organisms

*A. fumigatus* Af293, AfGFP (Loussert et al., 2010) and thirteen clinical isolates (YHCF 1-13) obtained from the Royal Hospital for Sick Children (Yorkhill Division), Glasgow, United Kingdom were used throughout this component of the study. Af293 and YHCF 1-4 were used for all fluorescent uptake assays and in quantitative PCR expression analyses. ATCC1163 was used for the *in vivo* expression analyses. *A. fumigatus* isolates were typically stored as stock spore suspensions in sterile Microbank™ beads (Pro-lab diagnostics) at -80°C and subsequently were sub cultured on Sabouraud dextrose agar (SAB) (Sigma-Aldrich) containing 4% glucose as appropriate at 37°C.

### 2.2.2 Harvesting and standardisation of conidia

*A. fumigatus* was grown on SAB containing 4% glucose as appropriate at 37°C for 72h. Spores were harvested in phosphate buffered saline (PBS) (Sigma-Aldrich) containing 0.025% (v/v) Tween 20 (Sigma-Aldrich) by flooding the plate with 5mL. After gentle rocking, the spore suspension was collected into universal tube and the spores were counted using a Neubauer haemocytometer. The standardised suspension was then adjusted to required conidial count in RPMI 1640 media (Sigma-Aldrich). All procedures were carried out in a class 2 biological safety cabinet (MDH Intermed).

### 2.2.3 *In vitro* biofilm formation

*A. fumigatus* biofilms were grown on commercially available flat bottom polystyrene microtitre plates (96 or 24 well plate) or Thermonox™ coverslips (13mm) (Corning Incorporated, Corning). Biofilms were formed by aliquoting 200µL or 1000µL of  $1 \times 10^5$  conidia/mL into 96 or 24 well plate. The plates were then incubated at 37°C for 8, 12 and 24 h. In each plate appropriate positive and negative control wells were added for quality control. After incubation period the medium was removed and the biofilms were washed with PBS to remove any non-adherent cells.

## 2.2.4 Microscopy

### 2.2.4.1 Fluorescent imaging of *A. fumigatus* biofilm

*A. fumigatus* GFP expressing strain (AfGFP) biofilms were formed on Thermanox™ coverslips in RPMI media for 8, 12 and 24 h using a seeding density of  $1 \times 10^5$  conidia/mL as detailed above. After incubation at 37°C, the coverslips were washed in sterile PBS and inverted onto a glass slide so the biofilm faced upwards. The biofilms were then coated with mounting media and covered with another fresh coverslip. Then the biofilms were visualised under a fluorescence microscope (Motic BA400) at an excitation and emission wavelength of 395nm and 475nm, respectively. One representative from each group was digitally captured using Colorview (Soft Imaging System).

### 2.2.4.2 Scanning electron microscopy

*A. fumigatus* biofilms were formed on Thermanox™ coverslips for 24 h using a seeding density of  $1 \times 10^5$  conidia/mL as detailed above. After incubation at 37°C, the coverslips were washed in sterile PBS. The biofilms were fixed by treating the biofilm with 0.1M cacodylic buffer fixative (pH 7.2 [Fisher Scientific]) containing 4% (v/v) formaldehyde and 1% gluteraldehyde (v/v) for 2h then washed 3 times in 0.1M cacodylic buffer and H<sub>2</sub>O, dried overnight and then dehydrated with a series of ethanol washes (70% (v/v) for 10min, 90% (v/v) for 10min, 100% (v/v) for 20min), and finally air dried overnight. The specimens were then coated with 40% gold - 60% palladium and observed with a Zeiss Evo scanning electron microscope in high vacuum mode at 6 -10 kV.

### 2.2.5 *Aspergillus fumigatus* phase dependant susceptibility testing

Voriconazole (Pfizer) was prepared in stock solutions of dimethyl-sulphoxide (DMSO, Sigma-Aldrich) and diluted to the required concentrations in RPMI. For making up the dilutions an aliquot of 100 $\mu$ L of voriconazole was added to the appropriate wells in a 96-well microtitre plate (Corning) and serially diluted two fold across adjacent wells of the plate, producing a concentration range of 0 - 256 mg/L. For testing the biofilm MIC, biofilms were allowed to grow for 8, 12 or 24 h and then washed with PBS as described above, and then serially diluted voriconazole in RPMI were transferred into the wells containing biofilm. The plate was then incubated for further 24 h at 37°C. The viability of the cells were assessed using an XTT assay or by alamarBlue<sup>®</sup> assay. The reduction in metabolically activity was assessed by comparing with untreated biofilm controls.

### 2.2.6 XTT reduction assay

2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay was performed as described somewhere else with minor modifications (Meletiadis et al., 2001a, Meletiadis et al., 2001b, Antachopoulos et al., 2006). In brief, XTT (Sigma-Aldrich) was prepared in stock by dissolving in PBS at a concentration of 0.5 g/L. The solution was then filter sterilised using vaccum filter with a pore size of 22 $\mu$ m and stored at -80°C. Before each XTT assay an aliquot of XTT stock was thawed and supplemented with menadione (Sigma-Aldrich) to a final concentration of 10  $\mu$ M. For testing the metabolic activity of the cells, a 100  $\mu$ L of XTT-menadione was added into each well and incubate the plate in dark at 37°C for 3h. After incubation, optical density was measured using plate reader (FLUOstar omega, BMG Labtech) at 492nm. The change in absorbance in the XTT assay directly correlates with the metabolic activity of the biofilm as previously described (Ramage et al., 2001a, Meletiadis et al., 2001a).

### 2.2.7 Ala-Nap uptake assay

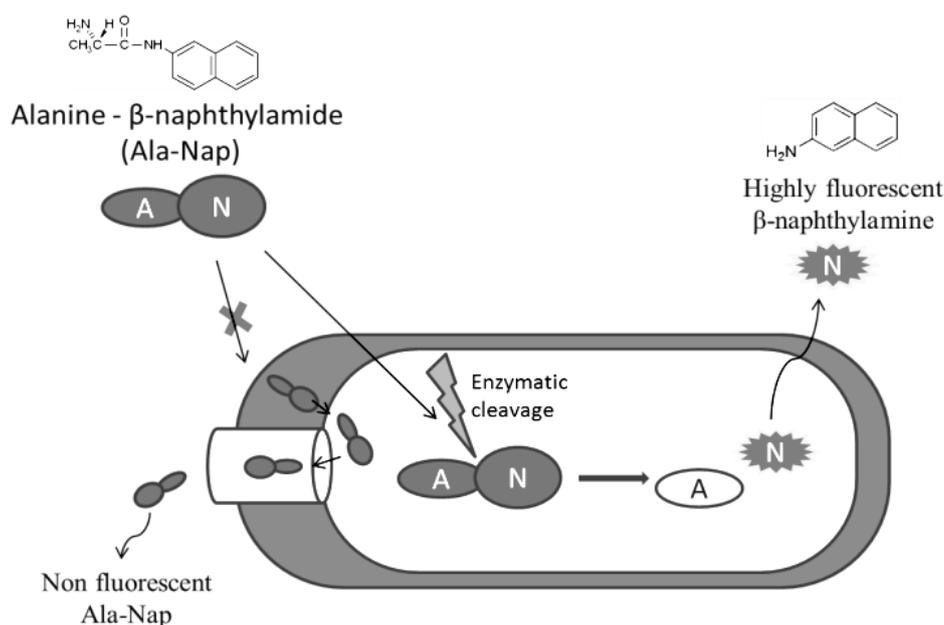
The efflux pump activity of *A. fumigatus* was assessed by using MC-005,556 (Ala-Nap) as previously described (Lomovskaya et al., 2001). MC-005,556 (Ala-Nap) is enzymatically cleaved inside the cells to produce highly fluorescent  $\beta$ -naphthylamine (Figure 2.1). Higher levels of fluorescence reflect low efflux pump activity and vice versa. *A. fumigatus* conidia was standardised to  $1 \times 10^5$ /mL in RPMI and conidial suspensions inoculated into black flat bottomed microtiter plate (Costar 3603, Corning) and incubated for 8 h at 37°C. The medium was then aspirated and any non-adherent cells removed by thoroughly washing the cells with buffer solution three times (K<sub>2</sub>HPO<sub>4</sub> [50mM], MgSo<sub>4</sub> [1mM], and glucose [0.4%]) at pH 7.0. The reaction was initiated by addition of Ala-Nap (Sigma-Aldrich) at a final concentration of 64 - 256 mg/L. Fluorescence (RFU) was quantified at 30s intervals at an excitation wavelength of 320nm and emission of 460nm over 1 h at 37°C using a fluorescent plate reader (FLUOstar Omega, BMG Labtech).

#### 2.2.7.1 Assessment of phase dependant efflux activity

An optimised alanine  $\beta$ -naphthylamine (Ala-Nap) fluorescent assay was performed on six isolates (Af293 and YHCF1-YHCF4) to assess whether different phases of *A. fumigatus* morphological development exhibited efflux pump activity, as described above. Selected isolates were washed and standardised to  $1 \times 10^5$  conidia/mL in RPMI as described above and standardised conidial suspensions inoculated into black flat bottomed microtitre plate (Costar 3603, Corning) and incubated for 8, 12 and 24 h at 37°C. The biofilms were then washed with assay buffer. The dry weight of each biofilm was also quantified for normalisation. Mature biofilms (24 h) were also disaggregated and washed three times to take account for lack of diffusion. The reaction was initiated by addition of Ala-Nap at a final concentration of 128 mg/L and fluorescence was quantified as described above. The change in relative fluorescence units (RFU) was normalised against the dry weight (mg) of each biofilm and presented as  $\Delta$ RFU/mg of biofilm.

### 2.2.7.2 Assessment of efflux activity with azole treatment

The effect of pre-treatment with voriconazole was also investigated with Ala-Nap assay. For this experiment a sub MIC level of voriconazole 0.0625 mg/L (for the strain Af293) was selected and used for pre-treatment. Biofilms were grown for 8 h as described above and treated them with voriconazole at a defined concentration. The medium was then aspirated and biofilm was washed with assay buffer to remove the voriconazole and other non-adherent cells. The reaction was initiated and fluorescence was measured like mentioned above. The change in relative fluorescence units (RFU) was normalised against the dry weight (mg) of the biofilm and presented as  $\Delta\text{RFU}/\text{mg}$  of biofilm.



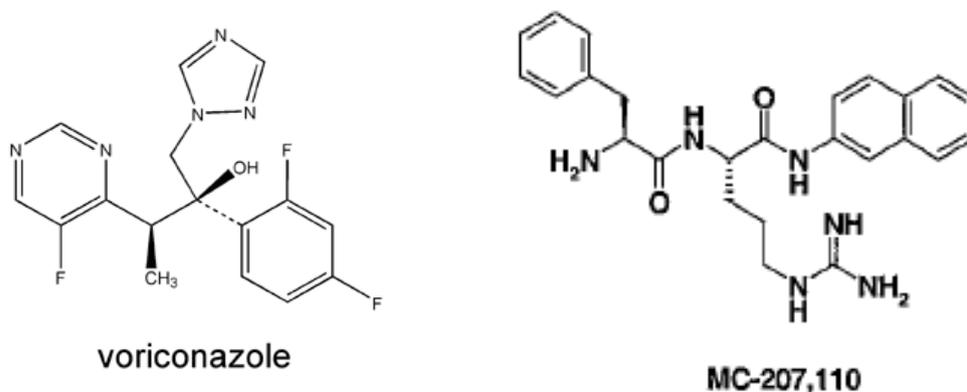
**Figure 2.1- Ala-Nap uptake assay.** The compound Ala-Nap is generally not fluorescent in media once it gets enzymatically cleaved inside the cell it produces a fluorescent compound called β-naphthylamine. It is a general substrate of efflux pumps so the efflux system actively engages in pumping out this compound before the enzymatic cleavage. Therefore, higher levels of fluorescence reflect low efflux pump activity and vice versa.

## 2.2.8 Assessment of azole sensitivity in the presence of an efflux pump inhibitor

To determine whether azole sensitivity was related to efflux pump activity, an efflux pump inhibitor (L-Phe-L-Arg- $\beta$ -naphthylamide [MC-207,110], Sigma-Aldrich) was used in combination with voriconazole to determine whether antifungal efficacy could be enhanced.

### 2.2.8.1 Checkerboard titration assay

For testing azole susceptibility in combination with MC-207,110, Af293 conidia were initially standardised to  $1 \times 10^5$  conidia/mL, inoculated into 96-well microtitre plates and incubated for defined periods (8,12 and 24 h) at 37°C, as described above. The medium was then aspirated and any non-adherent cells removed by thoroughly washing the cells 3 times with PBS. A checkerboard assay was prepared with both voriconazole and MC-207,110. Voriconazole was tested at 11 different concentrations (0.005 - 4 mg/L), while MC-207,110 was tested at 7 concentrations (8 - 512 mg/L). Voriconazole was dispensed alone in the first row and was combined with MC-207,110 in the remaining rows. MC-207,110 was also dispensed alone in the first column (Figure 2.2). After incubation period, an alamarBlue<sup>®</sup> colorimetric assay was used to assess viability, as per the manufacturer's instructions (Invitrogen). Following these initial experiments, a defined concentration of MC-207,110 was selected (64 mg/L) for all subsequent microdilution testing on the entire panel of strains, with voriconazole at 0.005 to 4 mg/L.



		Azole ( $\mu\text{g/ml}$ )												
		0	4	2	1	0.5	0.25	0.12	0.06	0.03	0.02	0.01	0.005	
MC-207,110-EPI ( $\mu\text{g/ml}$ )	0	Azole alone $\rightarrow$												
	512													
	256													
	128													
	64													
	32													
	16													
	8													

**Figure 2.2 - Checkerboard assay.** In checkerboard format eleven different concentrations of voriconazole was tested in combination with seven different concentrations of MC-207,110. Voriconazole alone was dispensed along the first row and MC-207,110 alone along the first column to find their individual activity against *A. fumigatus* biofilm. Remaining well were designed in the way getting different concentration combinations of drug and EPI.

### 2.2.9 RNA extraction from biofilms

The biofilms were grown in 24 well plate or cell culture flasks (Fisher Scientific) for 8, 12 and 24 h and washed with PBS to remove any non-adherent cells as described above. The biofilms were then scraped off from the plate surface using sterile cell scrapers (Corning) and transferred into 2mL screw cap tubes (Stratech) with 1mL of Trizol<sup>®</sup>. The cells were then mechanically disrupted using 200 $\mu$ L (0.5mm diameter) of silicon glass beads with a mini Bead Beater<sup>™</sup> (Biospec Products). The biofilm samples were bead beat for two cycles at 48 Hz for 30s and samples were placed on ice for 30s in between each cycle. The homogenized biofilm materials were maintained at room temperature for 5min and 100 $\mu$ L of 1-bromo-3-chloropropane (Fisher Scientific) was added to each tube followed by vortex for 30s. Incubated the tubes at room temperature for 15min and then centrifuged at  $\times 10000$ rpm for 15min at 4 $^{\circ}$ C using refrigerated microcentrifuge. The RNA containing upper aqueous layer was carefully transferred to a sterile RNase free microcentrifuge tube. The RNA was then precipitated by addition of equal volume of 100% isopropanol. The mixture was incubated at -20 $^{\circ}$ C for overnight to get the maximum RNA precipitates. After overnight incubation the precipitate was centrifuged at  $\times 10000$  rpm for 10min at 4 $^{\circ}$ C using refrigerated microcentrifuge. The supernatant was decanted and replaced with 500 $\mu$ L of 70% (v/v) ethanol to wash the formed pellet. The tubes were inverted for 3-4 times and centrifuged for 5min at  $\times 10000$ rpm at 4 $^{\circ}$ C. The ethanol was then discarded and air-dried for approximately 5min. Finally the pellet was re-suspended in an appropriate volume of RNase free water.

### 2.2.10 DNase digestion

The extracted RNA was cleaned up by on column DNase treatment using Qiagen RNase-free DNase kit following the manufacturer's instructions. The quantity and quality of RNA was then assessed using a NanoDrop<sup>™</sup> spectrophotometer ND-1000 (Labtech International Ltd) and stored at -80 $^{\circ}$ C until required.

### 2.2.11 cDNA synthesis

The high capacity RNA-to-cDNA master mix (Applied Biosystem) was used to reverse transcribe the RNA as per manufacturer's instructions. Briefly, the RT master mix was thawed and centrifuged them before use. Then 4 $\mu$ L of RT master mix or No-RT control mix was added to 100ng-1 $\mu$ g of total RNA and the volume of the reaction was made up to a final total of 20 $\mu$ L with RNase free water. All the reactions were carried out on ice. Then the reverse transcription reaction was performed using MyCycler PCR machine (Bio-Rad) with the following program. 25°C for 5min, 42°C for 30min, 85°C for 5min and hold at 4°C. The reaction volume was set to 20 $\mu$ L and the PCR tubes were loaded into the thermal cycler for reverse transcription run.

### 2.2.12 Real time PCR analysis

*A. fumigatus* Af293, YHCF1-YHCF4 biofilms were prepared in triplicate on polystyrene plates for selected time periods (8, 12 and 24 h) as described above. In addition, the effect of pre-treatment with voriconazole (1 mg/L) was investigated for Af293, YHCF1 and YHCF2 on each of these populations. RNA was extracted by TRIzol™ method and cDNA was subsequently synthesised as described above. The expression of the multidrug efflux pump genes, *AfuMDR* 1-4, was then assessed using quantitative RT-PCR using SYBR® GreenER™ (Invitrogen, Paisley, UK) according to the manufacturers' instructions. The primer sets in Table 2.1 were used, as previously described (Knutsen et al., 2006). Three independent replicate samples from each strain for each parameter were analysed in triplicate using MxProP Quantitative PCR machine and MxProP 3000 software (Stratagene). The PCR conditions for all primers as follows 5min at 95°C (initial denature), 30s at 95°C (denaturation), 30s at 50-65°C (annealing), 30s at 72°C (extension) for 40 cycles, followed by a final extension at 72°C for 10min. All PCR reactions were carried out in a total volume of 25 $\mu$ L. All the gene expressions were normalised to  $\beta$ -tubulin housekeeping gene according to the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

**Table 2.1- List of oligonucleotide primers used for real time PCR analysis of efflux pump gene expressions.**

Gene name - ID	Primer	Sequence
<i>β-tublin</i> - AFUA_1G10910	<i>β-tub</i> F	CAATGGCTCCTCCGATCTCC
	<i>β-tub</i> R	TCCATGGTACCAGGCTCG
<i>MDR1</i> - AFUA_5G06070	<i>MDR1</i> F	TTCCCTTGTTCAACAATTCTCTTCG
	<i>MDR1</i> R	TGACATAGACTGTGACAAACTCG
<i>MDR2</i> - AFUA_4G10000	<i>MDR2</i> F	TTTAGCTCCACCGGGTTTG
	<i>MDR2</i> R	TCGAAAGACCGAACATGCTTGA
<i>MDR2</i> - AFUA_3G03500	<i>MDR2</i> F	TCTGATGGCGGTCATCACT
	<i>MDR2</i> R	ATATCCATCCCCCAGGC
<i>MDR4</i> - AFUA_1G12690	<i>MDR4</i> F	GTCGCCGTTACTTTGAGAGC
	<i>MDR4</i> R	ATGAAGGCAACCACATAGGC

### 2.2.13 *In vivo* biofilm model

The *in vivo* analysis, diffusion chamber mouse model was performed by Dr P. Warn and colleagues in University of Manchester. All post-processing of the biofilms was performed by R. Rajendran. Biofilm samples were collected, homogenised and quickly placed in Trizol<sup>®</sup> solution and stored at -80°C until required.

#### 2.2.13.1 Biofilm chamber

A diffusion chamber kit (Millipore) was modified to create the biofilm chamber. Briefly, durapore polyvinylidene fluoride (PVDF) membrane (Millipore,) with pore size 0.45µm was fixed to one side of plexiglass rings (Millipore) and silicon sheet was fixed on the other side (Figure 2.3).

#### 2.2.13.2 Chamber Implantation

Chambers were autoclaved before implanting in mice. Male CD-1 mice were anaesthetized with ketamine and xylazine. The dorsal flank of mice was shaved and a small incision was made in the skin. The diffusion chamber was implanted subcutaneously in such a way that the silicon side of the chamber faced the skin and the durapore membrane was touching the body. The wound was closed using Ethicon black braided silk non-absorbable suture. Meloxicam (2mg/kg) was administered intra-peritoneally twice a day up to 72h post-surgery.

#### 2.2.13.3 *A. fumigatus* growth and azole treatment in diffusion chamber

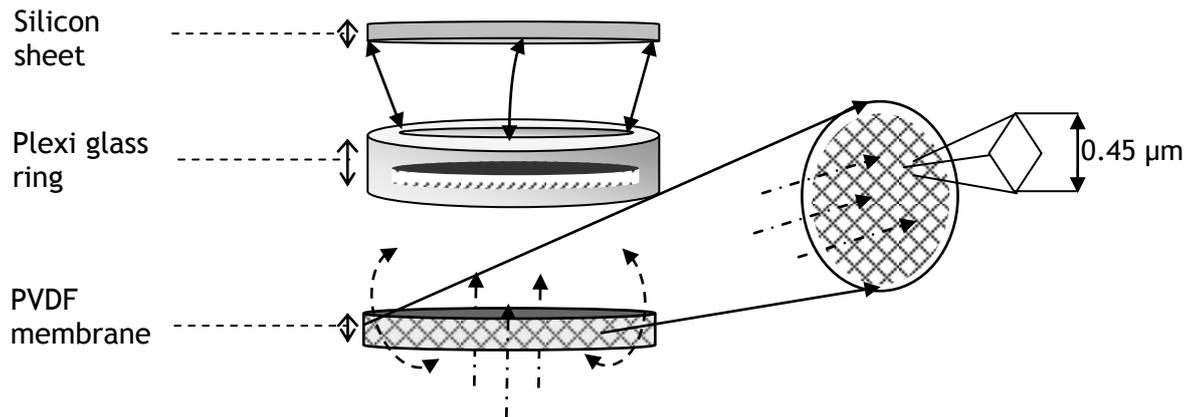
Once week after the surgery, *A. fumigatus* ATCC1163 ( $1 \times 10^5$  spores/mouse) was injected into the tissue chamber under isoflurane anaesthesia. Mice were treated with vehicle (control), low dose (LD 3 mg/kg) and high dose (HD 10 mg/kg) voriconazole orally. Mice, in at least triplicate, were euthanized at 0, 1, 4 and 24 h post-infection and the chamber was removed.

#### 2.2.13.4 *In vivo* biofilm RNA extraction

The collected chambers were opened and the biofilm materials were put in Trizol<sup>®</sup> and stored at -80°C. Each sample was thawed on ice and placed into 2mL screw cap tube. The RNA was then extracted by Trizol<sup>®</sup> - bead beating method as described above.

#### 2.2.13.5 *In vivo* gene expression analysis by real time PCR

The complementary DNA sequence of *in vivo* RNA samples was synthesised using High capacity RNA-to-cDNA master mix as described above. The expression of multi drug transporter gene (*AfuMDR4*) was then assessed using quantitative RT-PCR using SYBR<sup>®</sup> GreenER<sup>™</sup> as described above. The housekeeping gene  $\beta$ -tublin was used to calculate the relative expression of *AfuMDR4* according to the  $2^{-\Delta\Delta CT}$  method. Three animals were used as a baseline for comparison to vehicle control (n=3 at 1, 4, and 24 h).



**Figure 2.3- Biofilm chamber.** Diffusion chamber kit was modified as shown above to get the biofilm chamber. It contains a silicon sheet attached to plexi glass ring at one side and PVDF membrane at the other side of the ring, which favours the diffusion of nutrients and other molecules from host to the chamber and keeps *A. fumigatus* grown in them.

### 2.2.14 Efflux pump mutational studies

The *MDR* gene knockouts were made in University of Manchester with the guidance of Dr M. Bromley and Prof P. Bowyer.

#### 2.2.14.1 Design of oligonucleotide primers for the generation of gene KO cassettes

Typically eight primers are required to generate the KO cassette, two to amplify the 5' flank, two to amplify the 3' flank, two to amplify the selectable marker and finally two nested primers to amplify the final product. The research group in Manchester have optimised this procedure so that a generic selectable marker fragment can be used and hence only six primers are needed for each KO cassette. For designing the primers, firstly *MDR* genes were identified on the central *Aspergillus* data repository <http://www.cadre-genomes.org.uk/>. Then the gene sequence was extracted along with 1200bp of flanking sequence using the export data page (<http://www.cadre-genomes.org.uk/Aspergillusfumigatus/export> view) and pasted it into a word processor. The start site ATG and stop codons were identified in order to ensure the extracted sequence was in the correct orientation. If necessary the reverse complement of the output was obtained from CADRE using the website <http://www.bioinformatics.org/SMS/revcomp.html>, which aids downstream characterisation.

All the primers were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3/input.htm>). For designing the primers for amplifying flanking regions, 1200 bp of upstream flanking sequence was selected and pasted into primer 3. The design settings for primer 3 was set as follows: Product size range 1000-1200, Primer size Min 18: Opt 20: Max 22, Primer Tm Min 58: Opt 60: Max 62. After running primer 3, ideally picked primers those have 1-2 G's or C's in the last 3 bases. The primer marked left primer was named xxxxxxxP1 where the x represents the CADRE gene identifier. The primer marked right primer was attached with the linker sequence TAGTTCTGTTACCGAGCCGG(X) 18-22 to the 5' end gave primer xxxxxxxP2. For downstream primers, the 1200 bp of downstream flanking sequence was selected and repeated the primer design

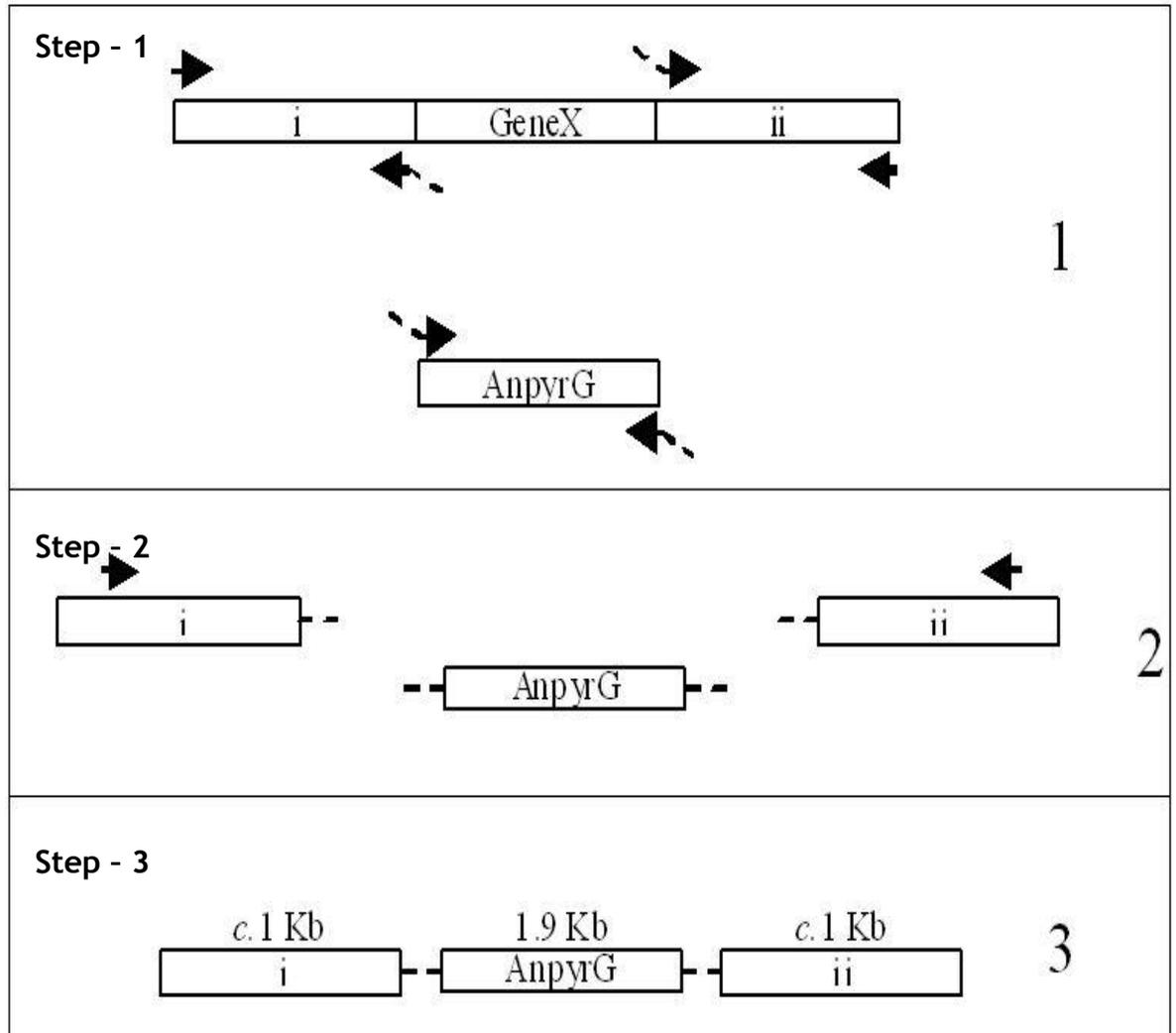
process. The primer marked left primer was attached with linker sequence GCTCTGAACGATATGCTCCC(X) 18-22 at the 5' end gave primer xxxxxxxP3. The primer marked left primer was named as xxxxxxxP4. In the Microsoft word document with the gene sequence, identified the location of primers P1 and P4. Then selected all sequence between P1 and P4 (not including primers P1 and P4) and identified the number of bases in the selected sequence which gave the value 'b'. The selected sequence was then pasted into primer3 and entered the setting as product size range (b - 200)-b. All other settings are as same as mentioned above. The resulting primers were named as xxxxxxxP5 and the right xxxxxxxP6.

Table 2.2- Gene knock out primers.

Gene name-ID	Primer	Sequence
<i>MDR1</i> - AFUA_5G06070	AFUA_5G06070 P1	TGGCCATACCTGAGGTTCTC
	AFUA_5G06070 P2	TAGTTCTGTTACCGAGCCGGTTGGTAACTG GCGGACAAT
	AFUA_5G06070 P3	GCTCTGAACGATATGCTCCCTCCTTCCCCCTT CTTCTCAT
	AFUA_5G06070 P4	GCTGTGCTGTTGCAAGGTAG
	AFUA_5G06070 P5	ACAAGAGGGACAGAGCAGGA
	AFUA_5G06070 P6	AGTAAAGCCTCTGCCGAACA
<i>MDR2</i> - AFUA_4G10000	AFUA_4G10000 P1	TCCTGGTGACCCTCTACACC
	AFUA_4G10000 P2	TAGTTCTGTTACCGAGCCGG GAGAGAAGATGCGGAATTGG
	AFUA_4G10000 P3	GCTCTGAACGATATGCTCCC GAGGCGCTCACATAGACTCC
	AFUA_4G10000 P4	CCCAAACACCATGAACATCA
	AFUA_4G10000 P5	GTGTATGAACGCCCTCTGGT
	AFUA_4G10000 P6	ACTGTCGAAAGCGCCAATAG

<i>MDR3</i> - AFUA_3G03500	P1	GTCGATATCCAGCCCACACT
	P2	TAGTTCTGTTACCGAGCCGG TTGGAACAGATGATGCTTGG
	P3	GCTCTGAACGATATGCTCCC AATCTCGTCCATGTCCCTTG
	P4	CTCCCTGCATTTTCAGTCACC
	P5	GCCGGCATTATAACTGCTTC
	P6	CCTTCTTCGAAGGCAAACAG
<i>MDR4</i> - AFUA_1G12690	AFUA_1G12690 P1	ATTGGTGGCTACCCAGTACG
	AFUA_1G12690 P2	TAGTTCTGTTACCGAGCCGG TCACCGATAAGCCAGGTAGG
	AFUA_1G12690 P3	GCTCTGAACGATATGCTCCC GACTGTGGGACTCTCGGTTC
	AFUA_1G12690 P4	AGGCTGTCTTCGAAAGTGGA
	AFUA_1G12690 P5	CCCACGCCAAGATACAGAAT
	AFUA_1G12690 P6	ACAGATACAGGGACGGATGG

Highlighted region shows the linker sequence in the primer.



**Figure 2.4 - Schematic representation of PCR fusion.** Step-1: the 5' flank (i), 3' flank (ii) and selectable marker (AnpyrG) were amplified in three separate reactions. Step-2: The amplified products have overlapping linkers, which permit fusion in a second round reaction. To increase the specificity of this second round reaction nested primers were used to aid amplification. Step-3: formation of gene knockout cassette was confirmed by gel electrophoresis.

### 2.2.14.2 PCR amplification of flanking genomic fragments

The amplification of each fragment was completed using AccuPrime *Taq* High Fidelity DNA polymerase PCR (Invitrogen) as described previously (Ben-Ami and Kontoyiannis, 2012). The PCR reaction was carried out with 50 $\mu$ L reaction volume and following PCR cycling conditions (Table 2.3).

**Table 2.3 - PCR cycling conditions for amplification of flanking regions.**

Cycle	Denature	Ramp	Anneal	Ramp	Extend	Ramp
1	94°C, 2min					
2-31	94°C, 20s	To 70°C at maximum rate; 70°C, 1 s; ramp to the annealing temperature at 0.1°C/s	55°C, 30s	To the extension temperature of 68°C at 0.2°C/s	68°C, 1 min	At maximum rate to 94°C

The PCR products were then cleaned using QIAquick PCR purification kit (Qiagen) and gel electrophoresed for product confirmation. The flanking regions were amplified using primers P1, P2, P3 and P4 (Table 2.2). Following to amplification of flanking regions fusion PCR was carried out with primers P5 and P6 (Table 2.2). The template consists of the two amplified flanking regions and selectable marker. The PCR amplification using primers P5 and P6 fused the three fragments into single knockout cassette. The fusion PCR reaction was done using the following PCR conditions (Table 2.4). Finally the fusion PCR product was cleaned and gel electrophoresed before transformation.

Table 2.4- Fusion PCR cycling conditions

Cycle	Denature	Ramp	Anneal	Ramp	Extend	Ramp
1	94 °C, 2min					
2-11	94 °C, 20s	To 70 °C at maximum rate; 70 °C, 1 s; ramp to the annealing temperature at 0.1 °C/s	55 °C, 30s	To the extension temperature of 68 °C at 0.2 C/s	68 °C, 5 min	At maximum rate to 94 °C
12-16	94 °C, 20s	To 70 °C at maximum rate; 70 °C, 1 s; ramp to the annealing temperature at 0.1 °C/s	55 °C, 30s	To the extension temperature at 0.2 °C/s	68 °C, 5 min extension time for first cycle	At maximum rate to 94 °C
17-26	94 °C, 20s	To 70 °C at maximum rate; 70 °C, 1 s; ramp to the annealing temperature at 0.1 °C/s	55 °C, 30s	To the extension temperature at 0.2 °C/s	68 °C, 5 min 20 s extension time for first cycle	At maximum rate to 94 °C

### 2.2.14.3 Transformation and screening

For gene knock out spores of Af293 were grown in 100 mL SAB broth containing 100µg/mL of ampicillin for 14-16 h at 37°C, the resulting mycelium was then harvested by filtration through Miracloth (Calbiochem) then digested in 15mL sterile 50mM CaCl<sub>2</sub>, 0.6 M KCl with 6% Glucanex (NovoNordiskFerment) at 37°C for 3 h at 100rpm to form protoplasts. These were filtered as before then centrifuged at 2000g for 10minutes at 4°C. The pellet was suspended in 50 mM CaCl<sub>2</sub>, 0.6M KCl and protoplasts were counted using an improved Neubauer haemocytometer (Weber Scientific International) then adjusted to give 1x10<sup>7</sup>/100µL aliquot. 3 - 4µg purified PCR product was added to each aliquot followed by incubation on ice for 25minutes prior to adding 25µL 60% polyethylene glycol (PEG6000). After 10 min incubation at RT, the mixtures were transferred to Vogels minimal media containing 0.6M KCl and 200µg/mL hygromycin and incubated at 37°C for 2 days or until the appearance of resistant colonies.

### 2.2.14.4 Gene knockout confirmation

After transformation of gene knockout cassette into protoplast and resistant colonies screening, the DNA was extracted from screened colonies and PCR was performed to confirm the gene knockout. The confirmation PCR was completed with primers in Table 2.5 using Thermal cycler (Biorad). The PCR product was then gel electrophoresed to confirm the gene knockout.

Table 2.5- Primers for confirming the gene knock out

Gene	Primer	Sequence
<i>MDR1</i> - AFUA_5G06070	<i>MDR1</i> L	GAACTGTCGCGGAAGAAGTC
	<i>MDR1</i> R	CCACCAATGCGGTAGTCTTT
<i>MDR2</i> - AFUA_4G10000	<i>MDR2</i> L	GGCCTACGTCAGTCTCAAGC
	<i>MDR2</i> R	AAAGCTCGAAAGACCGAACA
<i>MDR2</i> - AFUA_3G03500	<i>MDR2</i> L	ATGTGGGCGGTTATAGCTTG
	<i>MDR2</i> R	AAACATAAGGCGTGGACCAG
<i>MDR4</i> - AFUA_1G12690	<i>MDR4</i> L	CACTGAACGCAACTCCTGAA
	<i>MDR4</i> R	CTCGGATCCGGTACAACACT

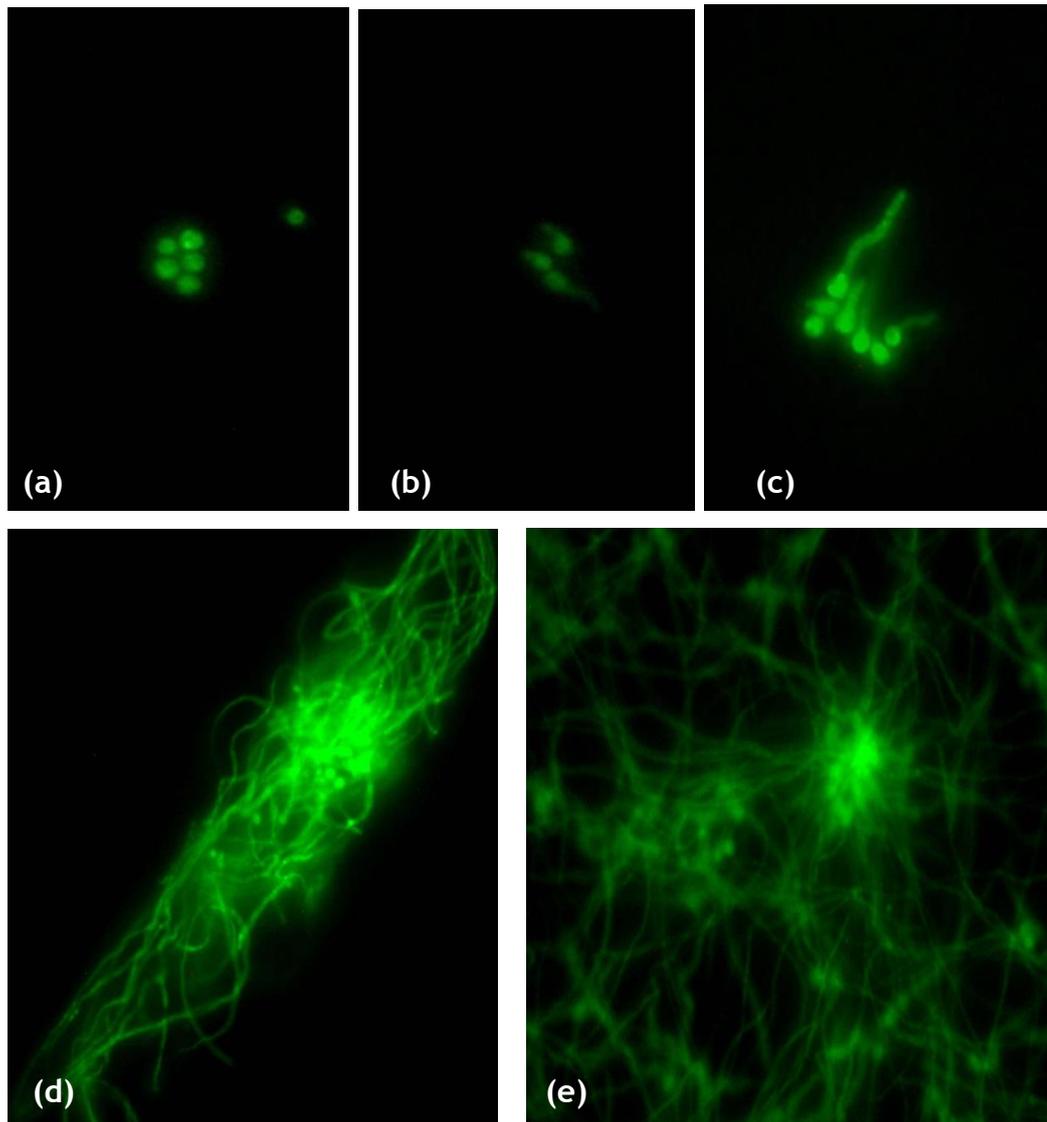
### 2.2.15 Statistics

Analysis of variance (ANOVA) and t-tests were used to investigate independent sample data (different strains or on samples from different animals). A Bonferroni correction for multiple comparisons was applied to the data where appropriate. SPSS (Version 11, Chicago, USA) was used for these analysis and GraphPad Prism (Version 4, La Jolla, USA) for the production of the figures.

## 2.3 Results

### 2.3.1 *Aspergillus fumigatus* biofilm formation

Fluorescence microscopy was used to visualise the phenotype of different phases of *A. fumigatus* biofilm formation, a GFP strain was grown on a coverslip and viewed under the fluorescent microscope. Figure 2.5 a-b shows resting conidia and germling formation (after 8 h incubation). With 12 h incubation, an elongated tube like structure from germlings was visualised indicates the initial hyphal formation (Figure 2.5c). A complex multicellular structure was imaged after 24 h incubation period, Figure 2.5 d-e shows free floating hyphal cells and mesh like biofilm formation respectively.



**Figure 2.5 - Different phases of *A. fumigatus* biofilm.** *A. fumigatus* GFP expressing strain (AfGFP) biofilms were formed on Thermanox™ coverslips in RPMI media for 1, 8, 12 and 24 h ( $1 \times 10^5$  conidia/mL). After incubation at 37°C, the coverslips were washed in sterile PBS and the biofilms were visualised under a fluorescence microscope (Motic BA400). One representative from each group was digitally captured using Colorview Soft Imaging System. (a) Round structured conidia, (b) germlings after 8 h, (c) initial hyphae after 12 h and (d-e) shows matured free floating hyphae and biofilm formation after 24 h incubation.

### **2.3.2 *A. fumigatus* biofilm exhibits phase dependant resistance to voriconazole**

For all fourteen *A. fumigatus* strains examined in this study, voriconazole consistently exhibited activity against germlings (8 h), with a  $sMIC_{90}$  range of between 0.0315 to 1 mg/L (Table 2.6). The  $sMIC_{90}$  of voriconazole increased by up to 16 to 256 fold against a monolayer of proliferating mycelia (12 h) compared to germlings. In those multicellular *A. fumigatus* populations where hyphae were densely intertwined (24 h), the voriconazole  $sMIC_{90}$  was  $\geq 256$  mg/L, which was  $> 512$  mg/L compared to germlings.

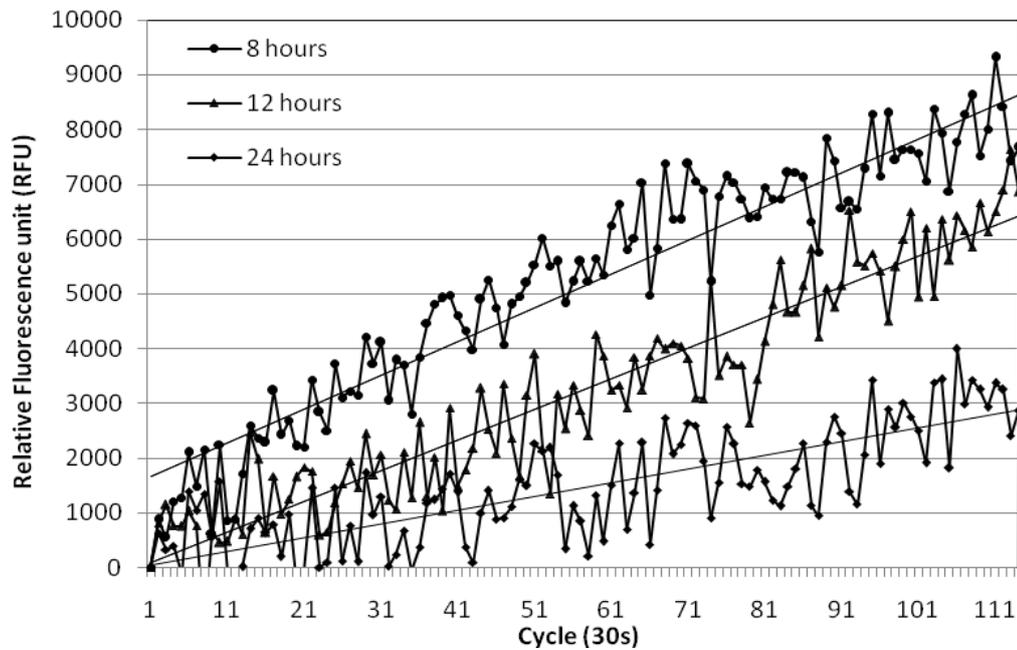
### **2.3.3 *A. fumigatus* exhibits phase dependant Ala-Nap uptake**

Analysis of efflux pump activity was assessed by quantifying the change in relative fluorescent units ( $\Delta RFU$ ) in 8, 12 and 24 h populations (Figure 2.6, Table 2.7). The data was then normalised by using biofilm dry weight ( $\Delta RFU/mg$ ). The data demonstrated that 8 h germlings exhibited the greatest levels Ala-Nap uptake ( $\sim 1150 \Delta RFU/mg$ ), i.e. low levels of efflux. Both 12 h and 24 h phases of growth demonstrated significantly lower levels of Ala-Nap uptake of  $\sim 600$  and  $\sim 410 \Delta RFU/mg$ , respectively (ANOVA;  $p < 0.0001$ ) (Figure 2.7).

**Table 2.6 - Antifungal activity of voriconazole against adherent *Aspergillus fumigatus* grown for 8, 12 and 24 h.**

Strain	Sessile minimum inhibitory concentration (sMIC <sub>90</sub> )		
	8 h	12 h ( $\Delta$ MIC <sup>a</sup> )	24 h ( $\Delta$ MIC <sup>a</sup> )
AF293	0.5	8 (16)	>256 (>512)
YHAF1	0.0625	1 (16)	>256 (>512)
YHAF2	0.0625	1 (16)	>256 (>512)
YHAF3	1.0	32 (32)	>256 (>512)
YHAF4	0.125	16 (128)	>256 (>512)
YHAF5	0.125	16 (128)	>256 (>512)
YHAF6	0.125	16 (128)	>256 (>512)
YHAF7	0.125	8 (64)	>256 (>512)
YHAF8	0.0312	4 (128)	>256 (>512)
YHAF9	0.0625	16 (128)	>256 (>512)
YHAF10	0.0625	32 (256)	>256 (>512)
YHAF11	0.0625	8 (64)	>256 (>512)
YHAF12	0.0625	8 (64)	>256 (>512)
YHAF13	0.0312	4 (128)	>256 (>512)

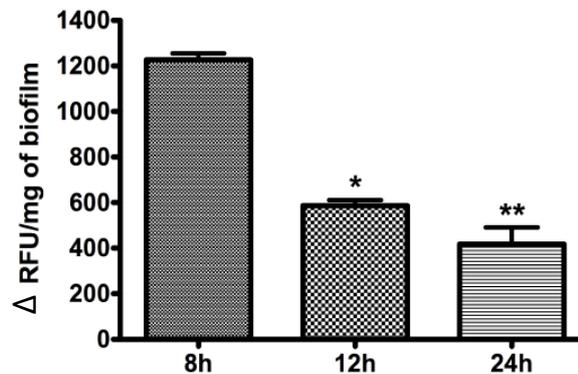
<sup>a</sup>Fold change in MIC compared to 8 h germling cells MIC.



**Figure 2.6 - Ala-nap uptake by *A. fumigatus* biofilms.** Fluorescence produced by differently grown *A. fumigatus* biofilm (8, 12 and 24 h). Standardised *A. fumigatus* conidia were incubated with Ala-Nap (128 mg/L), which is enzymatically cleaved to produce fluorescent  $\beta$ -naphthylamine. The change in fluorescence ( $\Delta$ RFU) were determined over 120 min at Ex320nm/Em460nm using a fluorescent plate reader. Increase in fluorescence indicates more ala-nap uptake and vice versa.

**Table 2.7- Assessment of efflux activity by Ala-Nap uptake assay.** Blank corrected raw data showing relative fluorescence produced with the respective phase of *A. fumigatus* growth.

Strain	Relative fluorescence unit (RFU)		
	8 h	12 h	24 h
AF293	40123.2	23422.2	17487.0
YHAF1	39405.6	21716.7	30319.5
YHAF2	39592.8	23081.1	11484.0
YHAF3	35973.6	24028.6	16356.0
YHAF4	36223.2	18874.2	15051.0



**Figure 2.7 - *Aspergillus fumigatus* displays phase dependant efflux activity.** Efflux activity of 8, 12 and 24 h biofilms. Standardised *A. fumigatus* conidia were incubated with Ala-Nap (128 mg/L) and resultant fluorescent production was measured using plate reader. The change in fluorescence of dry weight normalised biofilms ( $\Delta$ RFU/mg) was determined. High values indicate low efflux and vice versa. Five different strains were used and the experiments performed on three separate occasions. The error bars represent the standard deviation of the means. Ala-Nap internal cleavage was normalised ( $\Delta$ RFU/mg), and shown to be maximum after 8 h growth, whilst significantly reduced at 12 and 24 h, respectively (\* $p < 0.0001$ ). \* - 0.05, \*\* - 0.01.

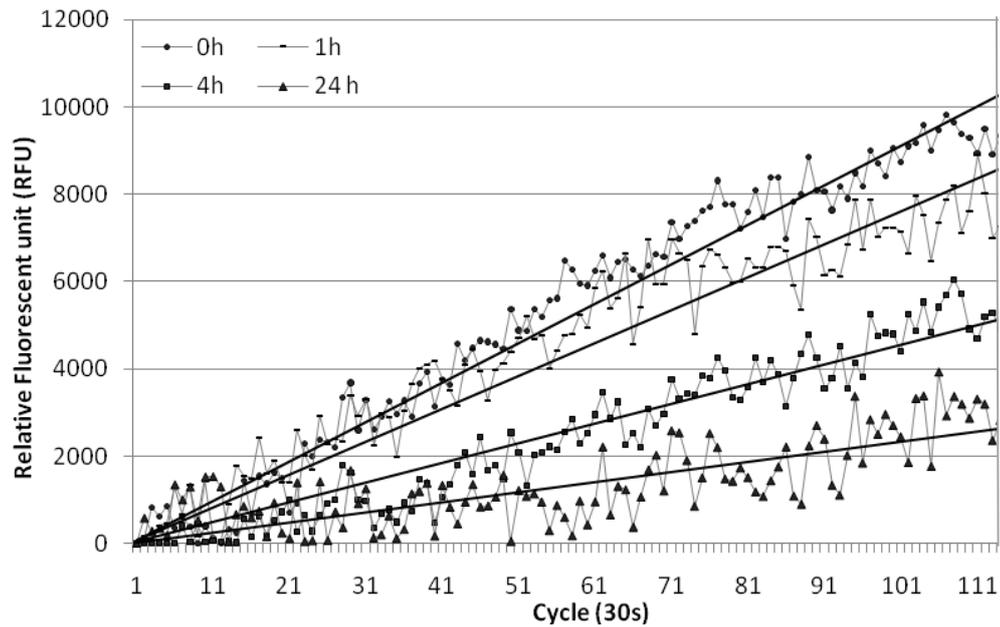
### 2.3.4 Efflux activity is induced by voriconazole treatment

The 8 h germling growth phase was subsequently examined to evaluate the effect of pre-treatment with voriconazole (0.0625 mg/L) on efflux activity (Figure 2.8a). This phase was selected as quantifiable efflux activity was constitutively low amongst the isolates examined, allowing quantification of any changes following voriconazole treatment. The data demonstrated that after voriconazole treatment the  $\Delta$ RFU/mg was reduced from ~1100 to ~980 after 1h, and significantly reduced to ~750 ( $p < 0.05$ ) and ~550 ( $p < 0.01$ ) after 4 and 8 h, respectively (Figure 2.8b).

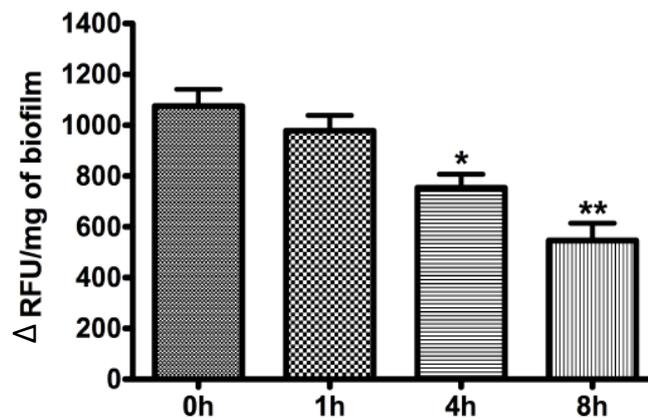
### 2.3.5 Competitive inhibition of efflux pumps improves *A. fumigatus* sensitivity to voriconazole.

An efflux pump inhibitor (EPI [MC-207,110]) was used to assess the contribution of efflux pumps to voriconazole sensitivity. EPI [MC-207,110] is a competitive substrate of efflux pumps. A checkerboard assay was initially developed to test combinations of voriconazole and EPI (Figure 2.9). It was demonstrated that EPI did not exhibit any antifungal activity at any of the concentrations tested (>512 mg/L). Therefore 64 mg/L was selected for all combinational experiments. In the presence of EPI the MIC range was significantly reduced from 2 to 8 fold (t-test;  $p < 0.05$ ) (Figure 2.10, Table 2.8)

A)

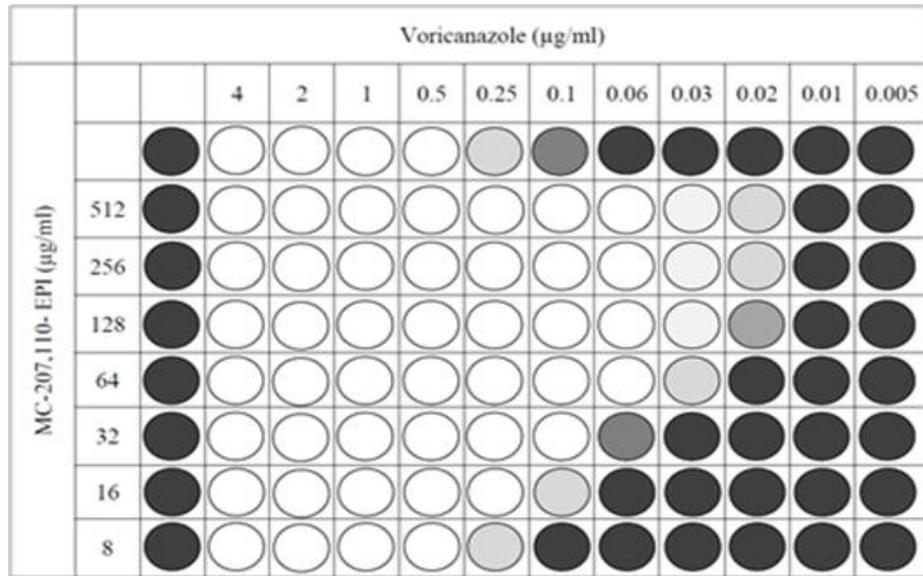


B)

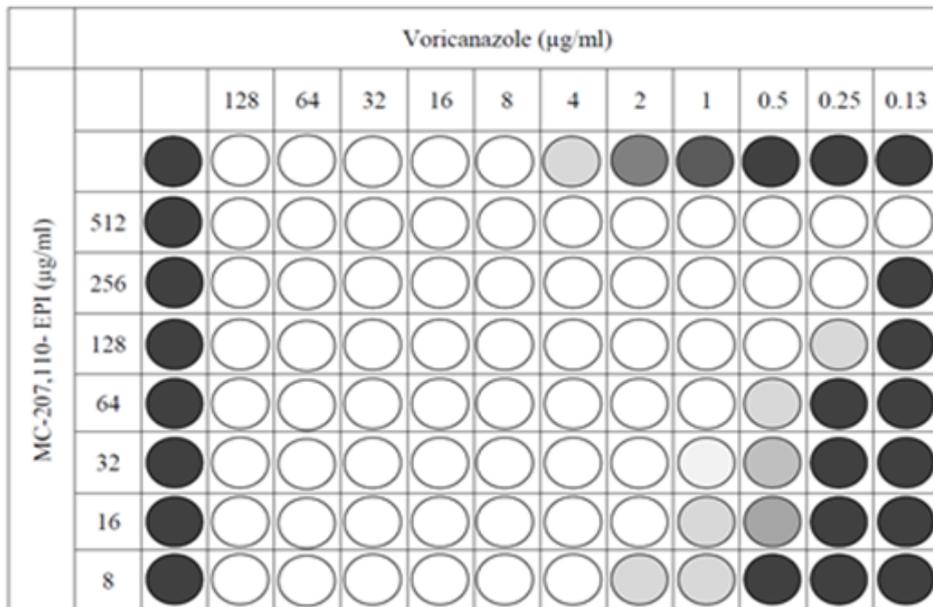


**Figure 2.8 - Efflux activity is induced by voriconazole.** *A. fumigatus* was grown in 96-well microtiter plates in RPMI at 37°C. (A) Fluorescence produced by 8 h *A. fumigatus* biofilm pre-treated with voriconazole (0.06251 mg/L) for 0, 1, 4 and 24 h (B) The change in fluorescence of dry weight normalised biofilms ( $\Delta$ RFU/mg) was determined. High values indicate low efflux and vice versa. The error bars represent the standard deviation of the means. (\* $p < 0.0001$ ). \* - 0.05, \*\* - 0.01.

(a)



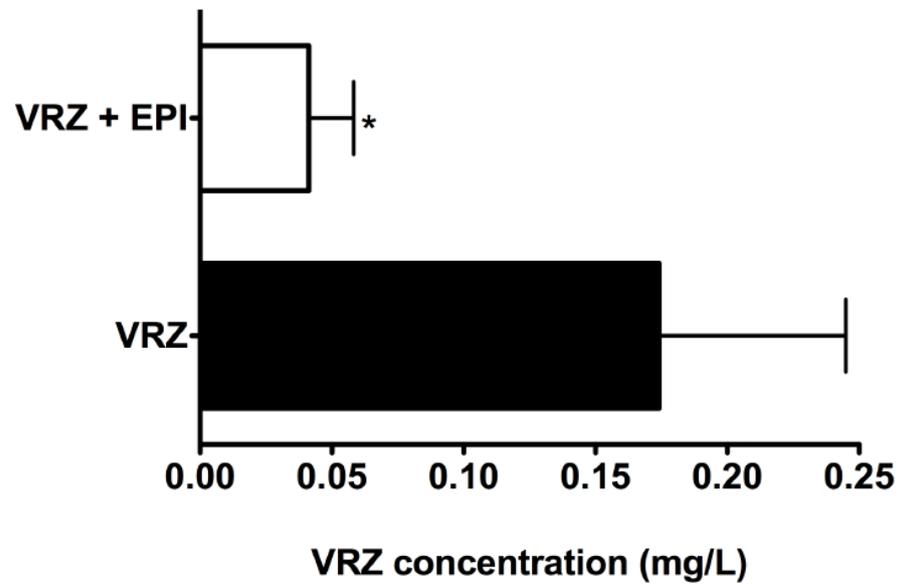
(b)



**Figure 2.9 - Pharmacological inhibition of efflux pumps enhances the efficacy of azoles against *A. fumigatus* biofilm.** *A. fumigatus* was grown in 96-well microtiter plates in RPMI at 37°C. After 8 h (a) or 12 h (b) cells were washed with PBS to remove non-adherent cells and fresh media was added with varying concentrations of the voriconazole (0-128  $\mu\text{g/mL}$ ) in combination with the efflux pump inhibitor (EPI) MC-207,110 (0-512  $\mu\text{g/mL}$ ) in a checkerboard format. ● represents growth in the well and ○ represents growth inhibition.

**Table 2.8 - MIC of 8 h *A. fumigatus* biofilm in the presence and absence of EPI.**

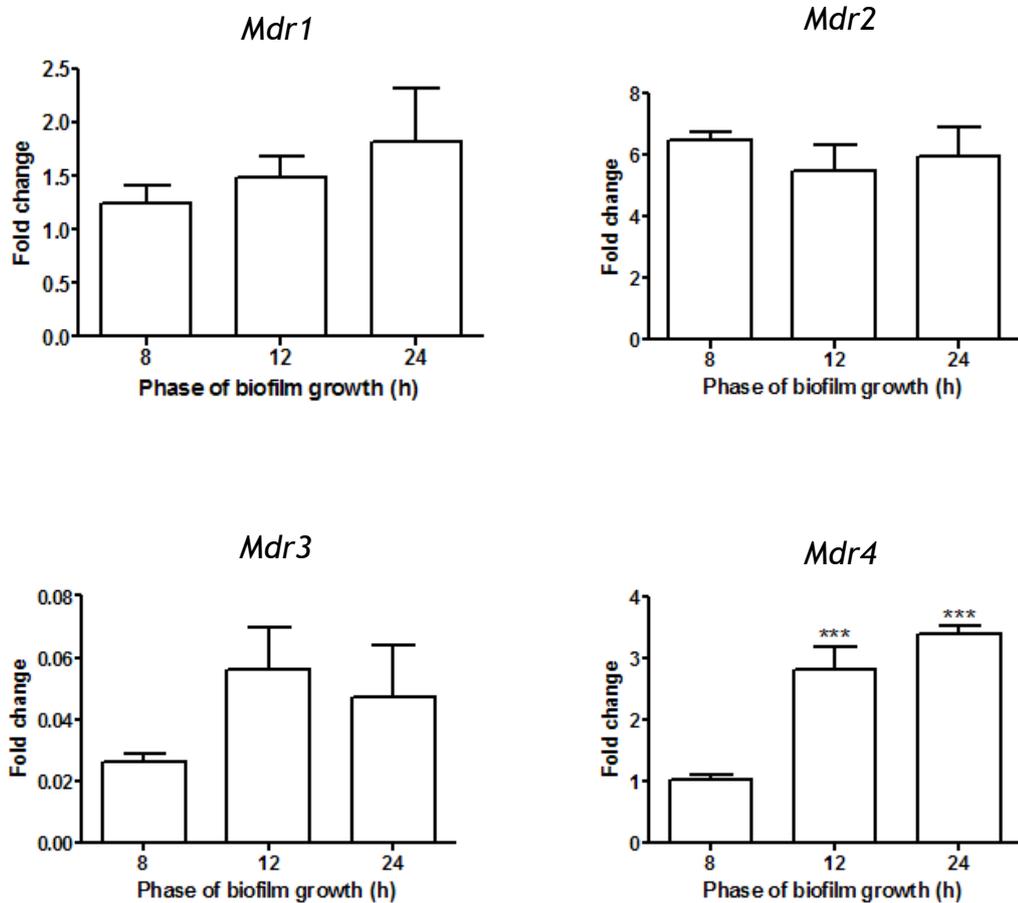
Strain	SMIC <sub>90</sub>		
	- EPI	+EPI	Fold change ( $\Delta$ MIC)
Af293	0.5	0.0625	8
YHCF1	0.0625	0.0312	2
YHCF2	0.0625	0.0156	4
YHCF3	1.0	0.25	4
YHCF4	0.125	0.0156	8
YHCF5	0.125	0.0156	8
YHCF6	0.125	0.0312	4
YHCF7	0.125	0.0156	8
YHCF8	0.0312	0.0078	4
YHCF9	0.0625	0.0156	4
YHCF10	0.0625	0.0156	4
YHCF11	0.0625	0.0078	8
YHCF12	0.0625	0.0156	4
YHCF13	0.0312	0.0078	4



**Figure 2.10 - *Aspergillus fumigatus* biofilm sensitivity to voriconazole is increased in the presence of an efflux pump inhibitor.** *A. fumigatus* biofilm were grown in flat bottomed 96-well plates for 8 h, washed and voriconazole (0.005 - 4 mg/L) added  $\pm$  MC-207,110 (64 mg/L). Viability was assessed using Alamar blue. Fourteen different strains were used and the experiments performed on three separate occasions. The data shows that the MICs of *A. fumigatus* are significantly reduced in the presence of an efflux pump inhibitor MC-207,110 by approximately 2-8 fold (t-test; \*p=0.0196). The error bars represent the standard deviation of the means.

### 2.3.6 *A. fumigatus* exhibits phase dependant efflux pump expression

The expression profile of four selected efflux genes (*AfuMDR1-4*) over different phases of biofilm formation were assessed in *A. fumigatus*. cDNA was produced for three different strains of *A. fumigatus* (one reference Af293 and two clinical strains - YHCF1-2) and samples from 8, 12 and 24 h tested in duplicate and expression normalised to  $\beta$ -tubulin using the  $2^{-\Delta\Delta Ct}$  methodology previously described (Livak and Schmittgen, 2001). All the graphs detailing the expression profiles of *AfuMDR1-4* assessed using real time RT-PCR are shown in Figures 2.11. Out of four efflux genes tested *AfuMDR2* was one of the highly expressed genes and *AfuMDR2* was the one expressed in lower level compared to others. The *AfuMDR1* and *AfuMDR4* genes showed a pattern of up regulation over different phases of biofilm formation. A significant up regulation of *AfuMDR4* gene was assessed with 12 and 24 h biofilms compared to 8 h ( $P < 0.0001$ ).



**Figure 2.11- Transcriptional analysis of efflux genes.** Quantitative RT-PCR was performed on each cDNA population using *B-tublin* as a housekeeping gene to calculate the relative expression of the genes according to the  $2^{-\Delta\Delta CT}$  method. The expression profile of efflux genes were expressed in fold change compared to housekeeping gene (a) *AfuMDR1*, (b) *AfuMDR2*, (c) *AfuMDR2* and (d) *AfuMDR4* was assessed using Quantitative RT-PCR. The error bars represent the standard deviation of the means. (\*\*\*) -  $P < 0.0001$ ).

### 2.3.7 *AfuMDR4* expression is associated with azole sensitivity

The *in vitro* expression of *AfuMDR4* transcripts were assessed in 8, 12 and 24 h phases of growth and compared to the fold increase in  $sMIC_{90}$  for five strains, and analysed as a fold-change in expression by normalising to the 8 h phase data (Table 2.9). Expression of *AfuMDR4* was shown to be differentially up-regulated in both the 12 and 24 h phases of growth in a strain dependant manner, with maximal up-regulation observed at the 12 h phase. The levels of up-regulation appeared to correlate with the change in  $sMIC_{90}$ , e.g. Af293 showed a 3.89 fold change in gene expression and a 16 fold change in MIC, whereas YHCF4 showed a 65.1 change in gene expression and a 128 fold change in MIC. At 24 h gene expression was reduced, which did not correlate with reduced MIC levels.

### 2.3.8 Efflux pump expression (*AfuMDR4*) is induced by *in vitro* voriconazole treatment.

Three populations were subsequently pre-treated with voriconazole and expression analysis of *AfuMDR4* performed at 1, 4 and 24 h post-treatment. Data was analysed at each time-point in comparison to matched untreated populations (Figure 2.12). These data demonstrated that *AfuMDR4* within the 8 h phase was significantly influenced by voriconazole exposure, increasing by 1.75- ( $p < 0.05$ ), 2.5- ( $p < 0.001$ ) and 4.2-fold ( $p < 0.001$ ) after 1, 4 and 24 h, respectively. *AfuMDR4* within the 12 h phase was also significantly influenced by voriconazole exposure, initially increasing 2.6- ( $p < 0.001$ ), 1.9- ( $p < 0.01$ ) and 4.4-fold ( $p < 0.001$ ) after 1, 4 and 24 h, respectively. *AfuMDR4* in the 24 h phase was also influenced by voriconazole exposure, but less dramatically, with an increase of 1.65- ( $p < 0.05$ ), 1.62- ( $p < 0.05$ ) and 1.13-fold ( $p > 0.05$ ) after 1, 4 and 24 h, respectively.

Table 2.9 - *A. fumigatus* exhibits phase-dependent resistance to voriconazole.

Strain	8 h		12 h		24 h	
	sMIC <sub>90</sub> ( $\Delta$ MIC <sup>a</sup> )	$\Delta$ MDR4 <sup>b</sup>	sMIC <sub>90</sub> ( $\Delta$ MIC <sup>c</sup> )	$\Delta$ MDR4	sMIC <sub>90</sub> ( $\Delta$ MIC <sup>c</sup> )	$\Delta$ MDR4
Af293	0.5 (8)	1	8 (16)	3.89	>256 (>512)	2.35
YHCF1	0.0625 (2)	1	1 (16)	4.37	256 (>512)	3.38
YHCF2	0.0625 (4)	1	1 (16)	2.31	>256 (>512)	2.72
YHCF3	1.0 (4)	1	32 (32)	31.60	>256 (>512)	4.50
YHCF4	0.125 (8)	1	16 (128)	65.10	>256 (>512)	14.80

<sup>a</sup>Fold change in the MIC following efflux pump inhibitor challenge.

<sup>b</sup>Fold change in expression of *AfuMDR4* compared to 8-h germling cells.

<sup>c</sup>Fold change in the MIC compared to the MIC for 8-h germling cells.

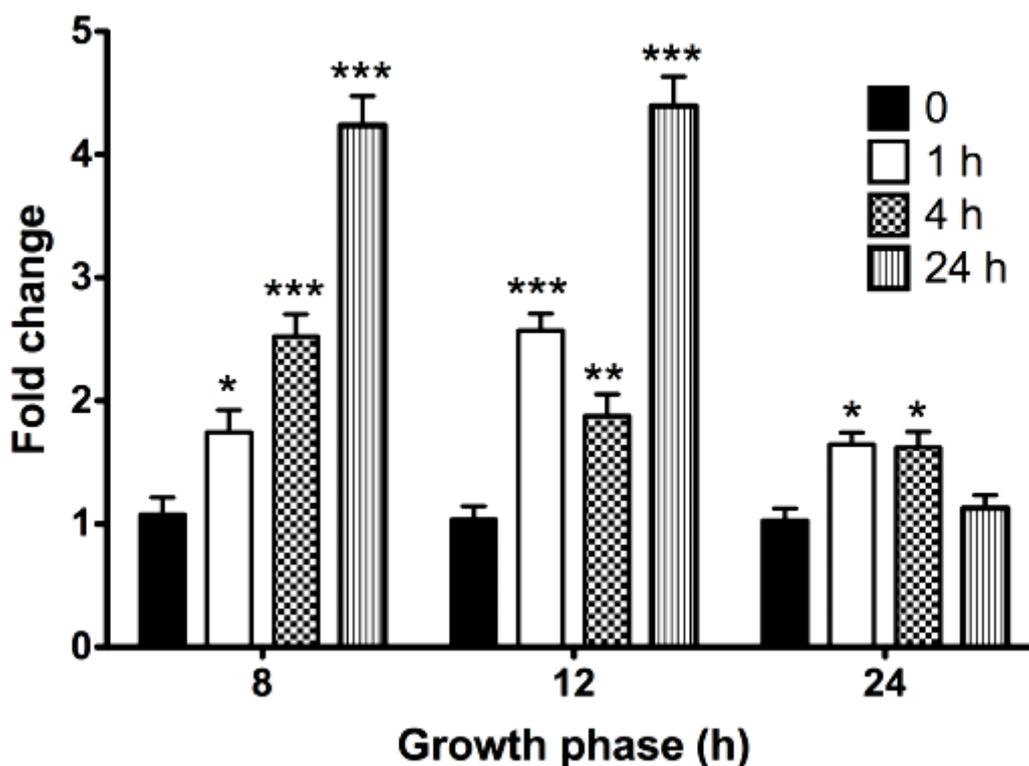
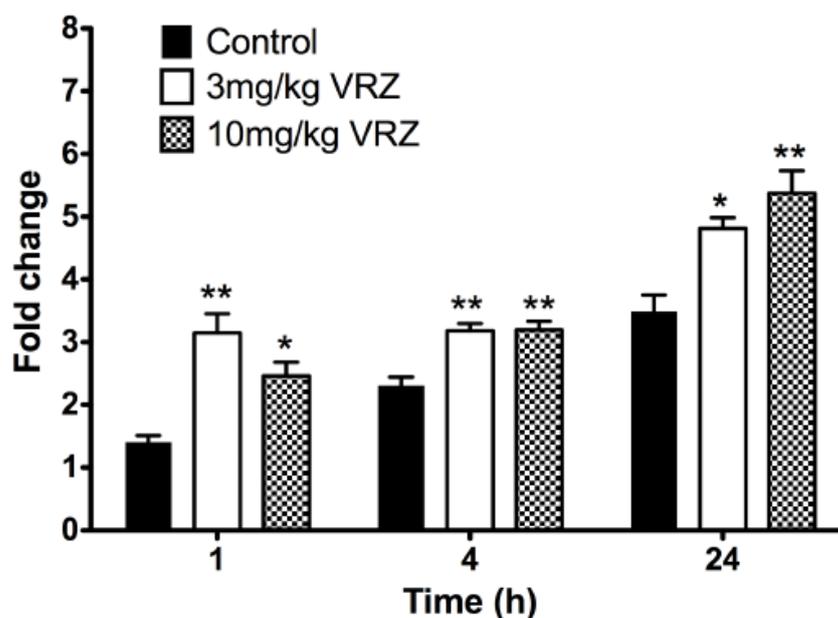


Figure 2.12 - *Aspergillus fumigatus* *AfuMDR4* expression is induced by exposure to voriconazole for 1, 4 and 24 h. Exposure to voriconazole induced *AfuMDR4* expression in the 8, 12 and 24 h phases of *A. fumigatus* growth. Quantitative RT-PCR was performed on each cDNA population using *B-tublin* as a housekeeping gene to calculate the relative expression of *AfuMDR4* according to the  $2^{-\Delta\Delta CT}$  method. Three isolates were tested on three separate occasions for each time point. The error bars represent the standard deviation of the means.

### 2.3.9 *Aspergillus fumigatus* exhibits phase dependant efflux pump expression (*AfuMDR4*) that is increased by voriconazole treatment *in vivo*.

The *in vivo* expression of *AfuMDR4* transcripts were assessed in vehicle control, 3 mg/kg (LD) and 10mg/kg (HD) voriconazole treated animals at 0, 1, 4 and 24 h post-treatment, and analysed as a fold-change in expression by normalising to the baseline control. Expression of *AfuMDR4* was shown to be significantly up-regulated according to ANOVA following treatment with voriconazole after 1, 4 and 24 h (Figure 2.13). A significant up-regulation was observed at 1 and 4 h with 3 and 10 mg/kg dose ( $p < 0.01$ ,  $p < 0.05$  and  $p < 0.01$ , respectively). At 24 h up-regulation of *AfuMDR4* was still observed ( $p < 0.05$  and  $p < 0.01$ , respectively).

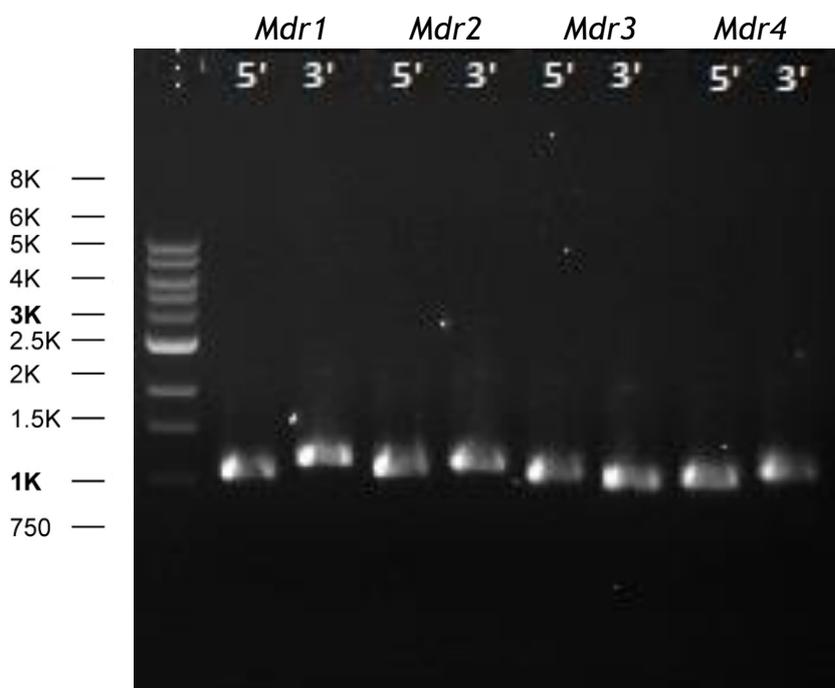


**Figure 2.13** -*Aspergillus fumigatus* *AfuMDR4* is expressed *in vivo* and is induced by voriconazole. Treatment with voriconazole (3 and 10 mg/kg) induced *AfuMDR4* expression 1, 4 and 24 h post treatment. Following removal of the subcutaneous *A. fumigatus* ATCC1163 biofilm and RNA processing, quantitative RT-PCR was performed on each cDNA population using *B-tublin* as a housekeeping gene to calculate the relative expression of *AfuMDR4* according to the  $2^{-\Delta\Delta CT}$  method. Three animals were used as a baseline for comparison to vehicle ( $n=3$  at 1, 4, and 24 h), 3 mg/kg ( $n=3$  at 1 and 4 h, with  $n = 5$  at 24 h) and 10 mg/kg ( $n=3$  at 1 and 4h, with  $n = 4$  at 24 h). The error bars represent the standard deviation of the means. \* $p < 0.05$ , \*\* $p < 0.01$ .

### 2.3.10 AfuMDR 1-4 mutational studies

#### 2.3.10.1 PCR amplification of flanking regions

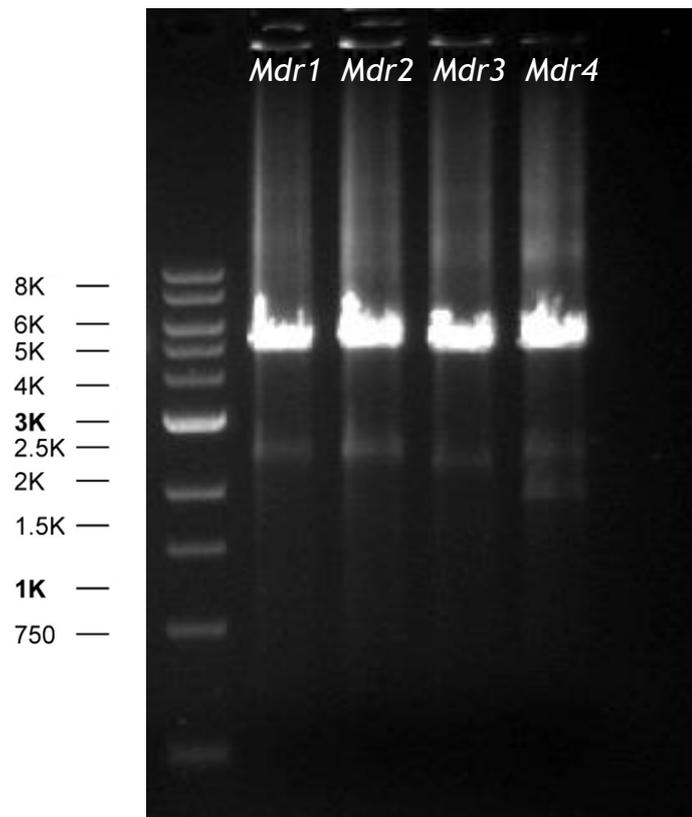
Knocking out of *AfuMDR1-4* genes involved two steps. In the first step, the flanking regions of the genes were amplified using the designed primers. The amplification product was then assessed for their size by comparing with a DNA ladder to confirm the amplification of correct target. Figure 2.14 show the gel electrograph with bands indicating the amplification of different flanking regions for *AfuMDR1-4* genes. The product size of 1000-1200bp indicates the correct amplification.



**Figure 2.14 - Confirmation of amplified flanking regions.** The amplified flanking regions of *AfuMDR1* (lane 2, 3), *MDR2* (lane 4, 5), *MDR3* (lane 6, 7) and *MDR4* (lane 8, 9) were gel electrophoresed to confirm the amplification. The bands at 1000-1200bp indicates the amplification of correct target.

### 2.3.10.2 Gene knockout cassette

The fusion PCR reaction was carried out with amplified flanking regions and a selectable marker to produce a gene knockout cassette. Figure 2.15 show the gel electrograph with bands indicating the formation of gene knockout cassette for *AfuMDR1* -4.



**Figure 2.15 - The fusion PCR product.** The amplified flanking regions and a marker sequence were fused together by a PCR reaction to form a gene knockout cassette. The bands in lane2-5 show the gene knockout cassettes formation. Lane 1- DNA ladder, lane 2 - *MDR1*, lane 3 - *MDR2*, lane 4 - *MDR2*, and lane 5 - *MDR4*.

### 2.3.11 Evaluation of *MDR* gene function

In order to confirm whether the differentially expressed transporters *AfuMDR1* - 4, played a functional role in azole resistance these genes were knocked out in *A. fumigatus* (Af293). Genes were knocked out and verified using PCR. In order to exclude random mutational effects from transformation two individual knockout transformants were selected for each gene knockout. After analysis and two rounds of single spore plating knockouts were tested for MIC to voriconazole in triplicate (Figure 2.16). The knockouts (*AfuMDR1*, 2 and 4) showed an MIC fourfold lower (0.063 mg/L) than that of the wild type strain (0.250 mg/L) for voriconazole reproducibly. The *AfuMDR3* knockout showed a reproducible two-fold lower MIC for voriconazole.

	4.000	2.000	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002
WT	○	○	○	○	○	●	●	●	●	●	●	●
$\Delta$ mdr1	○	○	○	○	○	○	○	●	●	●	●	●
$\Delta$ mdr2	○	○	○	○	○	○	○	●	●	●	●	●
$\Delta$ mdr3	○	○	○	○	○	○	●	●	●	●	●	●
$\Delta$ mdr4	○	○	○	○	○	○	○	●	●	●	●	●

**Figure 2.16 - MIC test with knockout strains and parental isolate.** MIC of voriconazole against wild type and knockout strains was determined by broth microdilution method. The first row indicates the concentrations of voriconazole in mg/L along each column. The dark circles indicate growth of *A. fumigatus* isolates and white circles indicate growth inhibition.

## 2.4 Discussion

*A. fumigatus* triazole resistance has become increasingly associated with the ergosterol biosynthetic pathway through mutation of the *CYP51A* gene locus. Increasing evidence suggests, however, that efflux pump mediated mechanisms may also be important determinants of resistance, especially given their characterised role in *C. albicans*. This study has demonstrated for the first time that efflux pump activity is associated with biofilm development and is also induced by triazole exposure, both of which contribute towards azole resistance.

This chapter demonstrated in a panel of clinical isolates using modified MIC methodologies that *A. fumigatus* grown in different phases of biofilm growth showed an increased resistance to voriconazole, a phenomenon previously demonstrated for Af293 by our group (Mowat et al., 2008b). Even when biofilm cells were disaggregated and standardised to similar cell counts using absorbance readings, a reduced sensitivity to VRZ was observed, which was also previously reported for *C. albicans* (Ramage et al., 2002a). Recent studies are in agreement with these observations, as it has been recently shown that triazole sensitivity is limited by complex filamentous growth (van de Sande et al., 2010). Whether this is because the physical quantity of drug is too low to elicit an effect or whether subtle transcriptional events occur in response to antifungal challenge, leading to reduced sensitivity, still remains to be determined. It is possible that the inoculum effect plays a role in limiting the effectiveness of VRZ (Lass-Flörl et al., 2003). A recent study reported that voriconazole treatment of *C. albicans* biofilms induced a calcineurin dependant response, resulting in changes in cell wall integrity that led to antagonism of the cell wall active micafungin (Kaneko et al., 2010). Moreover, global transcriptional analyses of voriconazole treated *A. fumigatus* mycelia demonstrated that over 2000 genes were differentially expressed, and amongst these were increased levels of transporter mRNA (da Silva Ferreira et al., 2006).

Based on this and previous microarray studies by Mowat E (Mowat E, PhD thesis, 2008), this chapter was decided to focus on efflux as a potential mechanism of resistance and analysed biochemical activity of efflux pumps in our model system. Active extrusion of a fluorescent molecule was shown to be elevated at 24 h and 12 h in comparison to 8 h germling cells. Voriconazole treatment of the 8 h germling cells resulted in a time dependant increase in efflux pump activity. The 12 h and 24 h cells were not tested as efflux was constitutively elevated, making differentiation of efflux activity difficult. This was potentially because of the role of extracellular matrix material, which has been shown to impede diffusion of molecules. Disruption and standardisation of the 24 h biofilm demonstrated an equivalent fluorescence to the undisrupted biofilm, suggesting that poor penetration of Ala-Nap was not a factor in the low levels of fluorescence detected in these assays. To demonstrate that efflux was involved in the reduced sensitivity to voriconazole, a broad spectrum efflux pump inhibitor, MC-207,110, a substrate of efflux pumps was used. These studies on the panel of isolates showed a significant MIC reduction to voriconazole of 2-8-fold.

Several studies have examined the expression of efflux pumps in *C. albicans* biofilms, where constitutive expression of *CaMDR1* was reported during early adhesion and in a phase dependant manner thereafter (Mukherjee et al., 2003, Mateus et al., 2004, Ramage et al., 2002a). However, to date no studies have reported this in *A. fumigatus* biofilms. In this study, the expression profile of *AfuMDR1-4* was assessed and found significant up regulation of *AfuMDR4* over the period of different phases of biofilm formation. Based on this result and previous work on itraconazole resistant mutants this chapter was decided to focus on *AfuMDR4*, which for these studies served as a quantifiable example (Nascimento et al., 2003). qPCR analysis demonstrated strain dependant up-regulation of this gene, particularly in the 12 h cells. Interestingly, those strains that exhibited the most elevated resistance and active biochemical efflux showed the highest levels of gene expression. Furthermore, voriconazole was shown to induce significant up-regulation of *AfuMDR4* transcripts in treated 8, 12 and 24 h cells in a time dependant manner. Ferreira and colleagues (2004) also observed azole-

constitutive and induced efflux pump expression on 24 h cultures following treatment with itraconazole. Here it was shown that *AfuMDR1-4* were differentially expressed following itraconazole treatment, and that *AfuMDR2* and *AfuMDR4* were preferentially induced on exposure to the azole, which has also been reported for *C. albicans*(da Silva Ferreira et al., 2004).

The clinical relevance of these transcriptional changes remains to be seen. Therefore, to evaluate whether efflux pump expression occurred *in vivo* we collaborated with Dr Peter Warn (University of Manchester) who devised an implanted *A. fumigatus* biofilm model. This was used to investigate the effects of voriconazole treatment on *AfuMDR4* expression from *A. fumigatus* biofilms. Expression was shown to be constitutive, and up-regulated in response to treatment with two different doses of voriconazole. Similarly, the expression of *CaMDR1* expression in a catheter based *C. albicans* biofilm model has been reported, but at a lower constitutive level (Nett et al., 2009). Moreover, the same group reported induction of *CaCDR1* and *CaCDR2* in their *in vivo* model following fluconazole treatment, but no change in the expression of *CaMDR1*(Lepak et al., 2006).

Disruption of transporter genes *AfuMDR1-4* in an *A. fumigatus* strain resulted in minor changes in azole sensitivity. *AfuMDR1*, 2 and 4 knockouts led to a fourfold reduction in MIC to voriconazole with 8 h grown germlings. It indicates the contributory role of transporter genes in azole resistance. However, this chapter demonstrate maximal strain dependant transcript expression at 12 h, yet voriconazole resistance increased to  $\geq 256$  mg/L for all strains tested. It is plausible that resistance is a highly regulated process, with efflux pump mechanisms playing an early role in resistance during colonisation, which is then reduced as extrapolymeric matrix is produced during biofilm maturation.

Collectively, the data presented herein, in addition to the available literature, support the hypothesis that efflux pumps are an important, but not exclusive, determinant of resistance to azoles (Morschhauser, 2010, Cannon et al., 2009). Their primary role may be for homeostasis within complex environments to protect themselves from acute toxicity, but within clinical environments, such as an aspergilloma, exposure to azoles drugs may enhance the levels of efflux pump expression, therefore either contributing towards or inducing clinical resistance. Indeed, expression of these pumps has been shown biochemically, indicating these transcripts are translated into protein. These observations may explain why treatment failure to azoles may occur clinically, and should raise doubt as whether sequential azole therapy is an appropriate treatment strategy for aspergillosis. Further studies are required to establish how *A. fumigatus* efflux pumps are regulated and whether inhibitors of these could augment azole therapies.

## Chapter 3

***Aspergillus fumigatus* extracellular  
DNA is a key component of the  
biofilm matrix**

### 3.1 Introduction

The previous chapter demonstrated phase dependant antifungal activity against *A. fumigatus* biofilms and association of efflux pumps. This work suggests that early resistance phenotypes were coincident with increased efflux pump activity. This correlation was not evident in mature biofilms, so this chapter hypothesises that ECM may play a greater role in the later phases of biofilm growth. ECM is a fundamental characteristic of biofilms, providing protection from hostile factors, including antimicrobial agents (Flemming and Wingender, 2010, Ramage et al., 2012). Independent investigations have shown that biofilm cells attaching to epithelial cells had increased levels of ECM, which were coincidental with decreased sensitivity to antifungal drugs (Seidler et al., 2008). Whilst the precise role of the ECM is not known, it is hypothesized that it plays a significant role in antifungal resistance by adsorbing the molecule and preventing its diffusion (Beauvais et al., 2007a). This is supported from data emerging from the *C. albicans* biofilm field, where it was demonstrated that ECM expression (specifically beta-glucans), encoded through *Fks1*, sequesters antifungal agents and reduces susceptibility (Nett et al., 2010a). The physical binding of amphotericin B by *C. albicans* biofilms and their ECM components was also established using absorbance spectra of amphotericin B (Vediappan et al., 2010). Given the role of ECM in decreased antifungal susceptibility the physical binding of antifungals by *A. fumigatus* biofilm matrix and their ECM components remain unclear.

In bacterial biofilms the typical ECM consists of exopolysaccharides, proteins, surfactants, lipids, water and nucleic acids (extracellular DNA [eDNA]). In *A. fumigatus* biofilms hydrophobic ECM is composed of galactomannan, alpha-1,3 glucans, monosaccharides, polyols, melanin and proteins (Beauvais et al., 2007a). Despite the growing body of knowledge regarding eDNA in bacterial biofilms, there is a scant knowledge concerning its existence and role in fungal biofilms. Recent studies have shown that eDNA is also an important component of *Candida* biofilms, including *C. albicans*, *C. tropicalis* and *C. parapsilosis* (Martins et al., 2010, Paramonova et al., 2009, Al-Fattani and Douglas, 2006).

In *C. albicans* eDNA has been shown to contribute towards maintenance and stability of mature biofilms, but not in their establishment (Al-Fattani and Douglas, 2006, Martins et al., 2010), and as a regulator of biofilm cells antifungal resistance (Martins et al., 2012a). These data suggest the potential therapeutic use of agents that affect the ECM. Remarkably, little is known about the presence and contribution of eDNA in *A. fumigatus* biofilms.

The purpose of this chapter was to assess the antifungal binding by different biofilm components and to investigate the presence and biological role of eDNA in *A. fumigatus* biofilm ECM.

This chapter has been published in a peer reviewed journal:

**Rajendran R, Williams C, Lappin DF, Millington O, Martins M, Ramage G.2013. Extracellular DNA release acts as an antifungal resistance mechanism in mature *Aspergillus fumigatus* biofilms. Eukaryot Cell. Epub ahead of print 2013 Jan 11.**

## 3.2 Materials and methods

### 3.2.1 Strains and conidial preparation

*A. fumigatus* Af293, clinical isolates (YHCF1 - YHCF5) obtained from the Royal Hospital for Sick Children (Yorkhill Division), Glasgow. An *A. fumigatus* transformant expressing green fluorescent protein (Af-GFP) was used for microscopy analysis, as described previously (Wasylnka and Moore, 2002). All the isolates were stored as previously described in section 2.2.1. All *A. fumigatus* strains were grown on Sabouraud dextrose agar at 37°C for 72 h. Conidia were then harvested as described in section 2.2.2. Conidia were then counted on a Neubauer haemocytometer and adjusted to a standardised suspension of  $5 \times 10^5$  conidia/ml in RPMI. All procedures were carried out in a class 2 biological safety cabinet (MDH Intermed).

### 3.2.2 Amphotericin B absorbance spectrum

The absorbance spectrum of amphotericin B (ampB) was used to assess the amount of ampB binding with *A. fumigatus* biofilm as described previously (Huang and Kao, 2012). The concentrations of ampB starting from 64 mg/L serially diluted down to 0.125 mg/L in DMSO was prepared in a 96 well plate (Costar). The absorbance spectrum at a wavelength range of 300-500nm of serially diluted ampB were measured using a plate reader (Fluostar Optima; BMG Labtech). Absorbance maxima at 415nm of ampB were used to plot the standard curve.

### 3.2.3 Extraction of amphotericin B from *A. fumigatus* biofilm

To assess the binding of amphotericin B (ampB) to *A. fumigatus* biofilm matrix, preformed biofilms on Thermanox™ coverslips (13mm) were exposed to ampB for different time periods (0-120min) and ampB was extracted from biofilm ECM as perviously described (Vediyappan et al., 2010). In brief, 1 ml of DMSO was added to the 5ml plastic bijoux containing the biofilms on coverslips. Then the bijoux were vortexed for 1min to dislodge the biofilm and incubated in the dark for 5min at room temperature. The mixture was then centrifuged at x10000rpm

for 10min and the supernatant containing ampB was collected in fresh 1.5ml micro centrifuge tubes. The absorbance spectra of each extract were then assessed as described above. For each experiment ampB untreated biofilms were added as controls.

### 3.2.4 Amphotericin B binding to fungal components

The binding of ampB with different fungal components including chitin,  $\beta$ -glucan, ergosterol and zymosan was assessed by an ampB binding assay. Commercially available purified  $\beta$ -glucan from *Saccharomyces cerevisiae* (4mg/mL) (Calbiochem), chitin from crab shells (10mg/ml) (Sigma), ergosterol (10mg/mL) (Sigma), zymosan (4mg/mL) (Sigma) and gelatin (10 mg/mL) (Sigma) were prepared in distilled water. Then 32 mg/L of ampB was added to each component and incubated at room temperature in the dark for 5 min. Then the mixture was centrifuged at x4000rpm for 1min to remove the unbound ampB. The pellet was then washed with PBS and ampB was extracted using 1 volume of DMSO. The extracts were then assessed for absorption spectra using a plate reader (Fluostar Optima; BMG Labtech).

### 3.2.5 Microscopy

Standardised conidia of Af293 or Af-GFP were inoculated in RPMI onto Thermanox™ coverslips (13mm) within a 24-well tissue culture plate, and incubated for 24 h at 37°C. These were gently rinsed with PBS and stained, according to manufacturer's instruction, with 5 $\mu$ M calcofluor white (Af293 only) (Invitrogen) that binds chitin and  $\beta$ -glucan of fungal cell walls, and 20 $\mu$ M propidium iodide (PI) (Sigma) that stains the DNA present within the biofilm. Biofilm growth and accumulation of eDNA were visualised under a fluorescence microscope (Motic BA400, Colorview system) or confocal laser scanning microscope (CLSM) (Leica SP5 laser scanning confocal microscope) at an excitation and emission wavelength of 350:400 for calcofluor white, 540:525 for propidium iodide, and 480:490 for GFP. One representative from each group was digitally photographed. Quantification of the z-stacks was performed using Volocity 3D Image Analysis Software (Perkin Elmer). For scanning electron

microscopy (SEM) representative biofilms grown and treated on Thermanox™ coverslips were processed and viewed under a scanning electron microscope as previously described (Section 2.2.4.2).

### 3.2.6 DNA extraction

ECM was extracted as previously described, with slight modifications (Beauvais et al., 2009). Briefly, standardised *A. fumigatus* conidia were inoculated into a 75cm<sup>2</sup> tissue culture flask (Nunc) containing RPMI, YPD and YNB at 37°C for 8, 12, 24, 48 and 72 h on a rocking platform. After incubation the biofilm was removed from the flasks using sterile cell scrapers and washed with PBS. The disaggregated biofilm was then treated with 0.2M EDTA to extract the ECM. The samples were then centrifuged at 10000g and the EDTA supernatant was recovered and filtered using 0.45µm syringe filter (Millipore). The eDNA from EDTA supernatant and genomic DNA from *C. albicans* and *A. fumigatus* cells were extracted using MasterPure™ Yeast DNA purification kit (Epicenter®), according to the manufacturer's instructions.

### 3.2.7 Quantification of ECM associated eDNA

Different phases of biofilm (8, 12 and 24 h) ± azetazolamide (128 mg/L, Sigma), a chitinase inhibitor (Schuttelkopf et al., 2010), were formed at 37°C either in microtiter plates or in tissue culture flasks as described above. The quantity of eDNA within the ECM was measured using a microplate fluorescence assay (MFA) using a DNA binding dye (SYBR Green I), as previously described (Leggate et al., 2006). Briefly, SYBR Green I (Invitrogen) was added to eDNA extract in a black well microtiter plate (Costar3603; Corning) in a ratio 1:1. Binding of this dye produces fluorescence in direct proportion with DNA concentration. The levels of eDNA were quantified using a fluorescence plate reader (Fluostar Optima; BMG Labtech) at the excitation and emission wavelength of 485 and 518 nm, respectively. The concentration of eDNA in the sample was quantified by using the DNA standard curve as previously described elsewhere (Leggate et al., 2006).

### 3.2.8 RAPD PCR

The PCR amplification of seven different genes was performed using Reddymix™ PCR master mix (Thermo scientific). Primers used for RAPD PCR amplification of *ANXC4*, *BGT1*, *CAT1*, *LIP*, *MAT1-2*, *SODB*, *ZRF2* and cycling conditions were previously described elsewhere (Bain et al., 2007). The following cycling conditions were used for PCR amplification. 95°C for 5 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min and a final elongation step at 72°C for 5 min. The PCR reaction was performed in a MyCycler PCR machine (Bio-Rad). The amplified samples were then separated using horizontal gel electrophoresis and the gel was observed under UV light excitation (Bio-Rad Gel Doc 2000, Bio-Rad Life Sciences).

**Table 3.1- List of primers used for RAPD PCR.**

Gene	Primer sequence
<i>ANXC4</i> F	GCGAGATAGCAACACTTCAGT
<i>ANXC4</i> R	GGATACTGTTGCCCTAGATTTG
<i>BGT1</i> F	GATCGGTTGCCAGTCTTTGA
<i>BGT1</i> R	AATGGACGCAGAATGAAACT
<i>CAT1</i> F	AGCTCAACCGTCGTGAT
<i>CAT1</i> R	TGCCATGCCCAGACATA
<i>LIP</i> F	CGCCTCACTTCTCCTCA
<i>LIP</i> R	TGCGAAATGGCTGACG
<i>MAT1-2</i> F	ACACCCTTCGACTTTCCA
<i>MAT1-2</i> R	GATCGCATAGTGTGAGTGG
<i>SODB</i> F	GCTCCAAGAGCTGCTAC
<i>SODB</i> R	AAGCGCTTCTCCACAGC
<i>ZRF2</i> F	CTCATCCAAGCTTGTTTCC
<i>ZRF2</i> R	GTACCGCATCACCATCAA

### 3.2.9 Evaluating biofilm formation and stability

Biofilms were formed in 96-well microtitre plates as previously described (Mowat et al., 2007a). DNase I from bovine pancreas (Sigma-Aldrich), referred to as DNase for simplicity, was prepared in 0.15 M NaCl supplemented with 5 mM MgCl<sub>2</sub> (Martins et al., 2012a). To evaluate the effect of DNase treatment on preformed biofilms the Af293 biofilms were grown for 24 h and treated with DNase (0.25, 1 and 4 mg/mL) at 37°C overnight, with an untreated buffered control included on each plate for comparison. For further controls, heat inactivated DNase (0.25 mg/L), RNase (0.25 mg/L) and proteinase K (0.25 mg/L) (Sigma-Aldrich) were tested under the same conditions. To assess the effect of DNase on biofilm formation the standardised 5x10<sup>5</sup> Af293 conidia were incubated with DNase (0.25, 1 and 4 mg/mL) at 37°C overnight. Finally, to assess the effect of exogenous DNA on biofilm formation standardised conidia were incubated with salmon sperm DNA (Sigma-Aldrich), *C. albicans* and *A. fumigatus* genomic DNA (4, 8 and 20 mg/L) at 37°C overnight. DNA was extracted as described above. After each treatment the biofilms were washed in PBS and biomass quantified using a crystal violet assay.

### 3.2.10 Crystal violet assay

Crystal violet assay was used to assess the biofilm biomass as described previously (Christensen et al., 1985, O'Toole and Kolter, 1998). For quantifying the biofilm biomass, the spent media was discarded and the biofilm washed twice with PBS. The biofilms were then air dried and 100µl of 0.5% crystal violet (Fisher Scientific) solution added. The plate was then incubated at room temperature for 5-10min and the crystal violet solution was removed from each well. The wells were then carefully washed under running water, until all unbound crystal violet was removed. Then 100µl of 90% (v/v) ethanol was added to each well to de-stain the biofilms. The ethanol was then transferred to a clean 96-well microtitre plate and the absorbance read at 570nm using an absorbance plate reader (Fluostar Optima; BMG Labtech). The ethanol mixture was diluted down appropriately before absorbance measurement for the experiments having higher biomass for better sensitivity. A negative control well

containing no *A. fumigatus* was included on every microtitre plate for quality control.

### 3.2.11 Biofilm susceptibility testing

DNase was used in combination with different classes of antifungals to determine whether antifungal efficacy could be enhanced. Amphotericin B, caspofungin and voriconazole were prepared according to the Clinical Laboratory and Standards Institute recommendations and used throughout these experiments (CLSI, 2008). For testing antifungal susceptibility in combination with DNase, a checkerboard assay was performed with 24 h preformed biofilms as described previously (Section 2.2.8.1). An assay was prepared with DNase (0 - 256 mg/mL) and either amphotericin B (0 - 16 mg/L) or caspofungin (0 - 512 mg/L) or voriconazole (0 - 512 mg/L) within these ranges in combination. The cells susceptibility ( $sMIC_{50}$ ) was determined at 50% reduction in metabolism compared to untreated controls using the 2, 3-bis (2-methoxy-4-nitro-5-sulfo-phenyl) - 2 *H* tetrazolium - 5-carboxanilide (XTT)- based reduction assay, as previously described (Section 2.2.6). Following these initial experiments, a defined concentration of DNase was selected (128 mg/L) for all subsequent microdilution testing on different phases of biofilms with the panel of strains (n=6).

### 3.2.12 Statistics

Analysis of variance (ANOVA) and t-tests were used to investigate independent sample data. A Bonferroni correction was made to the p value to allow for multiple comparisons and applied to the data where appropriate. SPSS (Version 11, Chicago, USA) was used for these analysis and GraphPad Prism (Version 4, La Jolla, USA) for the production of the figures. The Spearman's rho correlation coefficient ( $\rho$ ) was determined to investigate the relationship between the parameters measured. A p value of less than 0.05 was considered significant.

### 3.3 Results

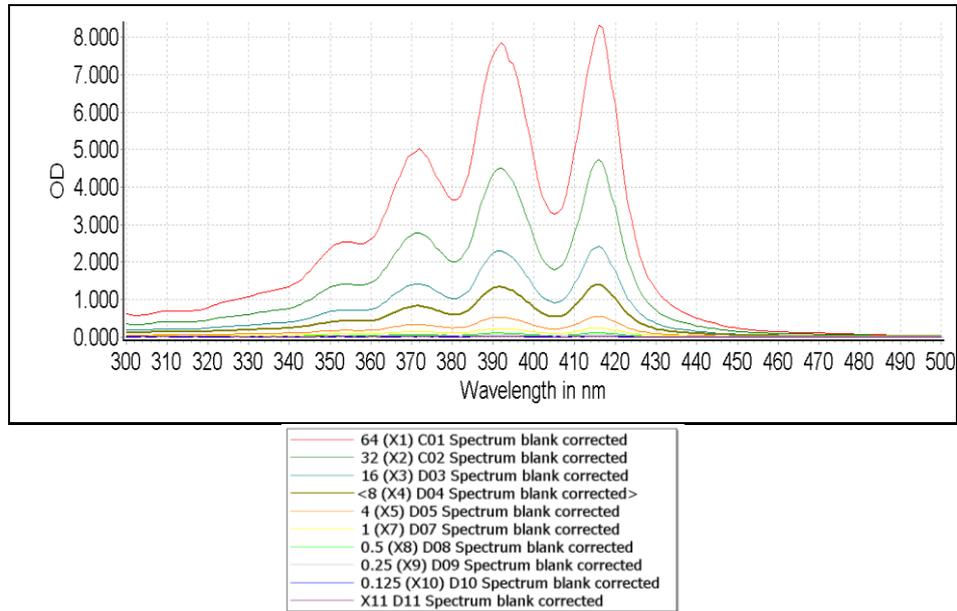
#### 3.3.1 Characteristic spectra of Amphotericin B

The absorption spectra of known quantities of ampB were scanned between wavelengths of 300 and 500nm. The absorbance spectrum of ampB in DMSO (Figure 3.1a) shows four typical peaks at approx. 350, 370, 390 and 415nm, with an absorption maximum at 415nm. This absorption pattern was detectable even at a concentration of as low as 0.125 mg/L of ampB. The standard curve of ampB in DMSO was obtained by plotting the ampB concentrations along the x-axis and the absorbance value measured at 415nm in the fourth derivative spectrum along the y-axis. Figure 3.1b shows an excellent correlation ( $r^2 = 0.98$ ) between ampB concentrations and the absorbance values of the fourth derivative.

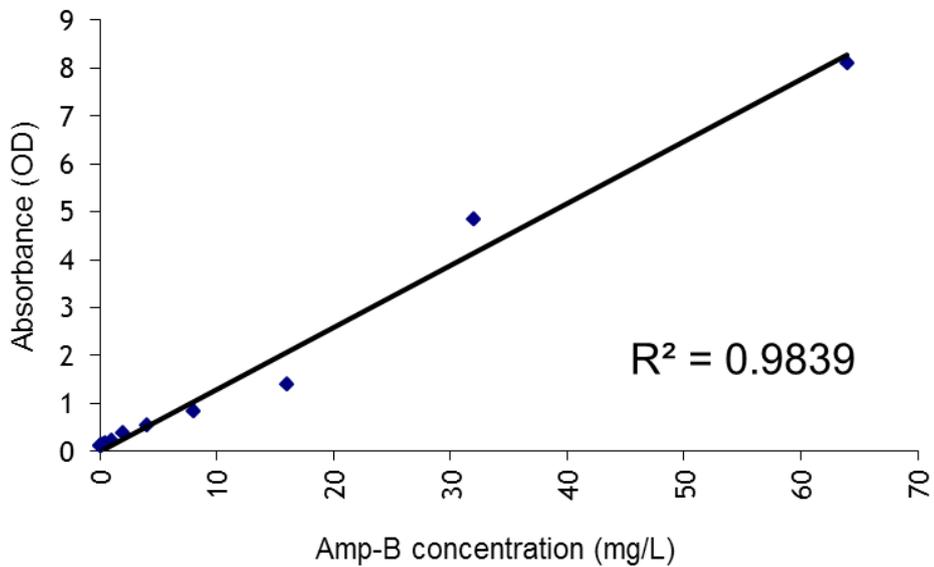
#### 3.3.2 Amphotericin B binds with fungal components

The binding of ampB with different fungal components was found using standardised ampB binding assay. Commercially available fungal components including chitin,  $\beta$ -glucan, ergosterol and zymosan were incubated with ampB (32 mg/L). The ampB was then extracted from fungal components using DMSO and ampB absorption spectra were assessed. Figure 3.2 show the absorption spectrum of extracted ampB. It shows ampB was bound to  $\beta$ -glucans, ergosterol and zymosan, and no measurable binding with chitin molecules. Approximately 3-5 mg/L out of 32 mg/L of added ampB was bound with  $\beta$ -glucans, ergosterol and zymosan in 5min.

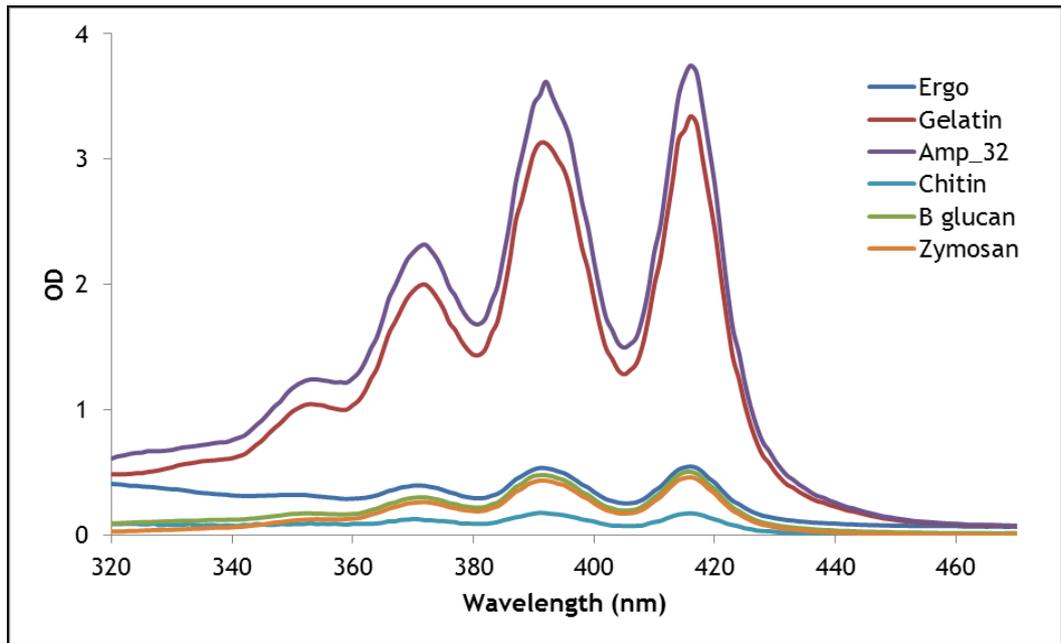
a)



b)



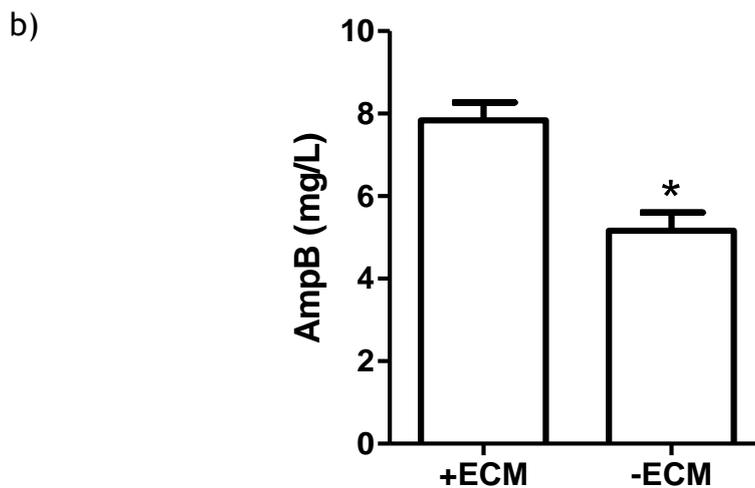
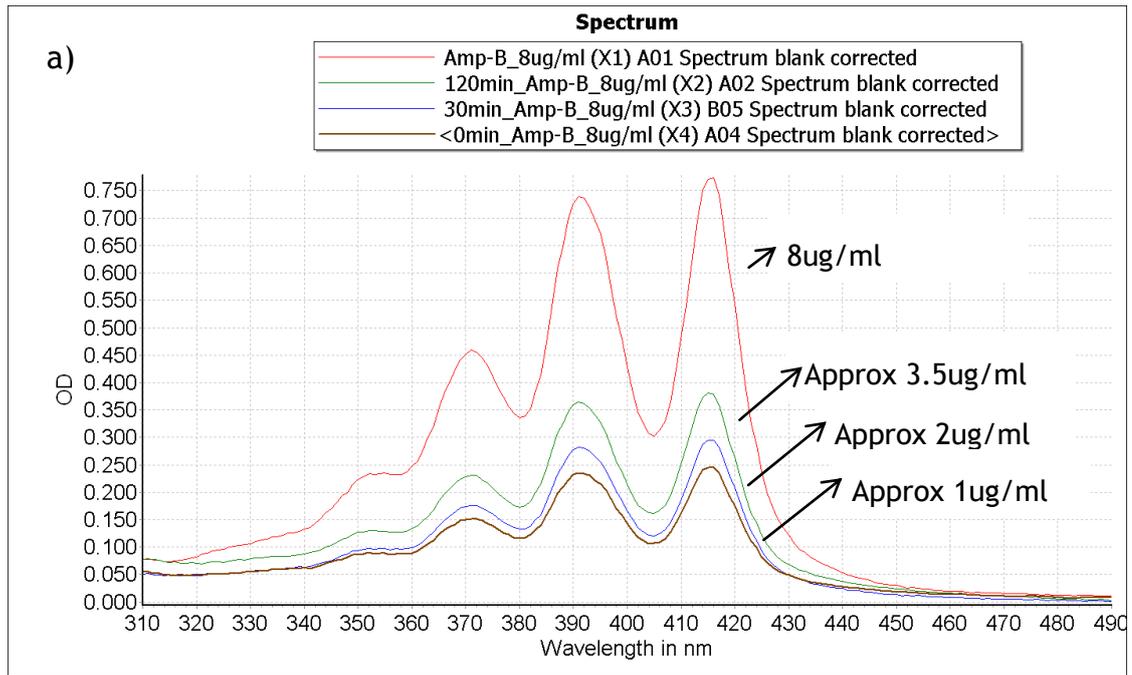
**Figure 3.1 - Characteristic spectra of amphotericin B.** a) absorption spectra of different concentrations (64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 mg/L) of ampB was measured between wavelengths of 300 and 500nm. b) Standard curve of ampB was plotted using ampB concentrations and absorbance values at 415nm.  $R^2$  value of 0.98 indicates linearity between ampB concentrations and absorbance values.



**Figure 3.2 - Amphotericin B binding with different fungal components.** Graph shows the absorbance spectra of ampB. Absorption spectrum of ampB extracts from ergosterol, chitin,  $\beta$ -glucan and zymosan was assessed between 320 and 470nm. The absorbance maxima at 415nm were used to quantify the amount of ampB bound with the components.

### 3.3.3 Amphotericin B binds with *A. fumigatus* biofilm matrix

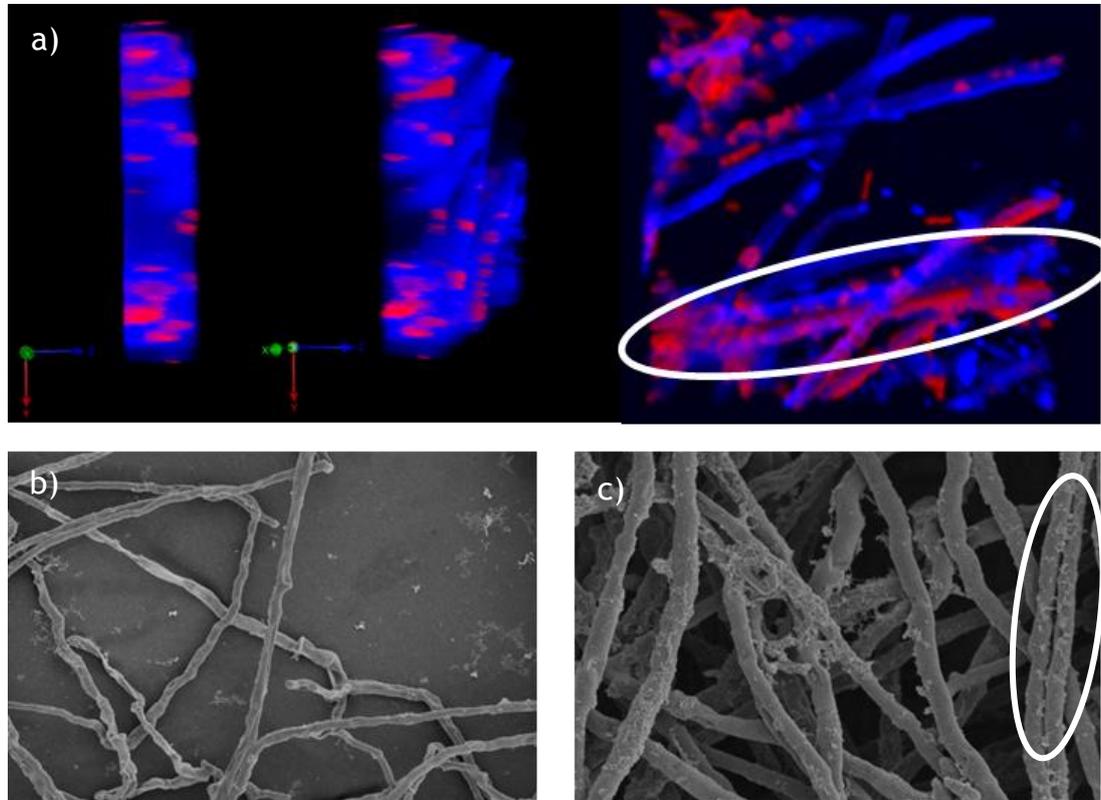
The *A. fumigatus* biofilms grown for 24 h were exposed to ampB (8mg/L) for 1, 30 and 120 min. After incubation period the ampB binding was assessed using absorption spectra. Figure 3.3 shows the spectra obtained with ampB extracts after each time point. Approx. 1 mg/L of AmpB was bound with biofilm after 1min, 2 mg/L in 30min and 3.5 mg/L in 120min. To find out whether the biofilm matrix plays a role in ampB binding, the binding was assessed in the presence and absence of biofilm ECM. The biofilms were treated with EDTA to remove the ECM and incubated with ampB (64 mg/L) for 1h. Figure 3.3b shows a significant reduction in AmpB binding in the absence of ECM compared to the EDTA untreated controls. Approx. 5 mg/L of AmpB was bound with no ECM biofilm and about 8 mg/L of ampB was bound with control biofilms.



**Figure 3.3 - Amphotericin B binds with *A. fumigatus* biofilm.** a) AmpB binding with 24 h grown *A. fumigatus* biofilm was measured after 1, 30 and 120min exposure to 8mg/L of Amp-B. Figure shows the absorption spectra of ampB extracts from each time point. b) The AmpB binding was assessed in the presence and absence of ECM. Biofilms were pretreated with EDTA to remove the ECM and the binding of AmpB was assessed using AmpB absorbance spectra. (\*  $p < 0.05$ ).

### 3.3.4 Presence of eDNA in *A. fumigatus* biofilm

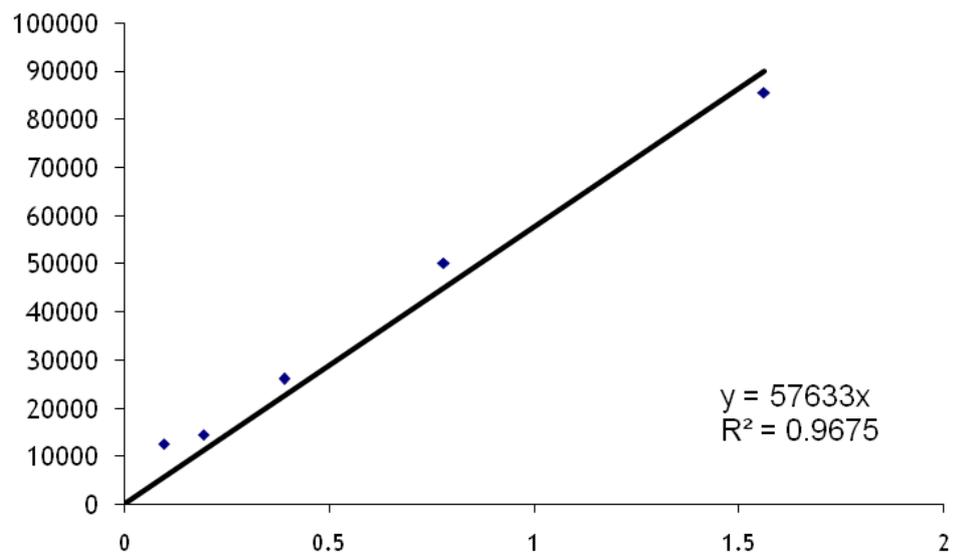
The presence of eDNA in bacterial biofilms is known to be associated with biofilm formation, stability and dispersal (Berne et al., 2010, Izano et al., 2008, Whitchurch et al., 2002). Due to the key structural and functional role of eDNA within bacterial biofilm ECM (Allesen-Holm et al., 2006), and more recently in *C. albicans* biofilms (Martins et al., 2010), it was hypothesised that eDNA might play a similar role in *A. fumigatus* biofilms. The visual inspection using CLSM of calcofluor white stained *A. fumigatus* biofilm counter-stained with PI showed the presence of eDNA. Besides staining dead cells PI can be used to map eDNA in biofilms (Allesen-Holm et al., 2006). At low magnification localised areas of diffuse red fluorescence were observed that were distinct from brightly stained dead cells. Higher magnification through the z-stacks of the biofilms indicated that eDNA co-localised with hyphae, which was located throughout the biofilm structure (Figure 3.4).



**Figure 3.4 - Presence of eDNA in *A. fumigatus* multicellular architecture.** a) CLSM images of 24 h grown *A. fumigatus* biofilms on Thermanox™ coverslip at 37°C. The different planer images of PI stained eDNA (diffuse red), which diffusely cover and intersperse calcofluor white (blue) stained hyphae (circled). (b-c) High magnification SEM images (x2000) of 24 h grown *A. fumigatus* biofilm showing the presence of some punctured hyphae covered and interspersed with ECM (circled). The images shown are representative of three independent experiments.

### 3.3.5 Standardisation of SYBR Green fluorescence assay

The DNA binding dye SYBR Green was used to quantify the amount of eDNA present in different phases of *A. fumigatus* biofilm formation. To standardise this assay different concentrations of salmon sperm DNA was prepared and stained with SYBR Green I. The subsequent fluorescence produced was quantified and plotted against DNA concentrations. Figure 3.5 show the DNA standard curve produced using SYBR Green fluorescence assay. The standard curve shows a good correlation ( $R^2 = 0.97$ ) between DNA concentrations and the fluorescence measurement. The quantity of eDNA in different biofilm samples was assessed by substituting fluorescence value as x in the equation  $y = 57633x$ .

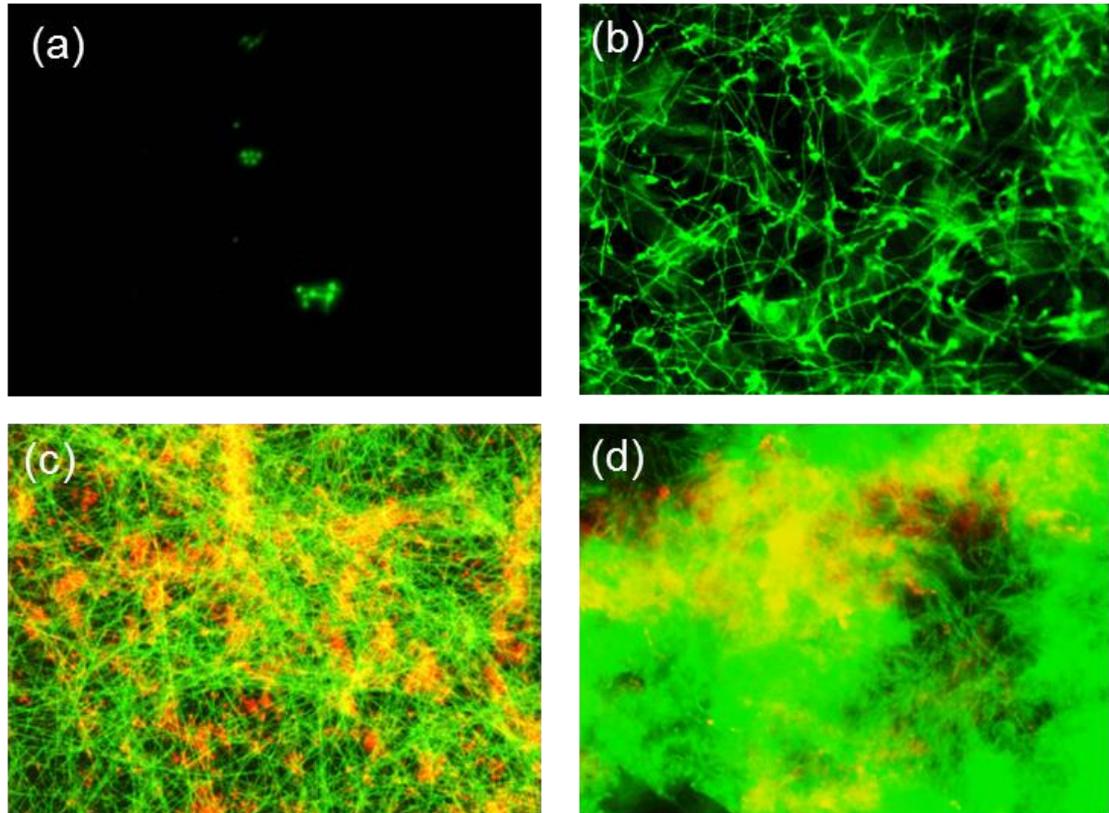


**Figure 3.5 - DNA standard curve from SYBR Green fluorescence assay.** Different concentrations of salmon sperm DNA was prepared in 96 well black plate and stained with SYBR Green I. The subsequent fluorescence was measured using fluorescence plate reader. Standard graph was then plotted using the fluorescence value (y-axis) and DNA concentrations (mg/L) (x-axis).

### 3.3.6 *Aspergillus fumigatus* releases extracellular DNA in a phase dependent manner.

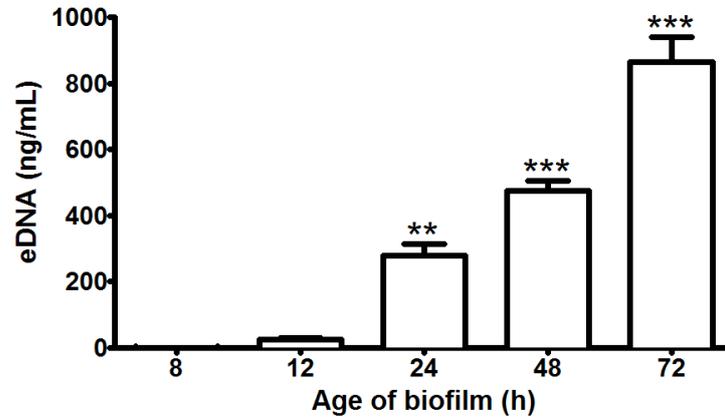
*A. fumigatus* (AfGFP) biofilm grown for 8, 12, 24 and 48 h stained with PI was investigated for the presence of eDNA using fluorescence microscopy. Figure 3.6 shows the presence of eDNA in different phases of biofilm formation. Red or Yellow appearance indicates the existence of eDNA in the biofilm. At the earliest time points at 8 h and 12 h (Figure 3.6 a-b) no or minimal PI fluorescence was observed and it increased over 24 and 48 h biofilms (Figure 3.6 c-d) in a time dependent manner.

Controlling for non-specific binding of PI to ECM was not possible, therefore to confirm that the staining observed was a result of eDNA this experiment aimed to quantify eDNA content throughout the biofilm. To do so Af293 biofilms grown for 8, 12, 24, 48 and 72 h were gently washed with EDTA to remove the ECM. Experiments were performed in triplicate on 3 separate occasions. The eDNA within the ECM was then quantified using the DNA standard curve from SYBR Green fluorescence assay. Significant release of eDNA was observed in a time dependent manner ( $p < 0.0001$ , ANOVA, Bonferroni's Multiple Comparison Post Test) (Figure 3.7). At the earliest time point (8 h) eDNA release was below detectable limits, whereas 4 h later 24ng/mL was detected. In comparison to 8 h biofilms, a significant increase of ECM associated eDNA was observed within biofilms of 24 h (279ng/mL,  $p < 0.01$ ), 48 h (473ng/mL,  $p < 0.001$ ) and 72 h (864ng/mL,  $p < 0.001$ ). The accumulation of eDNA released by *A. fumigatus* into ECM was also detectable by qPCR assay. The ct values reflect the quantity of eDNA present in each samples. A ct value of 24, 27, 31 and no-ct was obtained with 48, 24, 12 and 8 h biofilm samples.

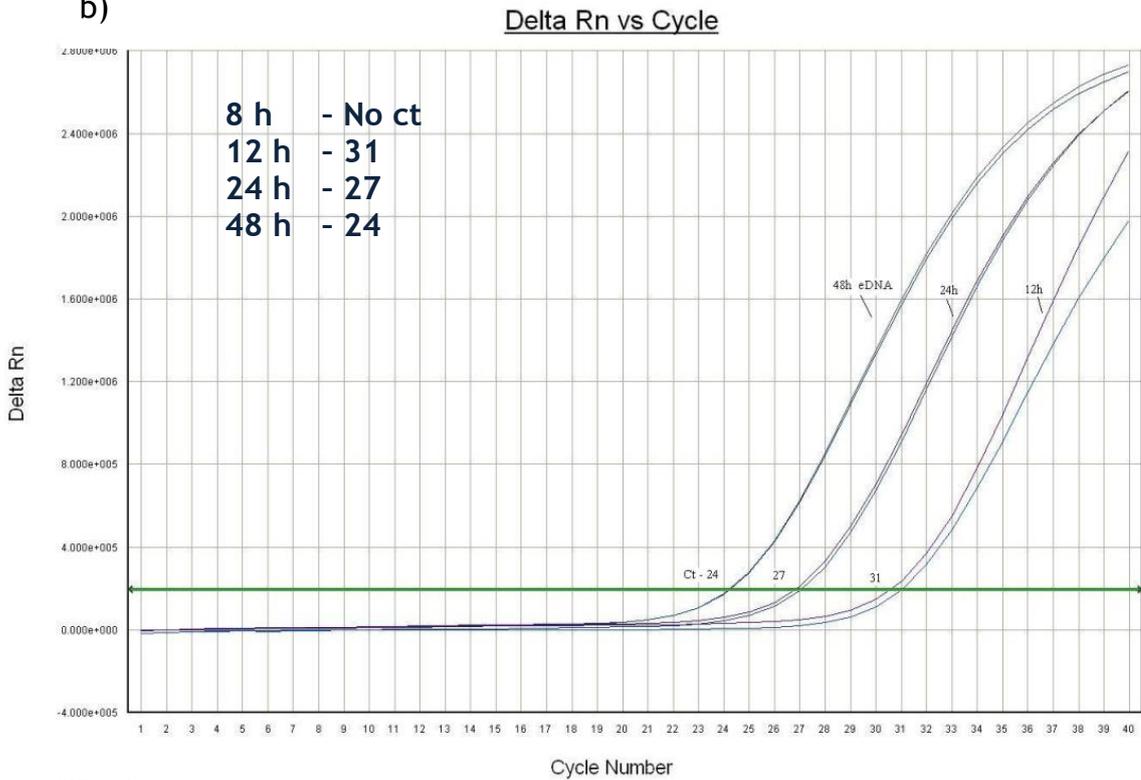


**Figure 3.6 - Phase dependent eDNA release in *A. fumigatus* biofilm.** Fluorescent micrographs (x20 magnification) of *A. fumigatus* (GFPstrain) grown for 8 h (a), 12 h (b), 24 h (c) and 48 h (d) on coverslips in a 24 well plate at 37°C. Biofilms were stained with DNA binding dye propidium iodide (PI). The colour saturated images of live GFP expressing cells (green) and PI stained eDNA (diffuse red) or dead cells (punctuate red) are shown. Yellow appearance indicates the presence of both live cells and eDNA. The images shown are representative of three independent experiments.

a)



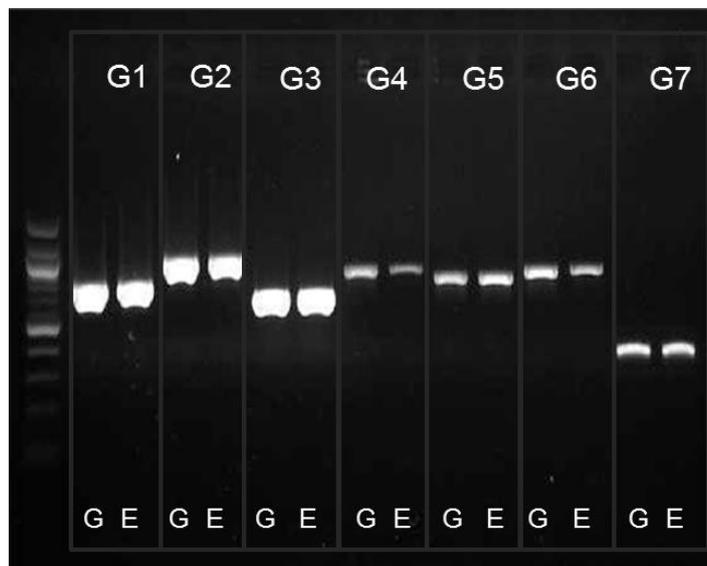
b)



**Figure 3.7 - The eDNA accumulation in biofilm ECM over time.** a) Extracted ECM were stained with DNA binding dye SYBR green I and resulting fluorescent production was measured using fluorescence microplate reader to assess the quantity of eDNA present. Significant increase in eDNA level was found starting after 24 h time point compared to 8 h baseline (\*\* $P < 0.001$ , \*\*\*  $P < 0.0001$ ), b) An amplification curve showing the ct values obtained for 8, 12, 24 and 48 h ECM samples.

### 3.3.7 Characterisation of eDNA released by *A. fumigatus*

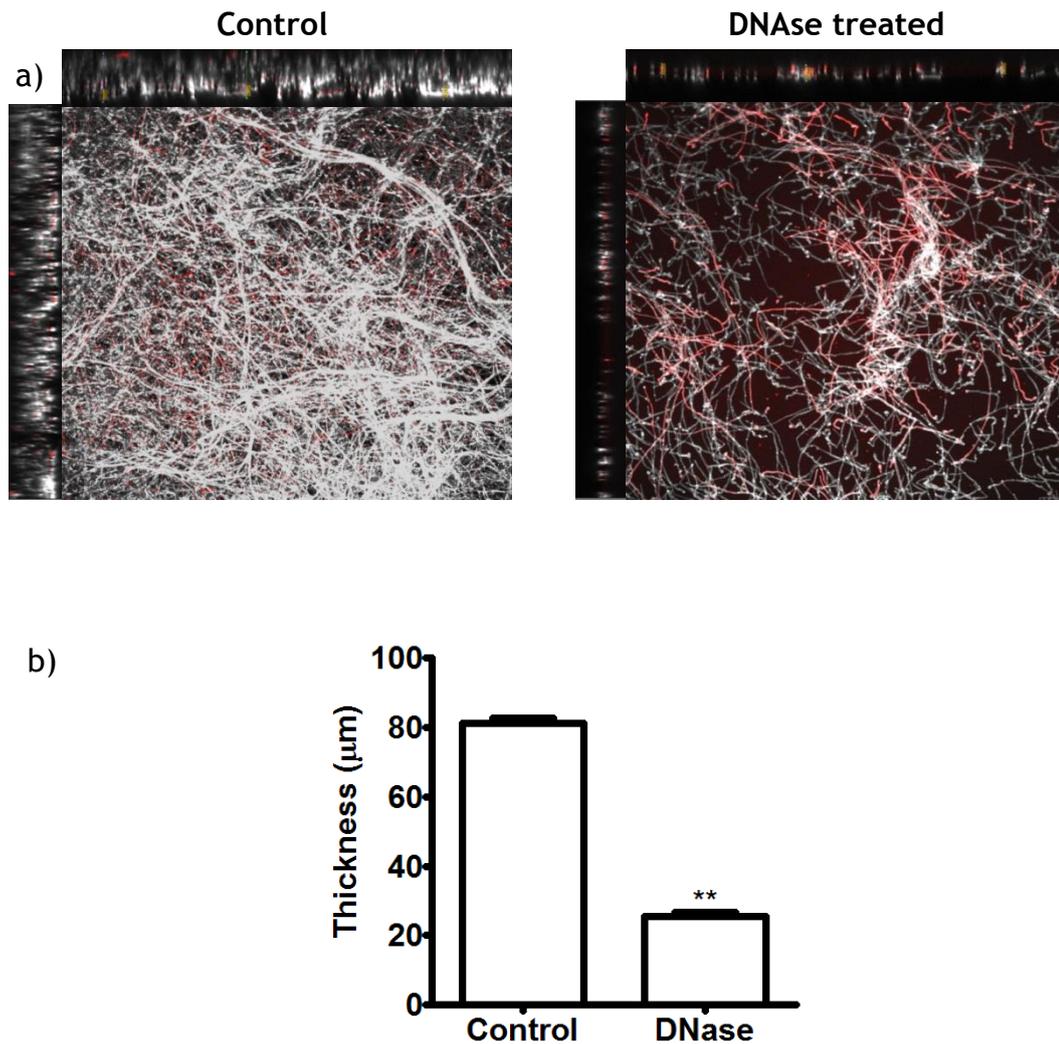
To investigate whether eDNA released from maturing biofilms was different from genomic DNA RAPD PCR analysis was performed using defined primer sets specific for 7 conserved genes (Martins et al., 2012b). Genomic DNA was extracted using standard methods and compared to eDNA extracted from ECM. Gel electrophoresis showed equivalent bands for all 7 genes amplified for both genomic and eDNA (Figure 3.8). This suggests that eDNA is a result of release from dying cells within the maturing biofilm. Microscopic examination of the hyphae within the biofilm was undertaken using SEM to investigate whether any structural defects were apparent. The SEM images showed occasional hyphae distributed throughout the dense filamentous structure had a punctured appearance at the hyphal tips coincidental with ECM extrusion (Figure 3.4 b). However, whether this event plays a direct functional role within the biofilm remained to be determined.



**Figure 3.8** - Random amplified polymorphic DNA PCR. RAPD PCR was carried out on extracted genomic (G) and extracellular (E) DNA samples to amplify seven different genes (G1 -*ANXC4*, G2-*BGT1*, G3-*CAT1*, G4-*LIP*, G5-*MAT1-2*, G6-*SODB* and G7-*ZRF2*) and the PCR products were electrophoresed on an agarose gel.

### 3.3.8 Extracellular DNA improves the structural integrity of *Aspergillus fumigatus* biofilms

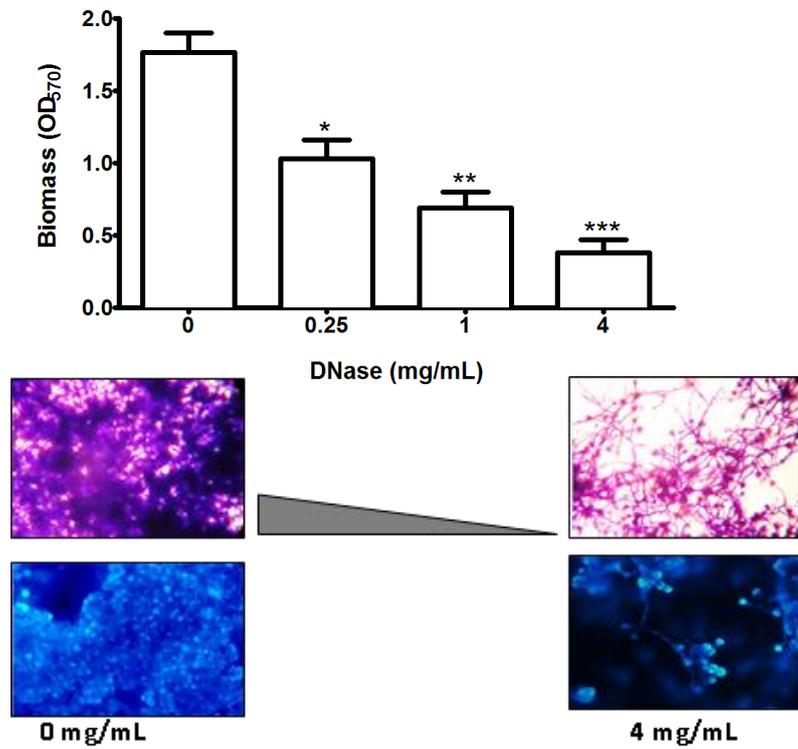
Given that as the biofilm matures eDNA release increases proportionally to the levels of ECM, then this suggests that it may play a specific role within the biofilm. Studies of *P. aeruginosa* have shown that eDNA plays a role in biofilm development (Whitchurch et al., 2002), indicating that this may also be the case for *A. fumigatus*. To test this hypothesis the addition of DNase to different phases of biofilm growth was performed, as has been described from studies of *C. albicans* (Martins et al., 2012a). Both CLSM and biomass assays were used to visualise and quantify the impact of 24 h DNase treatment. To control for the direct effect of DNase on cells the checkerboard assay demonstrated that no direct antimicrobial effect was observed at the concentrations tested. Structural integrity of the biofilm was markedly affected by the addition of DNase. CLSM imaging showed that the dense structure of mature *A. fumigatus* biofilms was extensively disaggregated, reducing the biofilm depth by approximately 57%, and leaving adherent germlings and a scattered number of hyphae compared to the untreated biofilm (Figure 3.9a). Quantitative analysis of biofilm depth was also shown to be significantly reduced by approximately 57% ( $p < 0.01$ ) (Figure 3.9b).



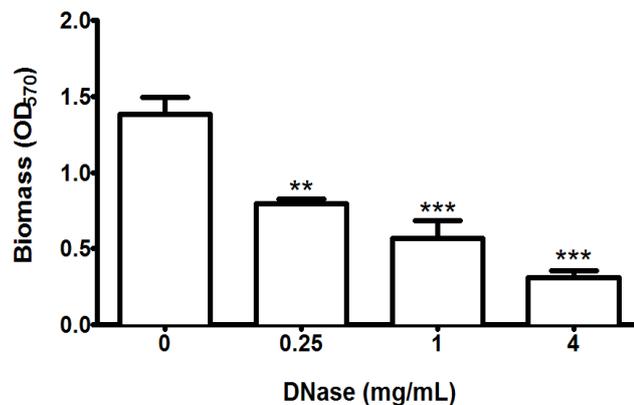
**Figure 3.9 - The eDNA play a role in biofilm stability.** (a) CLSM images of *A. fumigatus* biofilms (24 h) treated with vehicle or DNase (256 mg/L) for 24 h at 37°C. Biofilms were stained with calcofluor white and propidium iodide. The artificially coloured images of cells (white) and PI stained eDNA (red) or dead cells (punctuate red) are shown. The images shown are representative of three independent experiments. (b) Biofilm disruption was assessed by visualising the three dimensional architecture of biofilm under CLSM and thickness were analysed using volocity software (\*\*  $p < 0.002$ ).

Assessment of biomass by crystal violet assay demonstrated that the structural integrity of the preformed biofilm was shown to be markedly affected by the addition of DNase in a concentration dependant manner. Compared to the control biofilm 0.25 mg/mL of DNase significantly reduced the resultant biomass from an OD<sub>570</sub> 1.76 to 1.03 ( $p < 0.05$ ), 1 mg/mL to 0.69 ( $p < 0.01$ ), with a maximal effect observed with 4mg/mL (0.38,  $p < 0.001$ ) (Figure 3.10a). In contrast, treatment with heat inactivated DNase (0.25 mg/mL), RNase (0.25 mg/mL) and proteinase K (0.25 mg/mL) did not significantly reduce biofilm biomass compared to the vehicle control ( $p > 0.05$ ). To test its effect on biofilm development, conidia were treated as before and a significant reduction of biofilm biomass was also observed in a concentration dependent manner at concentrations of 0.25, 1 and 4 mg/mL ( $p < 0.0001$ , ANOVA with a Bonferroni Test *Post Hoc* for multiple comparisons) (Figure 3.10b). Compared to the biofilm development control 0.25mg/mL of DNase reduced the resultant biomass from an OD<sub>570</sub> 1.34 to 0.79 ( $p < 0.01$ ), 1 mg/mL to 0.57 ( $p < 0.001$ ), with a maximal effect observed with 4mg/mL (0.31,  $p < 0.001$ ).

a)



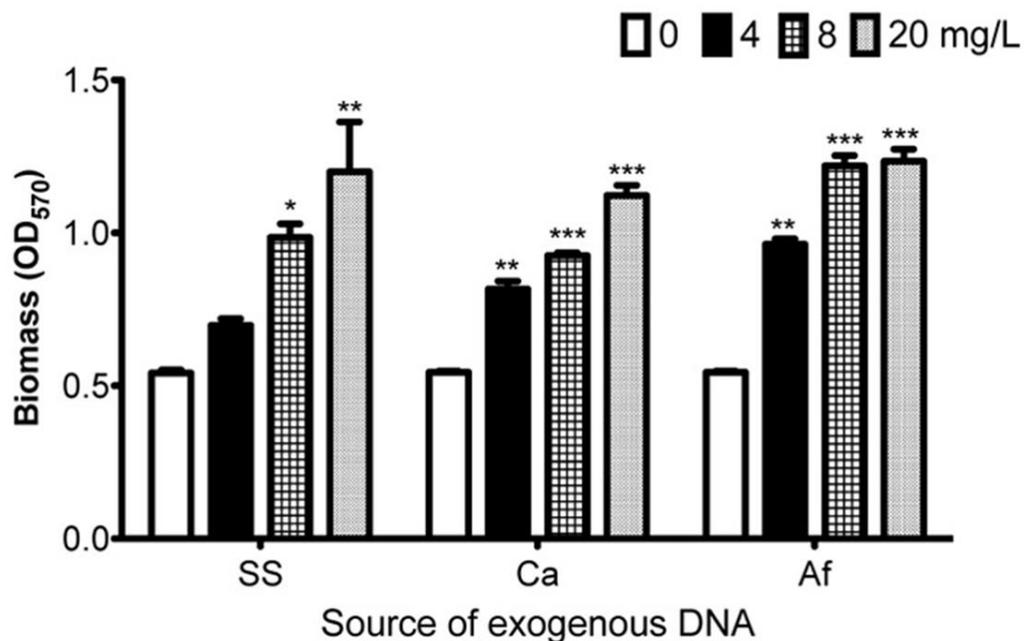
b)



**Figure 3.10 - The eDNA plays a role in biofilm formation and its stability.** The biofilm biomass assessed by crystal violet assay following DNase treatment is shown in the graph. (a) *A. fumigatus* conidia were incubated overnight with different concentration of DNase (0, 0.125, 1 and 4 mg/ml). The biomass data shows significant inhibition of the biofilm formation with eDNA digestion in a dose dependant manner compared to vehicle control. (b) The effect of DNase treatment on preformed biofilm was also assessed using crystal violet assay. Significant disruption of preformed biofilm is shown with DNase treatment compared to vehicle control (\*  $p < 0.02$ , \*\*  $p < 0.002$ , \*\*\*  $p < 0.0002$ ).

### 3.3.9 Addition of exogenous DNA improves biofilm formation

Given that DNase treatment led to architectural instability the next hypothesis was adding exogenous DNA during initial adhesion would positively promote biofilm formation, as has been shown to be the case for *C. albicans* biofilms. Addition of exogenous DNA from salmon sperm, *C. albicans* and *A. fumigatus* significantly increased the biofilm biomass at 4, 8 and 20 mg/L ( $p < 0.0001$ , ANOVA, Bonferroni Test *Post Hoc* for multiple comparisons) (Figure 3.11). The proportional increase in biofilm was 29%, 82% ( $p < 0.05$ ) and 122% ( $p < 0.01$ ) when 4, 8 and 20 mg/L of exogenous salmon sperm DNA was added. However, when *C. albicans* and *A. fumigatus* exogenous genomic DNA was added at 4, 8 and 20 mg/L the biofilm biomass increase was more notable, significantly increasing by 49% and 77% ( $p < 0.01$ ), 70% and 124% ( $p < 0.001$ ), and 106% and 126% ( $p < 0.001$ ), respectively.

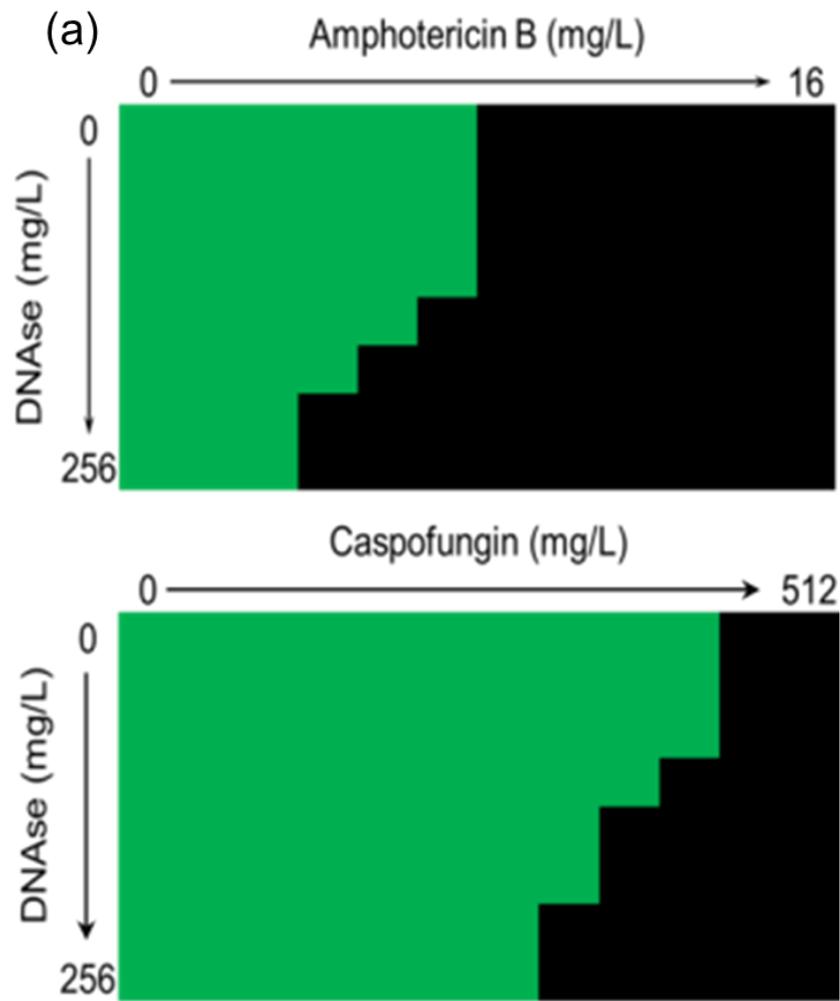


**Figure 3.11- Exogenous DNA enhances the *A. fumigatus* biofilm formation.** Addition of external DNA at concentrations of 0, 4, 8, 20 and 100 mg/L in RPMI growth medium showed significant increase in biofilm biomass with 24 h incubation in a dose dependant manner (\*  $p < 0.01$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.0001$ ).

### 3.3.10 Extracellular DNA release confers antifungal resistance upon of *Aspergillus fumigatus* biofilms

Numerous studies have shown the integral relationship between biofilm ECM and antifungal resistance. Given that eDNA is an important component of the ECM and that a recent study has shown in *C. albicans* biofilm sensitivity to antifungal agents is improved through the addition of DNase (Martins et al., 2012a). This experiment aimed to find whether the treatment of *A. fumigatus* biofilms with DNase in combination with different classes of antifungal agent would improve their activity. First, a checkerboard assay was conducted to test different combinations of DNase (0 - 512 mg/L) with amphotericin B (0 - 64 mg/L), caspofungin (0 - 256 mg/L) or voriconazole (0-256 mg/L). Antifungal testing using a checkerboard format on 24 h biofilms showed that the sessile minimum inhibitory ( $sMIC_{50}$ ) values of both amphotericin B and caspofungin treated cells decreased with an increase in DNase concentration (Figure 3.12 a-b). No significant change in MIC of azole in the presence of DNase was obtained. Based on this checkerboard data a fixed concentration of 128 mg/L was used for further experimental testing on different biofilm phases. It was shown that the susceptibility of 8 h biofilms was unaffected by antifungal and DNase treatment. However, the efficacy of both amphotericin B and caspofungin was improved as the biofilms matured to 12 h (1 - 2 fold), 24 h (8 - 16 fold) and 48 h (8 - 32 fold) (Table 3.2).

Spearman's rho correlation coefficient was calculated to test if the level of eDNA accumulation from *A. fumigatus* biofilms was correlated to the levels of antifungal sensitivity. Statistically significant correlations were observed between the level of eDNA accumulation in biofilms and the biomass ( $\rho=0.91364$ ;  $p=0.00003$ ), amphotericin B MIC ( $\rho=0.71729$ ;  $p=0.00864$ ) and caspofungin MIC ( $\rho=0.82664$ ;  $p=0.00092$ ). All correlations are shown in Table 3.3.



**Figure 3.12 - Effect of eDNA digestion on *A. fumigatus* biofilm susceptibility against antifungals.** Checkerboard assay was designed to test different concentration of DNase (0 to 256 mg/L) and amphotericin B (0-16 mg/L) and caspofungin (0- 512 mg/L) against 24 h biofilm. Green colour indicates growth of *A. fumigatus* below  $sMIC_{50}$  and dark color indicates the growth inhibition above  $sMIC_{50}$ .

Table 3.2 - sMIC (mg/L) of amphotericin B and caspofungin in the presence and absence of DNase (128mg/L)

## Amphotericin B

Strain	8 h		12 h		24 h		48 h	
	-	+	-	+	-	+	-	+
Af293	0.0625	0.0625	0.0625	0.0625	0.125	0.03125	1	0.125
YHCF1	0.015	0.015	0.031	0.015	0.125	0.01563	1	0.125
YHCF2	0.125	0.125	0.125	0.031	0.125	0.01563	2	0.125
YHCF3	0.031	0.015	0.0625	0.015	0.125	0.00781	1	0.0625
YHCF4	0.031	0.031	1	1	1	0.25	4	0.25
YHCF5	0.007	0.007	0.25	0.25	1	0.25	4	0.25

## Caspofungin

Strain	8 h		12 h		24 h		48 h	
	-	+	-	+	-	+	-	+
Af293	0.015	0.015	2	2	128	32	>256	64
YHCF1	0.062	0.03125	4	4	128	16	>256	64
YHCF2	0.062	0.062	8	4	64	8	>256	64
YHCF3	0.015	0.015	4	2	64	8	128	8
YHCF4	0.062	0.03125	4	2	64	16	>256	32
YHCF5	0.015	0.015	2	2	64	16	>256	32

Table 3.3- Correlation analysis

		<b>Biomass</b>	<b>AmpB</b>	<b>Caspo</b>
<b>eDNA</b>	R *	0.91364	0.71729	0.82664
	P <sup>#</sup>	0.00003	0.00864	0.00092
<b>Biomass</b>	R		0.58122	0.89598
	P		0.04748	0.00008

R\* = Spearman's  $\rho$  correlation coefficient

P<sup>#</sup> = Probability value based on 2-tailed test

P < 0.05 denotes significance

### 3.4 Discussion

The results of this study establish a novel serendipitous role for eDNA release in conferring antifungal resistance to *A. fumigatus* biofilms. *A. fumigatus* infections are typically difficult to manage with antifungal treatment and are associated with unacceptably high rates of mortality. It has been hypothesised that treatment failure may relate to the presence of innate resistance mechanisms associated with multicellular biofilms (Rajendran et al., 2011a, Ramage et al., 2012, Robbins et al., 2011). In the previous chapter the contributory role of efflux pumps in azole resistance was explained. It suggests that the efflux system playing an early role in resistance during colonisation, which is then reduced as extrapolymeric matrix is produced during biofilm maturation. Previous studies showed that in *C. albicans* biofilms extracellular matrix and their components such as  $\beta$ -1,3-glucans binds with antifungals and decreases the biofilm sensitivity to azole and polyene drugs (Gutierrez-Correa and Villena, 2003, Vedyappan et al., 2010). Here in this study the physical binding of amphotericin B with *A. fumigatus* biofilm components was investigated. The data show that amphotericin B physically binds with biofilm extracellular matrix and to fungal materials including  $\beta$ -glucans, chitin and ergosterol (Figure 3.2 - 3.3).

In addition, this study also investigated the presence of eDNA in *A. fumigatus* biofilm matrix. Given that eDNA has been shown to be an important component of biofilm ECM in both bacteria and fungi suggests that this may be conserved and possibly an active microbial biofilm process, but this has yet to be demonstrated. These data show for the first time that eDNA is a key structural component of maturing biofilms, released possibly through autolysis, which imparts protective effects against antifungal treatment as evidenced through the synergistic effects of DNase with either polyenes or echinocandins.

The presence and function of biofilm associated eDNA have been well documented in a variety of bacterial species (Allesen-Holm et al., 2006, Flemming and Wingender, 2010, Whitchurch et al., 2002), yet in fungal biofilms this is limited to *Candida* spp. (Al-Fattani and Douglas, 2006, Martins et al., 2010). Recent investigations of *C. albicans* have confirmed that despite the use of different growth media eDNA was present in mature biofilms, and based on digestion with DNase and the addition of exogenous DNA at different stages of biofilm development it was shown that eDNA contributes to the maintenance and stability of mature biofilms (Martins et al., 2010). Initially, a microscopy evaluation of mature *A. fumigatus* biofilms showed the diffuse presence of eDNA, which under closer inspection appeared to co-localised in between hyphae (Figure 3.4). Moreover, this could readily be detected and quantified. This study was also able to show an important structural role, as treatment with DNase led to disaggregation and destruction, whereas conversely the addition of exogenous DNA led to structurally superior biofilms. These series of investigations are the first to demonstrate the presence and critical function of eDNA as a biofilm ECM structural component, providing architectural stability to *A. fumigatus* biofilms.

In addition to providing structural support eDNA release may have a multifunctional role for *A. fumigatus*. The primary role ascribed to eDNA has been the regulation of biofilm formation and structure (Whitchurch et al., 2002). However, while investigations proceed to elucidate other functions (Grande et al., 2011), eDNA has been involved, for instance, in biofilm antimicrobial resistance (Tetz et al., 2009, Mulcahy et al., 2008). To further investigate alternative functional roles of eDNA its impact on antifungal treatment was evaluated. It has been shown that the ECM is a pivotal barrier for antifungal activity, as it is known to bind specific classes of antifungal agent and limit penetration of others in *C. albicans* (Al-Fattani and Douglas, 2004, Nett et al., 2010b). Moreover, it has been shown to have a functional role in *A. fumigatus* (Beauvais et al., 2007b, Ramage et al., 2011). The previous chapter hypothesised that the ECM was an important determinant of resistance based on differential efflux pump profiles, i.e. low relative efflux pump expression was

associated with high levels of azole and echinocandin resistance (Rajendran et al., 2011a). Given that eDNA is a key structural component of the ECM it was hypothesised that destabilizing it through the simultaneous addition of DNase would improve the penetration and sensitivity of antifungal agents, as has been shown for amphotericin B against pre-formed *C. albicans* biofilms (Martins et al., 2012a). Whilst DNase was shown to have no antifungal effect *per se*, using this in a checkerboard assay combined with antifungal agents increased the sensitivity of polyenes and echinocandins upon 24 h biofilms. In fact, this was clearly evident from analysis of different phases of biofilm using a fixed DNase concentration (Table 3.2). These data suggest that DNase treatment may offer an attractive chemotherapeutic strategy for the management of *A. fumigatus* infections.

Recently, the combined use of drugs (Espinel-Ingroff, 2009), or drugs with other agents such as enzymes (Kaplan, 2009), has received considerable attention. ECM degrading enzymes present broad-spectrum activity that is unlikely to induce antimicrobial resistance. The combined use of ECM degrading agents and antifungals has been previously tested *in vitro*. It was shown that *C. albicans* biofilm cells susceptibility to fluconazole and amphotericin B was increased by  $\beta$ -1,3-glucanase (Nett et al., 2007), whereas *A. fumigatus* biofilm cells susceptibility to polyenes was not changed by  $\alpha$ -1,3-glucanase (Beauvais et al., 2007b). Clinically, however, the DNase Pulmozyme® is currently used as a therapeutic agent for cystic fibrosis treatment as an adjunct to antibiotic treatment (Frederiksen et al., 2006). However, limitations to these chemotherapeutic approaches include the expense and increased risk of dispersing cells to distal sites of colonization (Kaplan, 2009).

To summarise, the data from this study demonstrate the physical binding of antifungal drug with ECM and this is the first report to show the presence of eDNA in *A. fumigatus*, which impacts antifungal resistance. These observations may allow us to improve the treatment of different forms of aspergillosis using agents, such as nucleases, allowing disaggregation of filamentous masses and improving penetration and efficacy of existing antifungal agents.

## **Chapter 4**

# **Investigating the Mechanisms of eDNA Release in *Aspergillus fumigatus* Biofilms**

## 4.1 Introduction

Recent studies have shown that extracellular DNA (eDNA) is a key component of both fungal and bacterial biofilms (Martins et al., 2010, Whitchurch et al., 2002, Flemming and Wingender, 2010), which is proposed to improve overall structural integrity. In *Pseudomonas aeruginosa* for example it was shown to contribute <1 to 2 % of the ECM composition. Moreover, bacterial biofilm studies have suggested that eDNA has a multifactorial purpose, namely as a nutrient source (Mulcahy et al., 2010), facilitator of genetic information exchange (Molin and Tolker-Nielsen, 2003), contributor to biofilm stability and dispersal (Whitchurch et al., 2002, Izano et al., 2008, Allesen-Holm et al., 2006, Berne et al., 2010), and as an antimicrobial resistance factor (Mulcahy et al., 2008, Tetz et al., 2009). Data from the previous chapter (Chapter-3) demonstrate the presence of eDNA within dense *A. fumigatus* biofilm populations, and that it plays a functional role in maintaining structural and architectural integrity and antifungal resistance. It also demonstrated that eDNA is most likely to be genomic DNA released from *A. fumigatus* during biofilm growth, but how this is regulated was unknown. Therefore this chapter aimed to undertake further studies to investigate a potential mechanism of release.

Mechanistically, cell death and lysis (Qin et al., 2007), quorum sensing (Spoering and Gilmore, 2006) and excretion from outer membrane vesicles (Kadurugamuwa and Beveridge, 1995) have been proposed to be involved in eDNA release in bacteria (Allesen-Holm et al., 2006, Kadurugamuwa and Beveridge, 1995). Autolysis is often cited as a key mechanism for the release of eDNA in bacterial biofilms (Qin et al., 2007). Evidence exists in eukaryotes from studies of *A. nidulans* that autolysis is regulated by chitinase activity (Shin et al., 2009, Emri et al., 2008, Molnar et al., 2006). In addition, chitinase activity was shown to be associated with cell wall biosynthesis at the growing tips of hyphae and hyphal remodelling of filamentous fungi (Adams, 2004, Bowman and Free, 2006, Selvaggini et al., 2004).

The molecular chaperone HSP90 regulates complex cellular circuitry in eukaryotes by stabilizing regulators of cellular signalling. The HSP90s have been proposed to be involved in cell wall biogenesis through the regulation of their client proteins Mkc1, Hog1 and Cek1 in *C. albicans* (Leach et al., 2012). The cell wall remodelling is mainly governed by the Mkc1 pathway by inducing the expression of remodelling enzymes such as chitinases (Heilmann et al., 2013). This study hypothesised that chitinases may play a role in autolysis of *A. fumigatus* biofilms that leads to the release of eDNA. Given the association of HSP90 proteins with chitinase expression it was hypothesised that inhibition of HSP90 by geldanamycin affects the chitinase regulated eDNA release.

The aim of this study was to investigate the mechanisms of eDNA release in *A. fumigatus* biofilms.

This chapter has been published in peer reviewed journals:

**Rajendran, R., Williams, C., Lappin, D. F., Millington, O., Martins, M. & Ramage, G. (2013)** Extracellular DNA release acts as an antifungal resistance mechanism in mature *Aspergillus fumigatus* biofilms. *Eukaryot Cell*.

Robbins, N., Uppuluri, P., Nett, J., **Rajendran, R.**, Ramage, G., Lopez Ribot, J. L., Andes, D. & Cowen, L. E. (2011) HSP90 governs dispersion and drug resistance of fungal biofilms. *PLoS Pathog*, 7, e1002257.

## 4.2 Materials and methods

### 4.2.1 Strains and conidial preparation

*A. fumigatus* Af293 and clinical isolates (YHCF1 - YHCF5) were obtained from the Royal Hospital for Sick Children (Yorkhill Division), Glasgow and used throughout these study. Chitinase deletion mutants  $\Delta Afchi1$ ,  $\Delta Afchi1chi2$ ,  $\Delta Afchi1chi2chi3$ ,  $\Delta Afchi1chi2chi3chi4$ ,  $\Delta Afchi1chi2chi3chi4chi5$  and their parental WT strain KU80 were generously provided by Jean Paul Latge (Institut de Pasteur, Paris, France). An *A. fumigatus* transformant expressing green fluorescent protein (Af-GFP) was used during microscopy, as described previously (Wasylnka and Moore, 2002). All the isolates were stored as previously described in section 2.2.1. All *A. fumigatus* strains were grown on Sabouraud dextrose agar at 37°C for 72 h. Conidia were then harvested as described in section 2.2.2. Conidia were then counted on a Neubauer haemocytometer and adjusted to a standardised suspension of  $5 \times 10^5$  conidia/ml in RPMI. All procedures were carried out in a class 2 biological safety cabinet (MDH Intermed).

### 4.2.2 Microscopy

Standardised conidia of Af293 were inoculated in RPMI onto Thermanox™ coverslips (13mm) within a 24-well tissue culture plate, and incubated for 24 h at 37°C. These were gently rinsed with PBS and stained, as described in section 3.2.5, with 5µM calcofluor white (Af293 only) (Invitrogen) and 20µM propidium iodide (PI) (Sigma). Biofilm growth and accumulation of eDNA were visualised under a fluorescence microscope (Motic BA400, Colorview system) or confocal laser scanning microscope (CLSM) (Leica SP5 laser scanning confocal microscope) at an excitation and emission wavelength of 350:400 for calcofluor white, 540:525 for propidium iodide, and 480:490 for GFP. One representative from each group was digitally photographed. Quantification of the z-stacks was performed using Volocity software (Perkin Elmer). For SEM representative biofilms grown and treated on Thermanox™ coverslips were processed and viewed under a SEM as previously described (Section 2.2.4.2).

### 4.2.3 Quantification of eDNA release

The quantity of eDNA released by different *A. fumigatus* isolates were assessed using a fluorescence DNA binding dye assay, as previously described (Section 3.2.7). Briefly, SYBR Green I (Invitrogen) was added to conidial suspension in a black well microtiter plate (Costar3603; Corning) in a ratio 1:4. Binding of this dye produces fluorescence in direct proportion with eDNA concentration. The levels of eDNA were quantified using a fluorescence plate reader (Fluostar Optima; BMG Labtech) at the excitation and emission wavelength of 485 and 518 nm, respectively every 10 min for the period of 24 h. The concentration of eDNA in the sample was quantified using the DNA standard curve.

### 4.2.4 Evaluating biofilm formation

Biofilms were formed in 96-well microtitre plates as previously described (Section 2.2.3). To assess the effect of class III chitinase gene mutants on biofilm formation, standardised conidia ( $5 \times 10^5$ ) of  $\Delta Afchi1$ ,  $\Delta Afchi1chi2$ ,  $\Delta Afchi1chi2chi3$ ,  $\Delta Afchi1chi2chi3chi4$ ,  $\Delta Afchi1chi2chi3chi4chi5$  and their parental WT strain KU80 were allowed to form biofilm in 96 well plate in RPMI for 24 h at 37°C. In addition, to evaluate the effect of a chitinase inhibitor, azetazolamide (Sigma-Aldrich) treatment on biofilm formation, Af293 biofilms were grown for 24 h in the presence and absence of azetazolamide (128 mg/L) at 37°C overnight. After each experiment the biofilms were washed in PBS and biomass quantified using a crystal violet assay as described in section 3.2.10.

#### 4.2.5 Quantitative gene expression

*A. fumigatus* Af293, YHCF1-YHCF4 biofilms were prepared in triplicate on tissue culture treated flasks as described previously (Section 2.2.3). RNA was extracted by mechanical disruption in TRIzol<sup>®</sup> (Invitrogen) as described previously (Section 2.2.9 and 2.2.10) and purified using an RNeasy MinElute clean up kit (Qiagen), as per the manufacturer's instructions. RNA was quantified and the quality determined using a NanoDrop spectrophotometer<sup>™</sup> (ND-1000, ThermoScientific). At first, cDNA was synthesised with high capacity RNA to cDNA master mix (Applied Biosystems) using a MyCycler PCR machine (Bio-Rad) as described in section 2.2.11 and stored at -20°C for expression analysis. The expression of a panel of Class III chitinase genes (*Chit1-5*) was then assessed using quantitative RT-PCR using SYBR<sup>®</sup> GreenER<sup>™</sup> (Invitrogen), according to the manufacturers' instructions. Primer for class III chitinase genes and *TEF1* and PCR cycling conditions were standardised previously by Alcazar-Fuoli and colleagues (Alcazar-Fuoli et al., 2011). Primers for *HSP90* were previously published by Marcin G Fraczek and colleagues (Fraczek et al., 2010). Table 4.1 show all the genes and primer sequences used in this chapter. The following PCR cycling conditions were used for amplification of chitinase genes, initial incubation at 95°C for 3 min then 40 cycles of 95°C for 5 s, 58°C for 5 s, 72°C for 15 s, 78°C for 4 s and 78°C for 1s. For the PCR amplification of *HSP90* gene the cycling conditions of 5min at 95°C (initial denature) then 40 cycles of 95°C for 30s, 55°C for 30s (annealing), and 72°C for 30s, followed by a final extension at 72°C for 10min was used. The individual gene expression levels were then calculated using  $2^{-\Delta Ct}$  method for different phases and normalised to the *TEF1* house keeping gene.

Table 4.1 - Primers used to quantitatively investigate chitinase expression

Gene	Primer	Sequence
<i>Afu5g03760</i>	<i>ChitA</i> - F	CAACCACCGTGACGTTGACA
	<i>ChitA</i> - R	TTCAGAAAAGCTGCCGTTGC
<i>Afu5g03530</i>	<i>ChitB</i> - F	TACCCTGACGCTGCCGATAA
	<i>ChitB</i> - R	AACCGTCTTGGCCCAGTTGT
<i>Afu3g07110</i>	<i>ChitC</i> - F	TGCGATTTGGGTGCAGTTCT
	<i>ChitC</i> - R	TTTCCTGGCAGACCGATGAA
<i>Afu7g05140</i>	<i>ChitD</i> - F	CAACCAGCTCCGCTCCTTGT
	<i>ChitD</i> - R	GCAGGCCGCAGTAGTTGTTG
<i>Afu8g00700</i>	<i>ChitE</i> - F	CGGCTGGGACTTTGACATTG
	<i>ChitE</i> - R	AAACTGCGCCTTGGTGACAA
<i>Afu1g06390</i>	<i>Tef1</i> - F	CCATGTGTGTCGAGTCCTTC
	<i>Tef1</i> - R	GAACGTACAGCAACAGTCTGG
<i>Afu5g04170</i>	HSP90 - F	TGACCAAGGCTGATTTGATC
	HSP90 - R	CAACAAGGTAAGCAGAGTAG

#### 4.2.6 Quantifying chitinase activity

Chitinase activity of *A. fumigatus* at different phases of biofilm formation was assessed using a fluorometric chitinase assay kit (Sigma, UK), as per manufacturer's instructions. In brief, supernatants were collected from different time points, and an appropriate volume of each sample was incubated with a substrate working solution (4-Methylumbelliferyl *N*-acetyl- $\beta$ -D-glucosaminide) at 37°C for 30 min. Fluorescence was then quantified at an excitation and emission wavelength of 360nm and 450nm, respectively. Appropriate positive and negative controls included in the kit were added in each plate. Finally, chitinase activity was calculated in units (U/mL). One unit of chitinase activity is defined as release of 1  $\mu$ mole of 4-methylumbelliferone from the substrate per min at pH 5.0 at 37°C. In addition, the chitinase inhibitor, azetazolamide was also tested for inhibition of activity using this assay.

#### 4.2.7 Biofilm susceptibility testing

For testing the effect of chitinase inhibition on antifungal susceptibility the biofilms (Af293 and 5 clinical isolates) formed in the presence of azetazolamide (128 mg/L) were treated with amphotericin B (0-32 mg/L) and the cells susceptibility ( $sMIC_{50}$ ) was determined at 50% reduction in metabolism compared to untreated controls using the 2, 3-bis (2-methoxy-4-nitro-5-sulfo-phenyl) - 2 *H* tetrazolium - 5-carboxanilide (XTT)- based reduction assay, as previously described (Section 2.2.6).

For testing the effect of Hsp90 inhibition by geldanamycin on antifungal susceptibility a checkerboard assay was employed. Amphotericin B, caspofungin and voriconazole were prepared according to the Clinical Laboratory and Standards Institute recommendations and used throughout these experiments (CLSI, 2008). Af293 conidial inoculum ( $1 \times 10^5$  conidia/mL) was dispensed into flat bottomed 96-well microtitre plates and incubated for 8 or 24 hours at 37°C. Biofilms were gently washed twice with PBS and each antifungal agent and geldanamycin were diluted to working concentrations in RPMI, which were tested either alone or in combination in a checkerboard format. Antifungal agent

dilutions were from 512 mg/L down to 0 with the following concentration steps in mg/L: 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5. Geldanamycin dilutions were from 100 µg/ml down to 0 with the following concentration steps in mg/L: 100, 25, 12.5, 6.25, 3.125, 1.5625. The cells susceptibility (sMIC<sub>50</sub>) was determined using the XTT- based reduction assay, as previously described (Section 2.2.6).

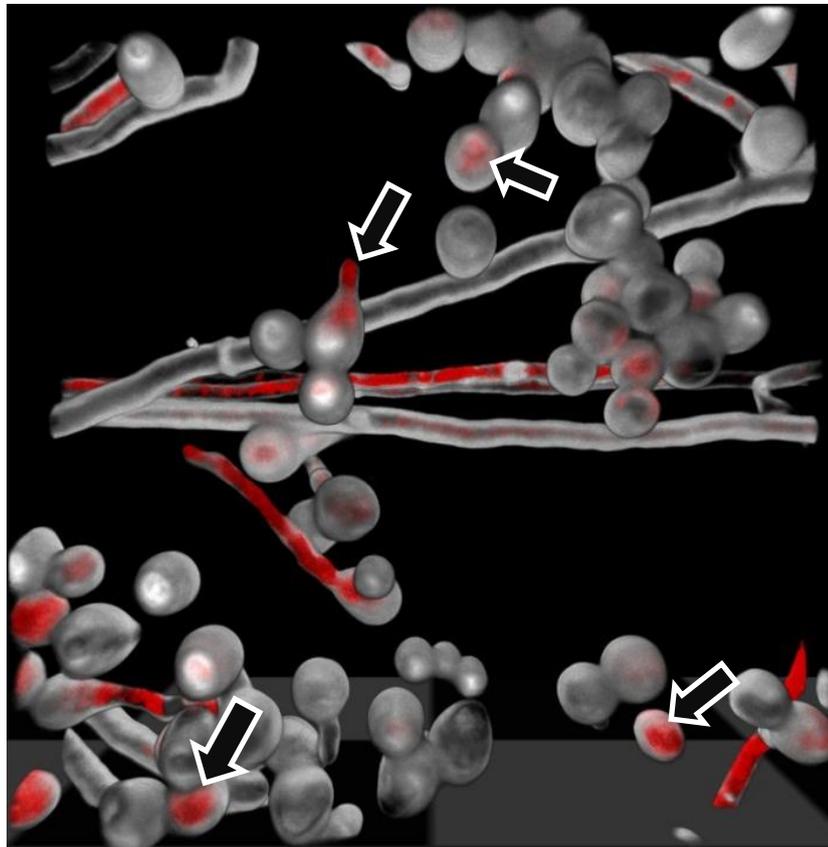
#### **4.2.8 Statistics**

Proportional data (fold change) were transformed to natural logarithm values before statistical tests were undertaken. Analysis of variance (ANOVA) and post hoc Bonferoni test was used to investigate independent sample data across a number of groups. The ANOVA and Dunnett t-test post hoc was used when a number of groups were compared only with the control group. Alternately when only two groups were under comparison a t-test was utilized. SPSS (Version 11, Chicago, USA) was used for these analysis and GraphPad Prism (Version 4, La Jolla, USA) for the production of the figures. The Spearman's rho correlation coefficient (R) was determined to investigate the relationship between the parameters measured. A p value of less than 0.05 was considered significant.

## 4.3 Results

### 4.3.1 Extracellular DNA release during *A. fumigatus* hyphal formation

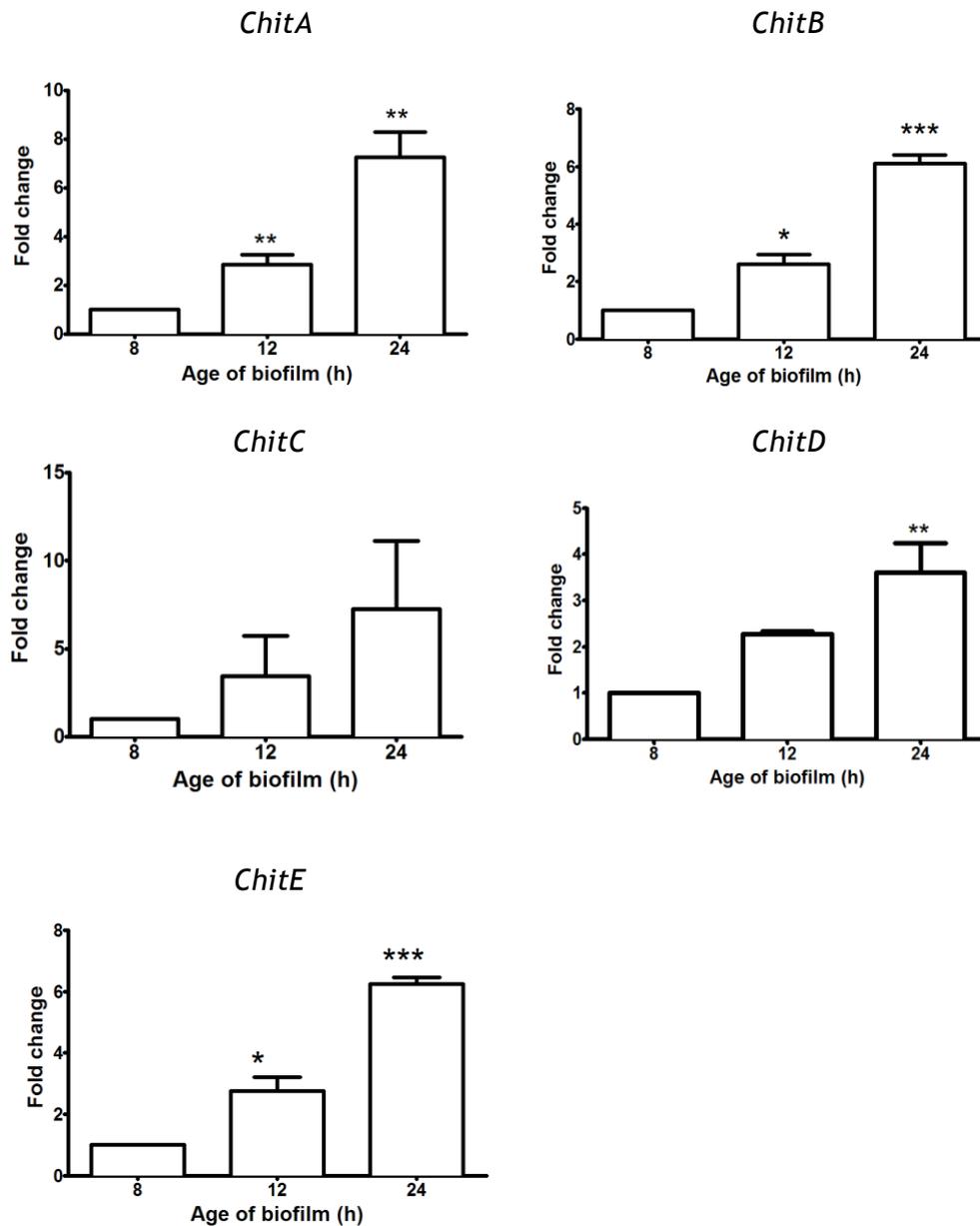
The visual inspection using CLSM of calcofluor white stained *A. fumigatus* biofilm counter-stained with PI showed the presence of eDNA. Besides staining dead cells PI can be used to map eDNA in biofilms (Allesen-Holm et al., 2006). At higher magnification localised areas of diffuse red fluorescence were observed at sites of germination of conidia and growing hyphal edges, which were located throughout the biofilm structure (Figure 4.1).



**Figure 4.1-** Conidial germination and hyphal growth release eDNA. CLSM image of *A. fumigatus* biofilm (24 h). Biofilm was stained with calcofluor white and propidium iodide. The artificially coloured image of cells (white) and PI stained eDNA (red) is shown. Black arrow indicates the eDNA localization on sites of conidial germination and hyphal growth.

### 4.3.2 Transcriptional analysis of chitinase genes

A transcriptional analysis of five different type III chitinases genes was done using real-time qPCR. The graphs in Figure 4.2 show the expression profile of the selected chitinase genes over different phases of *A. fumigatus* biofilm formation. These data showed that all transcripts (Class III - *ChitA*, *B*, *D* and *E*) except for *ChitC* were significantly up-regulated in a phase dependant manner ( $p < 0.05$  [8<12<24 h]). Expression levels compared to 8 h ranged from a 2.6 to 3.43-fold increase at 12 h and 3.6 to 7.25-fold increases at 24 h (Figure 4.2).



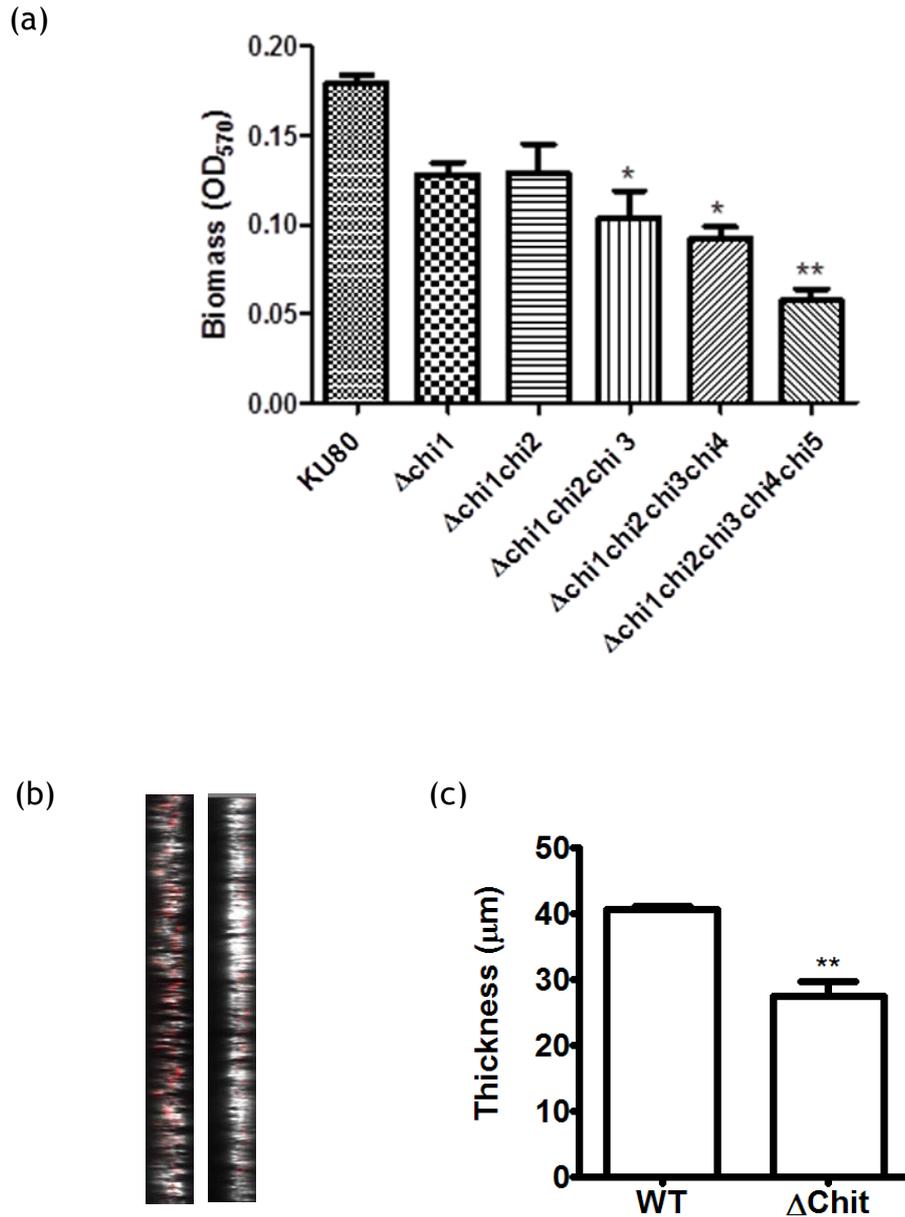
**Figure 4.2 - Phase dependant chitinase gene expression.** Quantitative real time PCR was carried out to assess the expression of five different class III chitinase genes (*ChitA-E*) over different phases. The individual gene expressions were calculated and normalised to the *Tef1* house keeping gene. All the results presented are expressed in fold change in expression compared to 8 h baseline. Statistically significant up regulation of gene expression was found for *ChitA,B,D* and *E* with 12 or 24 h time point compared to 8 h (\*  $p < 0.01$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.0001$ ).

### 4.3.3 Disruption of chitinase gene affects the biofilm formation

To determine the effect of deletion of chitinase genes on biofilm formation the 24 h biofilm biomass of  $\Delta Afchi1$ ,  $\Delta Afchi1chi2$ ,  $\Delta Afchi1chi2chi3$ ,  $\Delta Afchi1chi2chi3chi4$ ,  $\Delta Afchi1chi2chi3chi4chi5$  and WT strain KU80 was assessed. Using crystal violet biomass assay it was demonstrated that the chitinase mutants  $\Delta Afchi1chi2chi3$ ,  $\Delta Afchi1chi2chi3chi4$  and  $\Delta Afchi1chi2chi3chi4chi5$  had significantly reduced biofilm formation compared to their parental WT strain KU80 (\* $p < 0.05$ , \*\*  $P < 0.01$ ) (Figure 4.3a). To confirm this, biofilm thickness of the quintuple mutant was analysed using Volocity software on CLSM images. The data displayed a significant reduction in biofilm thickness from 40 $\mu$ m in WT strain to around 30 $\mu$ m in quintuple chitinase mutant ( $\Delta Afchi1chi2chi3chi4chi5$ ) (Figure 4.3b-c).

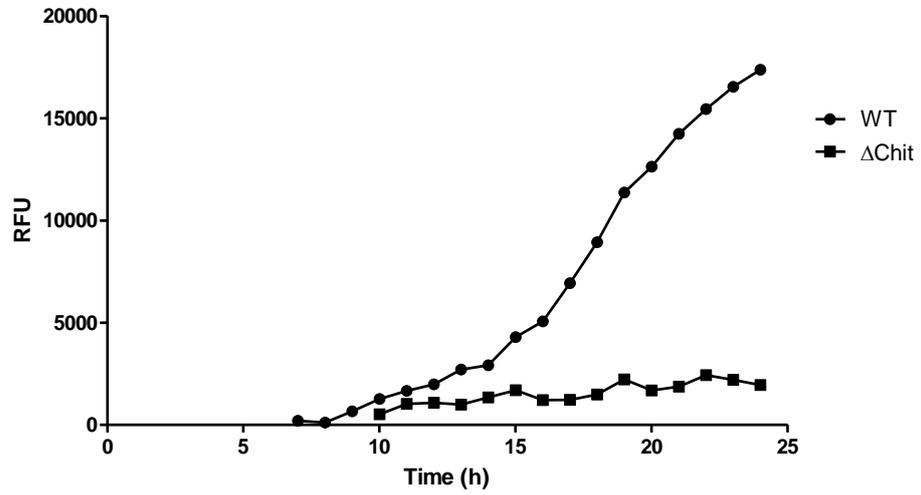
### 4.3.4 Chitinase gene knockout affects the eDNA release

To determine whether this biomass defect was a consequence of reduced eDNA release fluorescence assay was used to quantify the release of eDNA by chitinase mutants in real time in comparison to their parental WT strain. It was shown that release was significantly impeded throughout growth (Figure 4.4a). The eDNA release by  $\Delta Afchi1chi2chi3chi4chi5$  was around 2 ng/mL with 24 h biofilm this is significantly lower compared to eDNA release by parental WT strain KU80 of around 4 ng/mL (\* $p < 0.05$ ) (Figure 4.4b). The growth kinetics of the quintuple mutant and KU80 was also assessed to see whether there was any difference in their growth rate. The growth curve shows no difference between the mutant and KU80 till 16h but after that time point there was a slight difference between these strains. The quintuple mutant  $\Delta Afchi1chi2chi3chi4chi5$  showed a delay in reaching stationary phase compared to their parental strain KU80 (Figure 4.4c).

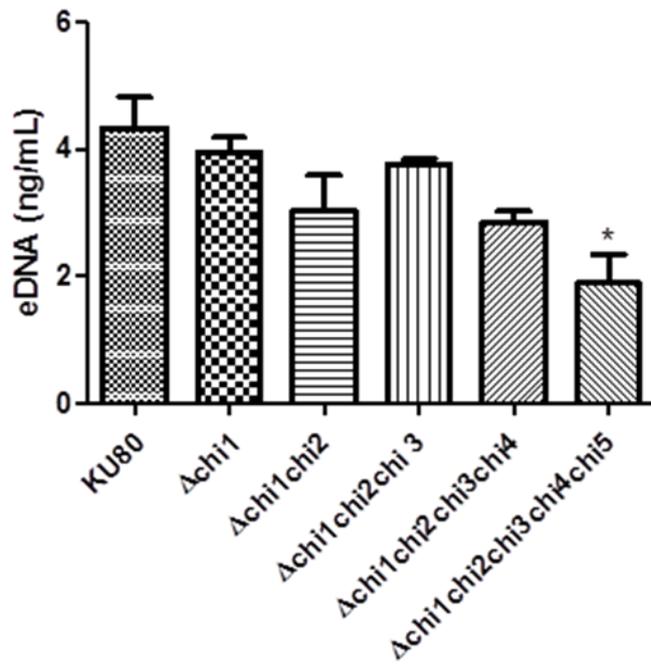


**Figure 4.3- Knocking out chitinase gene affects the biofilm formation.** *A. fumigatus* KU80 and chitinase deletion mutants  $\Delta Afchi1$ ,  $\Delta Afchi1chi2$ ,  $\Delta Afchi1chi2chi3$ ,  $\Delta Afchi1chi2chi3chi4$  and  $\Delta Afchi1chi2chi3chi4chi5$  biofilms grown for 24 in a 96 well plate at 37°C. (a) Assessment of overall biofilm biomass by crystal violet assay showed significant reduction in biomass with  $\Delta Afchi1chi2chi3$ ,  $\Delta Afchi1chi2chi3chi4$  and  $\Delta Afchi1chi2chi3chi4chi5$  compared to parental WT (KU80). (b-c) CLSM imaging and analysis of biofilm thickness using Volocity software of WT and quintuple chitinase mutant ( $\Delta Chit-\Delta Afchi1chi2chi3chi4chi5$ ) biofilm shows a significant reduction in biofilm thickness with  $\Delta Chit$ . Data was analysed by analysis of variance (ANOVA) and Bonferroni correction for multiple comparisons applied (\* $p < 0.05$ , \*\*  $p < 0.01$ ).

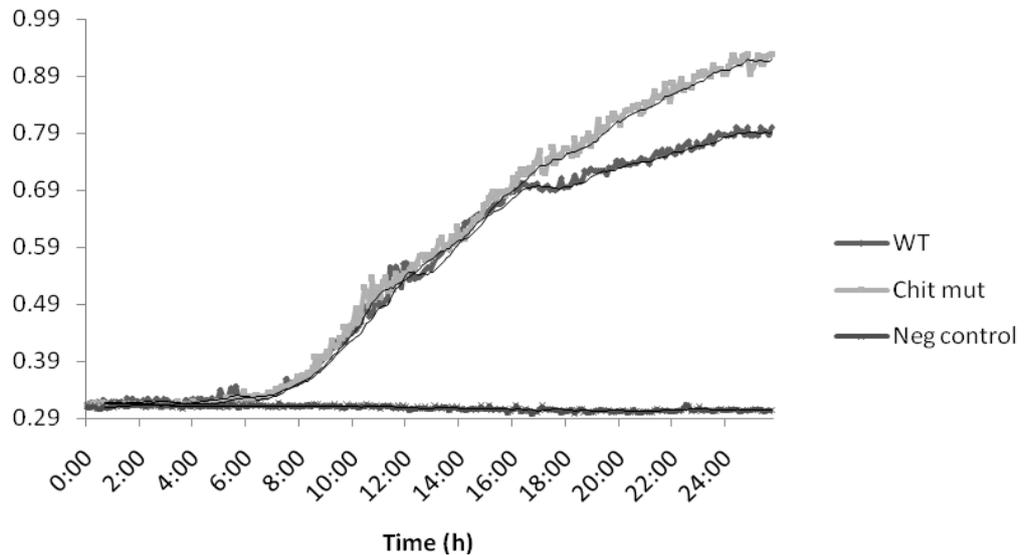
(a)



(b)



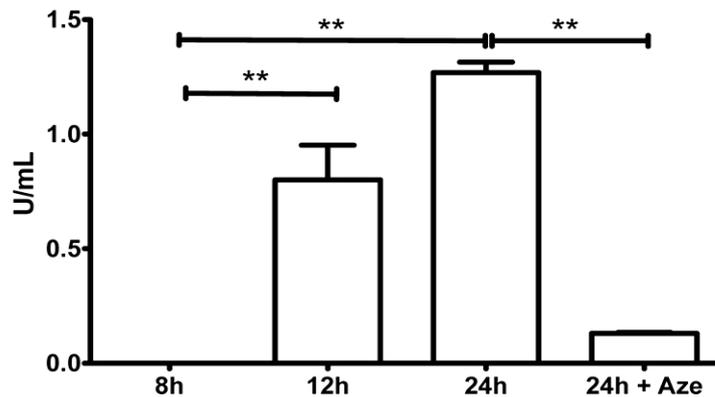
(c)



**Figure 4.4 - Chitinases contribute to eDNA release.** *A. fumigatus* KU80 and chitinase deletion mutants  $\Delta Afchi1$ ,  $\Delta Afchi1chi2$ ,  $\Delta Afchi1chi2chi3$ ,  $\Delta Afchi1chi2chi3chi4$  and  $\Delta Afchi1chi2chi3chi4chi5$  were grown in RPMI for 24 h in a 96 or 24 well plate at 37°C. (a) The eDNA release by WT and  $\Delta Afchi1chi2chi3chi4chi5$  were assessed in real time by measuring the relative fluorescence (RFU) produced by DNA binding dye SYBR<sup>®</sup> Green I using a OMEGA fluorescence plate reader, (b) the biofilms formed by WT and chitinase mutants were gently washed with EDTA to collect the ECM, a SYBR<sup>®</sup> Green fluorescence assay was used to assess the eDNA within the ECM. The bar graph shows the amount of eDNA present in ECM of WT and chitinase mutants. Experiments were performed in triplicate on 3 separate occasions, (c) Growth curve shows the quintuple chitinase mutant has a prolonged exponential phase compared to WT strain. Data was analysed by analysis of variance (ANOVA) and Bonferroni post test for multiple comparisons applied (\* $p < 0.05$ ).

#### 4.3.5 Phase dependant chitinase activity in *A. fumigatus*

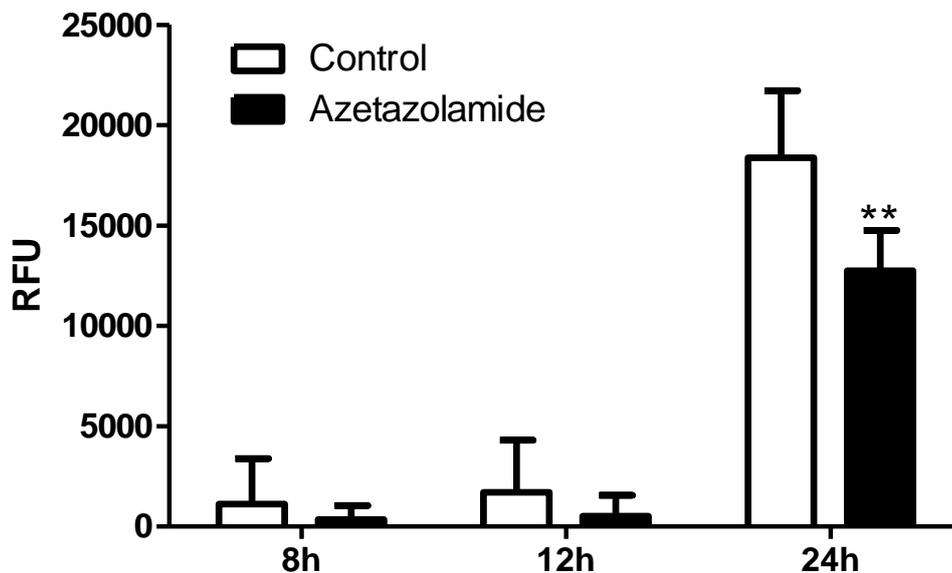
The chitinase activity of *A. fumigatus* over the period of biofilm formation was assessed using a biochemical assay. It was shown that as the biofilm matured the levels of chitinase activity significantly increased ( $p < 0.01$ ). No activity was observed for the 8 h phase, but at 12 and 24 h, 0.80 and 1.26 Units/mL were detected. Addition of azetazolamide was shown to significantly reduce activity of the 24 h biofilm to 0.13 Units/mL ( $p < 0.01$ ) (Figure 4.5), indicating that chitinase activity is associated with biofilm maturation.



**Figure 4.5 - Chitinase activity in *A. fumigatus* biofilm.** Chitinase activity was assessed using a commercially available fluorometric assay for 8, 12 and 24 h biofilms, and in the presence of azetazolamide (24 h + Aze). Data was analysed by analysis of variance (ANOVA) and Bonferroni post test for multiple comparisons (\* $p < 0.05$ , \*\*  $p < 0.01$ ).

#### 4.3.6 Effect of azetazolamide on eDNA release

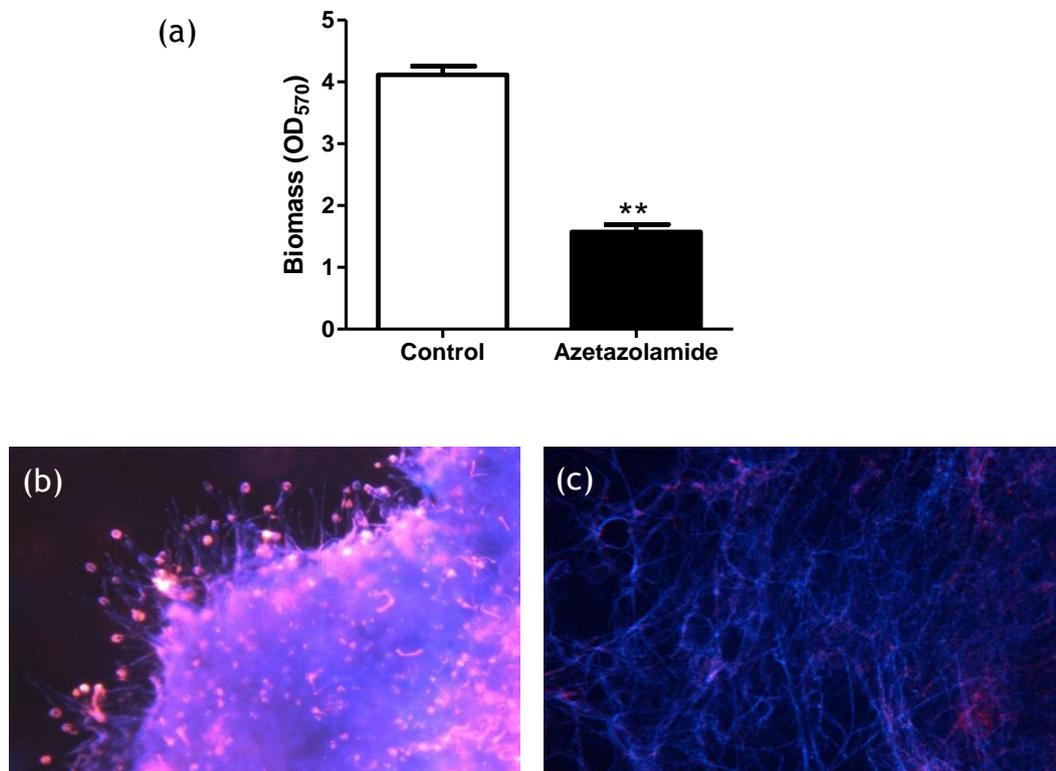
To test whether chitinase activity is associated with eDNA, chitinase activity was biochemically influenced using a chitinase inhibitor, azetazolamide (Schuttelkopf et al., 2010). The eDNA release was assessed for each phase of biofilm growth  $\pm$  azetazolamide (128 mg/L) using a SybrGreen I fluorescence assay. It was shown that for both 8 and 12 h biofilms azetazolamide did not significantly affect eDNA release, however, in 24 h biofilms a significant reduction of eDNA release was shown ( $p > 0.01$ ) (Figure 4.6).



**Figure 4.6 - Inhibition of chitinase activity reduces extracellular DNA release in mature biofilms.** eDNA release by *A. fumigatus* (Af293) was assessed in the presence of chitinase inhibitor (azetazolamide) for 8, 12 and 24 h using the SybrGreen I fluorescence assay. Each bar indicates SybrGreen fluorescence over different phases of biofilm formation. Error bar shows the standard deviation of the experiment. \*\*  $p < 0.001$ .

### 4.3.7 Inhibition of chitinase activity affects biofilm formation

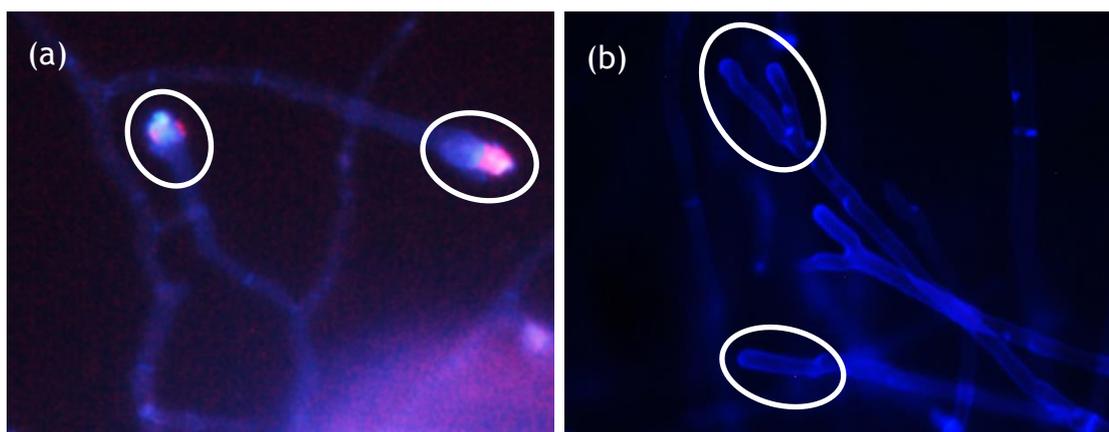
To investigate whether biofilm development was affected by chitinase activity, biofilms were grown in the presence of azetazolamide and biofilm biomass was assessed by crystal violet assay. It was demonstrated that the resultant biomass was significantly impeded ( $p < 0.01$ ) (Figure 4.7a). These data were confirmed by fluorescence microscopy, where it was shown that biofilms formed in the presence of azetazolamide were substantially compromised in their ECM and presence of eDNA (Figure 4.7 b-c).



**Figure 4.7 - Inhibition of chitinase activity affects *A. fumigatus* biofilm formation.** (a) Conidia were incubated with azetazolamide (128 mg/L) for 24 h and the resultant biomass was assessed using a crystal violet assay. Data was analysed by analysis of variance (ANOVA) and Bonferroni test *post hoc* for multiple comparisons (\*\*  $p < 0.01$ ),. (b-c) Fluorescent micrographs of *A. fumigatus* biofilm controls (b) or grown in the presence of azetazolamide (c) on Thermanox™ coverslips. These were stained with calcofluor white and propidium iodide and imaged using Colorview software at x200.

#### 4.3.8 Effect of azetazolamide on *A. fumigatus* growth

Given the role of chitinase in cell wall biogenesis and sporulation in filamentous fungi the effect of chitinase inhibitor on *A. fumigatus* morphology was assessed (Minari et al., 2002, Husni et al., 1998, Fukuda et al., 2003). Interestingly, fluorescence micrograph at higher magnification of the hyphae showed a co-localisation of eDNA at the hyphal tip compared to those treated with azetazolamide (Figures 4.8a-b). These data suggest that chitinases are an important factor in eDNA release from *A. fumigatus* biofilms, presumably through autolysis. Whether this process is regulated specifically or is a serendipitous effect of autolysis used to provide protection to the community of cells is at this time unknown.



**Figure 4.8** - Fluorescent micrographs of *A. fumigatus* biofilm (48 h) controls (a) or grown in the presence of azetazolamide (b) on Thermanox™ coverslips. These were stained with calcofluor white and propidium iodide and imaged using Colorview software at x400.

#### 4.3.9 Effect of azetazolamide on biofilm MIC

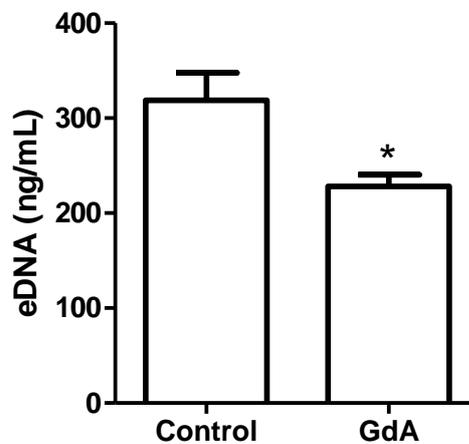
The MIC of 24 h biofilms formed in the presence of azetazolamide was assessed by broth micro dilution method. The MIC of amphotericin B was investigated and showed that biofilms formed in the presence of azetazolamide showed an increased sensitivity ranging from 0.0625 to 0.125 mg/L compared to 0.125 to 1 mg/L (1-8 fold) (Table 4.2).

**Table 4.2 - MIC of amphotericin B against 24 h biofilm formed in the presence and absence of azetazolamide (mg/L).**

Strain	Control	+ Azetazolamide	Fold change
Af293	0.125	0.03125	4
YHCF1	0.125	0.125	0
YHCF2	0.125	0.0625	2
YHCF3	0.125	0.03125	4
YHCF4	1	0.125	8
YHCF5	1	0.25	4

#### 4.3.10 HSP90 regulates the eDNA release in *A. fumigatus*

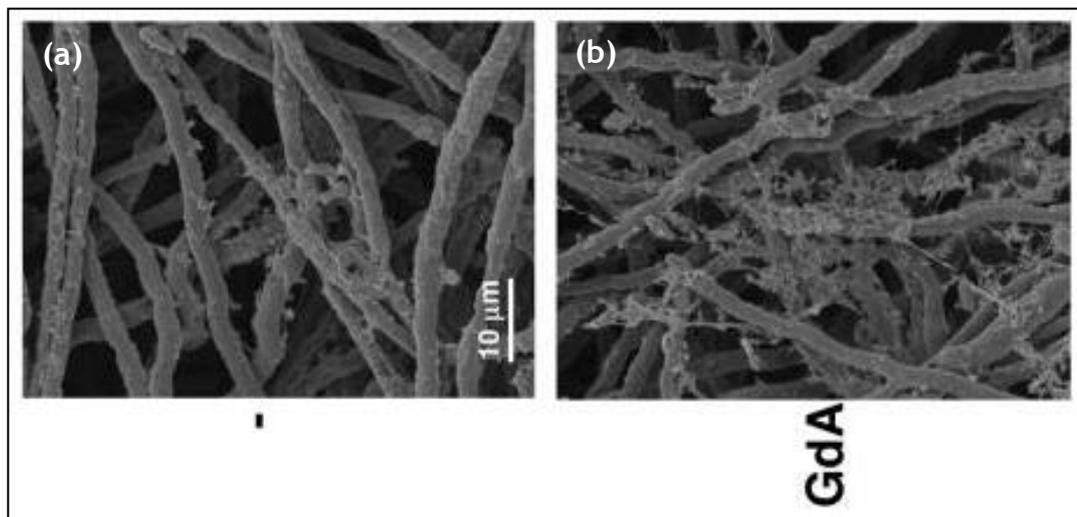
To test whether HSP90 is associated with eDNA release, HSP90 was biochemically depleted using a HSP90 inhibitor, GdA. The eDNA release was assessed for each biofilm growth  $\pm$  GdA using a SybrGreen I fluorescence assay. It was shown that GdA treatment significantly reduces the eDNA release from around 300ng/mL in untreated controls to around 200 ng/mL ( $p < 0.05$ ) (Figure 4.9).



**Figure 4.9 - HSP90 inhibition affects eDNA release by *A. fumigatus*.** Biofilms were treated with GdA or vehicle and ECM extracted for eDNA analysis. Extracted ECM were stained with DNA binding dye SYBR green I and resulting fluorescent production was measured using fluorescence microplate reader to assess the quantity of eDNA present. Significant decrease in eDNA level was observed with HSP90 inhibition (\* $p=0.04$ ).

#### 4.3.11 Inhibition of HSP90 affects the biofilm morphology

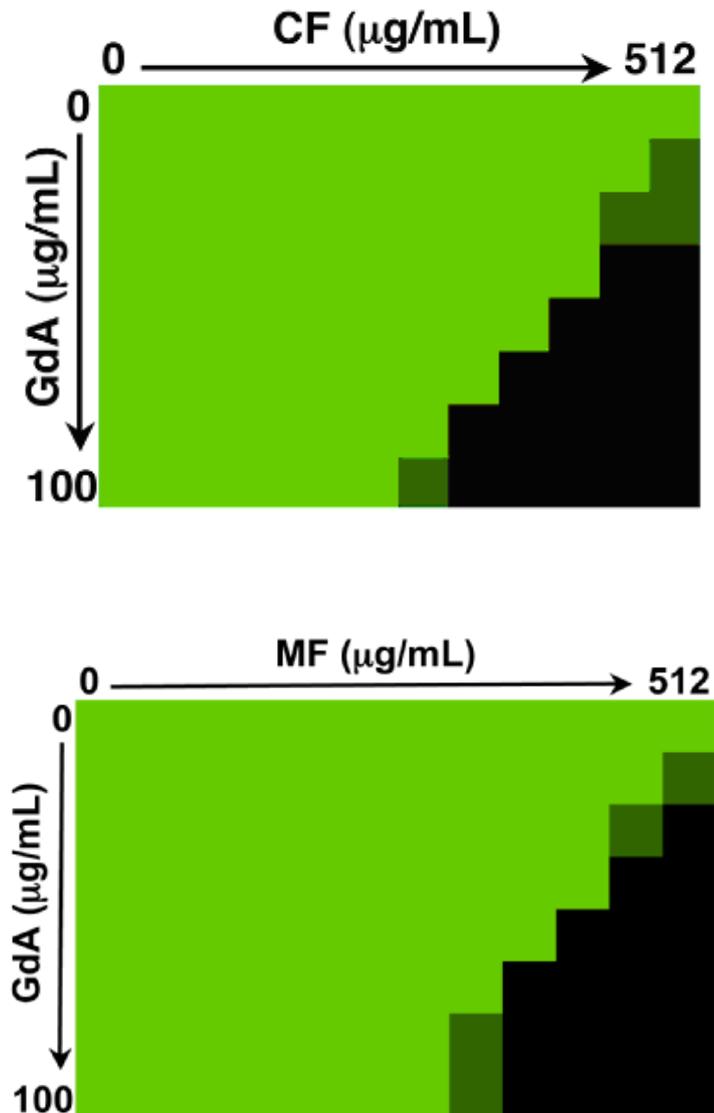
To test the effect of HSP90 inhibition on biofilm morphology biofilms were treated with an HSP90 inhibitor GdA. Scanning electron microscopy revealed striking architectural changes of *A. fumigatus* biofilms upon GdA treatment. Upon HSP90 inhibition increased hyphal and matrix production was observed compared to untreated control biofilms (Figure 4.10 a-b).



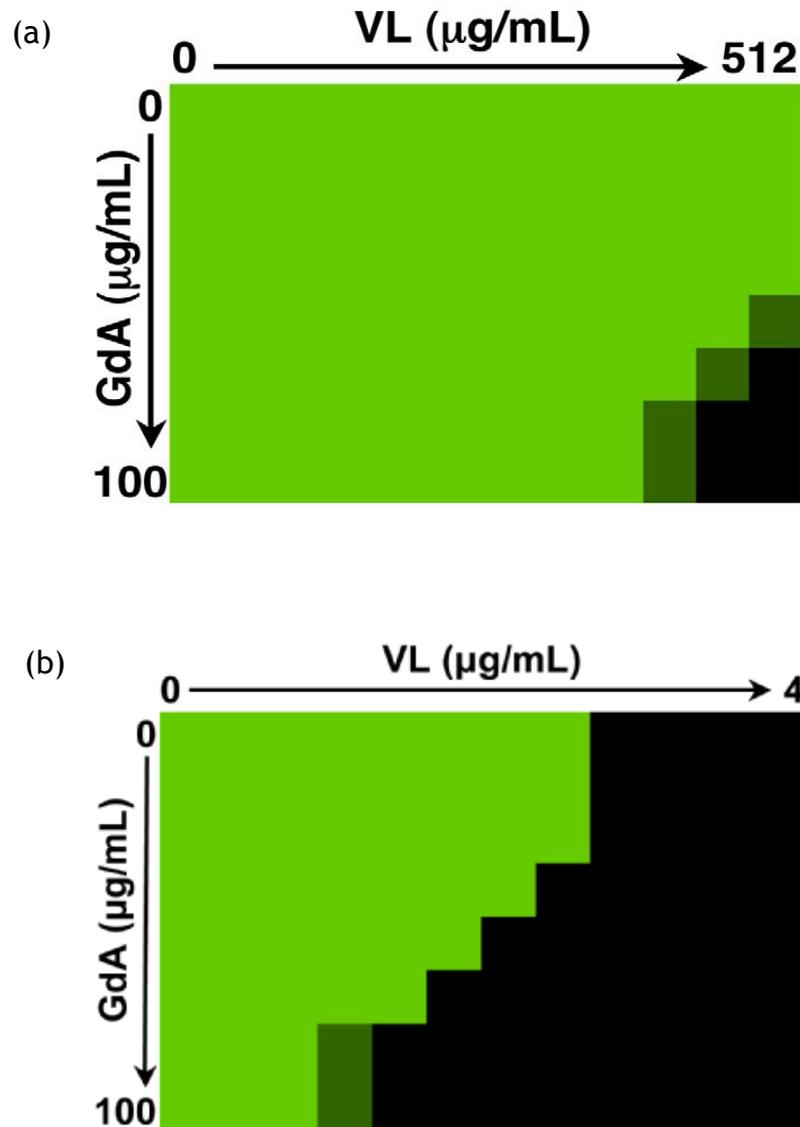
**Figure 4.10 - Pharmacological inhibition of HSP90 by geldanamycin affects biofilm biomass.** *A. fumigatus* cells were left untreated (a), or treated with 50 mg/L GdA (b) for 24 hours. Following GdA exposure, biofilms were fixed and imaged by scanning electron microscopy. Biofilms treated with GdA show increased biofilm extracellular matrix indicated by arrow. Crystal violet data also show a significant increase (\*  $p=0.01$ ) in biofilm biomass with GdA treatment.

#### 4.3.12 HSP90 is required for drug resistance of *A. fumigatus* biofilms

Since HSP90 was shown to be associated with eDNA release it was interesting to find the effect of HSP90 inhibition on drug resistance of *A. fumigatus* biofilms. After 24 hours of growth, *A. fumigatus* biofilms were subjected to a gradient of concentrations of the echinocandins caspofungin or micafungin, or the azoles voriconazole, in addition to a gradient of concentrations of the HSP90 inhibitor geldanamycin in 96 well microtiter plates under static conditions. Metabolic activity was assessed using the XTT reduction assay after an additional 24 hours. The biofilms were completely resistant to all the antifungal drugs tested and GdA individually, though the combination of GdA with many of the antifungals was effective in reducing biofilm development. Geldanamycin displayed robust synergy with both caspofungin and micafungin (Figure 4.11), with an FIC value of 0.375 for both drugs (Table 4.3). Geldanamycin also enhanced voriconazole activity (Figure 4.12a), with more potent effects observed when drugs were added to biofilms after only 8 hours of growth (Figure 4.12b).



**Figure 4.11- Pharmacological inhibition of HSP90 enhances the efficacy of echinocandins against *A. fumigatus* biofilms.** *A. fumigatus* was grown in 96-well dishes in RPMI at 37°C. After 24 hours cells were washed with PBS to remove non-adherent cells and fresh medium was added with varying concentrations of caspofungin (CF), micafungin (MF) and the HSP90 inhibitor geldanamycin (GdA) in a checkerboard format. Drug treatment was left on for 24 hours. Metabolic activity was measured by XTT assay. Bright green represents *A. fumigatus* growth, and black represents growth inhibition.



**Figure 4.12- Pharmacological inhibition of HSP90 enhances the efficacy of azoles against *A. fumigatus* biofilms.** *A. fumigatus* was grown in 96-well microtiter plates in RPMI at 37°C. (a) After 24 hours cells were washed with PBS to remove non-adherent cells and fresh media was added with varying concentrations of the voriconazole (VL) in combination with the HSP90 inhibitor geldanamycin (GdA) in a checkerboard format. (b) After 8 hours cells were washed with PBS to remove non-adherent cells and fresh media was added with varying concentrations of the azole voriconazole (VL) in combination with GdA in a checkerboard format. Drug treatment was left on for 24 hours. Metabolic activity was measured by XTT assay. Bright green represents *A. fumigatus* growth, and black represents growth inhibition.

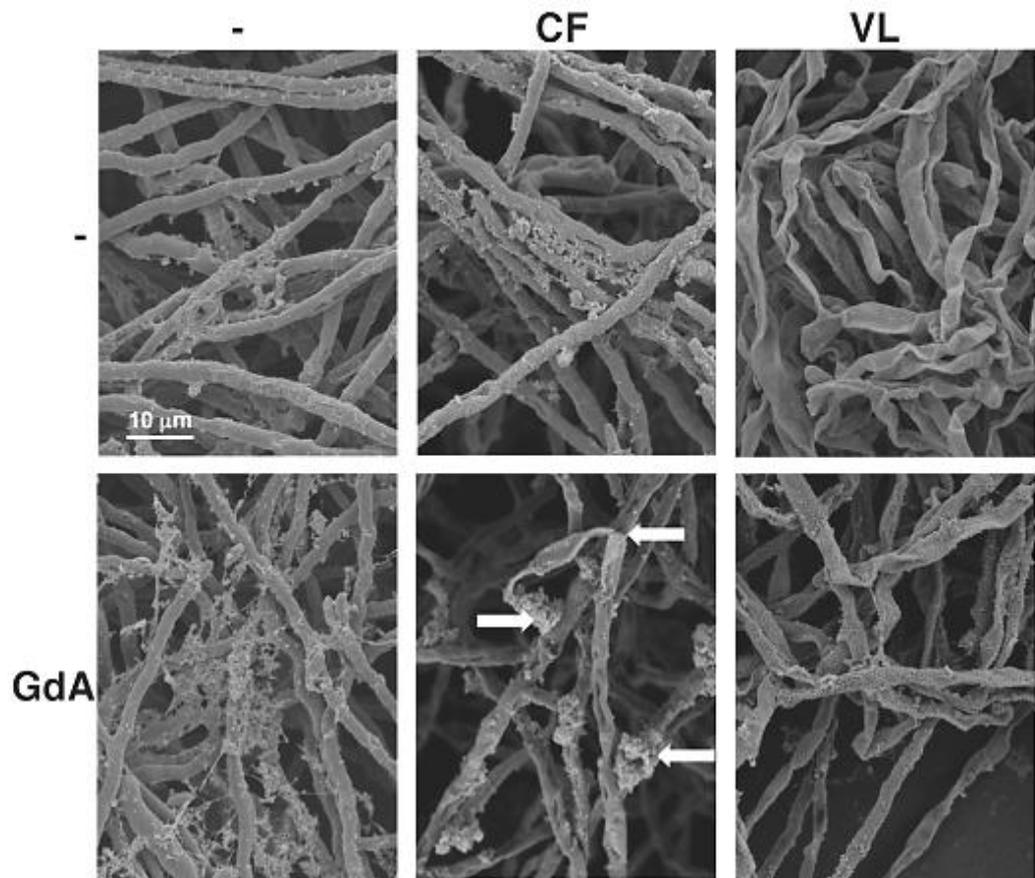
**Table 4.3 - Inhibition of HSP90 has synergistic activity with echinocandins against *A. fumigatus* biofilms**

Antifungal concentration range (µg/mL)	GdA concentration range (µg/mL)	FIC index <sup>a</sup>
Micafungin, 64-512	25-100	0.375
Caspofungin, 128-512	12.5-100	0.375

<sup>a</sup>FIC index (MIC<sub>50</sub> of drug A in combination)/(MIC<sub>50</sub> of drug A alone) + (MIC<sub>50</sub> of drug B in combination)/(MIC<sub>50</sub> of drug B alone). A FIC of <0.5 is indicative of synergism.

#### 4.3.13 Inhibition of HSP90 enhance the biofilm damage by antifungal treatment

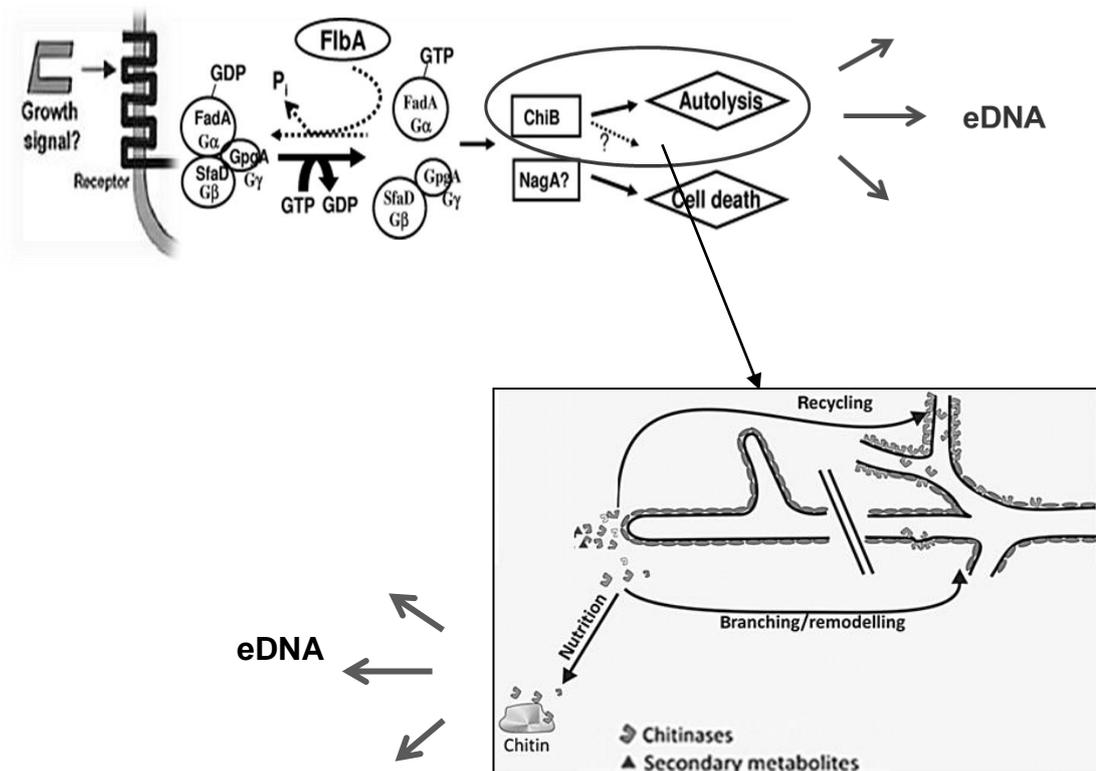
Next, given HSP90's role in regulating fungal morphogenesis impact of drug treatment on morphology of *A. fumigatus* biofilms were assessed. Treating biofilms with caspofungin alone resulted in minimal damage, however, the addition of both caspofungin and geldanamycin caused numerous burst and broken hyphae throughout the biofilm (Figure 4.13). Finally, voriconazole treatment resulted in a flat ribbon-like morphology, and the addition of geldanamycin induced further cell damage (Figure 4.13). Taken together, these results indicate that inhibition of HSP90 induces changes in morphology of *A. fumigatus* biofilms, in addition to enhancing the efficacy of azoles and echinocandins against these otherwise recalcitrant cellular structures.



**Figure 4.13 - Pharmacological inhibition of HSP90 affects biofilm morphology.** *A. fumigatus* cells were left untreated, or treated with 32 µg/mL CF or 256 µg/mL VL in the absence and presence of 50 µg/mL GdA for 24 hours. Following drug exposure, biofilms were fixed and imaged by scanning electron microscopy. Biofilms treated with antifungal show increased cellular damage in the presence of GdA. The white arrows indicate burst and broken hyphae in the biofilms treated with CF and GdA.

## 4.4 Discussion

In the previous chapter the extracellular DNA was demonstrated to be an important component of *A. fumigatus* biofilm ECM. Following these initial findings the aim of this investigation was set out to determine how exactly eDNA becomes a key structural component of the biofilm ECM in *A. fumigatus*. Although not well understood, mechanisms of eDNA release in eukaryotic cells include active secretion, necrosis and apoptosis (Tamkovich et al., 2008), with the possibility of more than one pathway being involved (Fink and Cookson, 2005). Bacterial autolysis is a commonly described mechanism of eDNA release within biofilms, which has been shown to improve *Enterococcus faecalis* and *Neisseria meningitides* biofilm development (Lappann et al., 2010, Thomas et al., 2008). In fungi, such as *A. nidulans*, the molecular basis of autolysis has been investigated. It has been shown that the chitinase *ChiB* plays a pivotal role in fungal autolysis in *A. nidulans* under carbon starvation (Pocsi et al., 2009, Szilagyi et al., 2010). *ChiB* is necessary to orchestrate the expression of autolytic hydrolases, with disruption of these genes leading to a reduced production of the cell wall-degrading hydrolase enzymes. Moreover, a recent functional analysis of the fungal/plant class 3 chitinases in *A. fumigatus* concluded that these endogenous GPI anchored enzymes may play a role by providing an energy source through autolysis (Alcazar-Fuoli et al., 2011). Fungal chitinases are secreted enzymes that have been shown to play a role in the digestion of exogenous chitin or utilization of fungal chitin during autolysis for energy. It was hypothesised in this study that as a consequence of autolysis eDNA is released thus conferring structural support for the development and maintenance of complex biofilm architecture.



**Figure 4.14 - Proposed mechanisms of eDNA release.** Chitinase activity was previously shown to be associated with autolysis process via FlbA pathway (Shin et al., 2009). The hydrolytic enzyme chitinase is also established to be a part of hyphal remodelling process in filamentous fungi. In this study the chitinase regulated autolysis process was proposed to be involved in the eDNA release in *A. fumigatus*.

Analysis of microarray data from different phases of *A. fumigatus* biofilm development published by Mowat E in her thesis revealed an up-regulation of class 3 chitinases (*Chit A-E*) (Mowat, 2008). Targeted real-time qPCR analysis of the five specific chitinases described by Alcazar-Fuoli and colleagues also showed a growth phase dependent upregulation of each chitinase (Alcazar-Fuoli et al., 2011). Using the same quintuple chitinase disruption mutant described in this paper it was tested whether its biofilm phenotype was defective. It was shown to have a significantly reduced biomass and showed a reduction of eDNA release (Figure 4.3-4.4). This study, and others, have demonstrated that this mutant has a similar growth rate to the wild type (Alcazar-Fuoli et al., 2011). These data indicate that an autolytic event within the complex biofilm is the most plausible explanation for the release of eDNA within the ECM.

It was suspected from our data that class III chitinases play a role in this process during cell wall remodeling. However, there are 18 members of the chitinase family, which are distributed in two subclasses of fungal/plant (class III) and fungal/bacterial (class V) families. This functional redundancy creates difficulty in specifically relating chitinase activity to eDNA release. However, when chitinase activity was inhibited biochemically then eDNA release was significantly affected. Functional and gene-expression studies in *A. nidulans* recently demonstrated that the  $\beta$ -1,3-endoglucanase *EngA* is also involved in autolytic cell wall degradation resulting from carbon starvation (Szilagy et al., 2010). Therefore, autolysis appears to be due to the production of cell wall-degrading enzymes, which are co-ordinately controlled in a complex manner. Within the biofilm there is a buildup of toxic secondary metabolites, and nutrients may be limited by diffusion through the ECM. This may provide the environmental stresses that induce the expression of these cell wall remodeling enzymes in order to liberate nutrients from the cellular community.

In a stressful environment fungal cells activate heat shock proteins (Hsp90). HSP90 is a key molecular chaperone in stress response stabilizing diverse regulators of cellular signalling in eukaryotes (Taipale et al., 2010, Pearl and Prodromou, 2006). Given the chitinase regulated autolysis pathway is associated with eDNA release and Hsp90 is involved in the antifungal resistance by regulating various cellular process. It was hypothesised that depletion of HSP90 could affect the eDNA release as well. Results presented establish a potentially novel role for HSP90 in eDNA release and drug resistance of *A. fumigatus* biofilms. Treating the *A. fumigatus* biofilms with HSP90 inhibitor appears to increase matrix production (Figure 4.9) compared to untreated controls. Future studies will dissect the molecular mechanisms by which HSP90 regulates biofilm matrix production.

Given the eDNA is a significant component of biofilm ECM and HSP90 inhibition upregulates matrix production, a study was designed to assess eDNA release in the presence of a HSP90 inhibitor. From the previous chapter it was shown that *A. fumigatus* biofilms possess elevated eDNA content in ECM, which affects antifungals from reaching its intracellular target. The ~28% reduction in matrix eDNA was observed upon HSP90 depletion (Figure 4.10) likely contributes to reduced antifungal resistance. HSP90 could regulate eDNA levels by directly or indirectly affecting chitinase autolysis pathway.

Given Hsp90 regulates diverse signalling cascades, it could also affect biofilm drug resistance in a multitude of other ways, such as by regulating remodelling of the cell wall and cell membrane (Mukherjee et al., 2003, Nett et al., 2007), signalling cascades important for matrix production (Nett et al., 2010b, Nobile et al., 2009), or the function of contact-dependent signalling molecules that initiate responses to surfaces (Kumamoto, 2005). Future studies will determine on a more global scale the impact of cellular state on HSP90 client protein stability, and the complex circuitry by which HSP90 regulates biofilm drug resistance. The results of checkerboard assays showed that compromising HSP90 function dramatically improves the efficacy of antifungals. Pharmacological inhibition of HSP90 enhances the efficacy of both azoles and

echinocandins against *A. fumigatus* biofilms (Figure 4.10-4.11). The synergy between HSP90 inhibitors and echinocandins is more pronounced than that with azoles, consistent with findings in the planktonic cellular state (Cowen et al., 2009). The results suggest that HSP90 is a novel regulator of matrix eDNA levels. For *A. fumigatus* the reduction in matrix eDNA levels upon HSP90 depletion provides a mechanism by which HSP90 might govern biofilm echinocandin resistance. Thus, inhibition of HSP90 enhances the efficacy of antifungals against biofilms formed by the fungal pathogen of humans separated by ~1 billion years of evolution, suggesting that this combinatorial therapeutic strategy could have a broad spectrum of activity against diverse fungal pathogens.

To summarise, the expression of chitinase activity in *A. fumigatus* was shown to be associated with eDNA release. This study demonstrates the inhibition of chitinase activity and HSP90 decreases the eDNA release, which impacts upon antifungal resistance. These observations may allow us to improve the treatment of different forms of aspergillosis using agents, such as azetazolamide (chitinase inhibitor) and geldanamycin (HSP90 inhibitor) allowing disaggregation of filamentous masses and improving penetration and efficacy of existing antifungal agents.

## Chapter 5

**Developing an *in vivo* model to evaluate *Aspergillus fumigatus* adaptive biofilm resistance mechanisms**

## 5.1 Introduction

*Aspergillus fumigatus* is the most frequent cause of invasive fungal infection in immuno compromised patients. Although a variety of factors are thought to contribute to the emergence of fungal infections, including a number of environmental or ecological changes, genetic changes resulting in pathogen evolution are crucial (Morens et al. 2004). The adaptation of fungal cells to the host environment is one of the important factors associated with their evolution (Scheffer 1991).

Adaptation by biofilm cells to azole antifungal treatment was investigated using a diffusion chamber mouse model, as outlined in chapter 2. However, this is problematic as the use of animal models are expensive, labour intensive, time consuming and are not without ethical concern. Therefore, this study aimed to develop an alternative *in vivo* model for studying *A. fumigatus* infection. Invertebrate insects, such as *Drosophila melanogaster*, *Manduca sexta*, *Bombyx mori* and *Galleria mellonella*, respond to microbial infection in a similar way to vertebrate animals (Cahill et al., 1997, Duchini et al., 2002, Patterson, 1999, Singh et al., 2003, Libanore et al., 2002). These therefore represent potential alternative models, thereby reducing the need for mammalian testing.

Larvae of the wax moth *G. mellonella* is an ideal model pathogen-host system, due to its ease of purchase, maintenance, inoculation and ability to generate ethical data more quickly. It has been used previously as an effective infection model for the study of different bacteria, such as *Pseudomonas aeruginosa* and the *Burkholderia cepacia* complex (Sambatakou et al., 2006b, Carvalho et al., 2008), and in fungal infections, including *C. albicans* and *Aspergillus* species (Mastella et al., 2000, Montoya et al., 2003, Singh and Husain, 2003).

The innate host immune response from *G. mellonella* against microbial infections has similarities similar to mammalian immune responses, with hemocytes present within the hemolymph of larvae functioning in a similar way as neutrophils (Perfect et al., 2001). Although *G. mellonella* do not exhibit any adaptive immune responses, they do show powerful resistance against invading pathogens (Vermes et al., 2000). Monitoring some of the humoral immune

responses from the insect, such as melanisation and production of antimicrobial peptides, are good indicators of virulence and pathogenesis.

Previous studies showed the use of *G. mellonella* infection model for antifungal efficacy testing (Kunst et al., 2006, Addrizzo-Harris et al., 1997, Akbari et al., 2005). Though, they have never been used as a model to study the adaptive resistance mechanisms in *A. fumigatus*, such as those described in the preceding chapters. Whilst *in vitro* modelling has been useful in the context of *A. fumigatus* biofilms investigations; *in vivo* models provide a more representative insight into these events. Therefore, investigating the correlation between *in vitro* expression profiles and *in vivo* expression profiles in *G. mellonella* will help determine whether this model is useful for the study of adaptive resistance mechanisms.

The aim of this chapter therefore was to develop an invertebrate *G. mellonella* infection model for *A. fumigatus* and to use this model for transcriptional analysis of different resistance genes in response to antifungal challenge, and to ascertain whether this model accurately represents clinical biofilm infection.

## 5.2 Materials and methods

### 5.2.1 Strains and conidial preparation

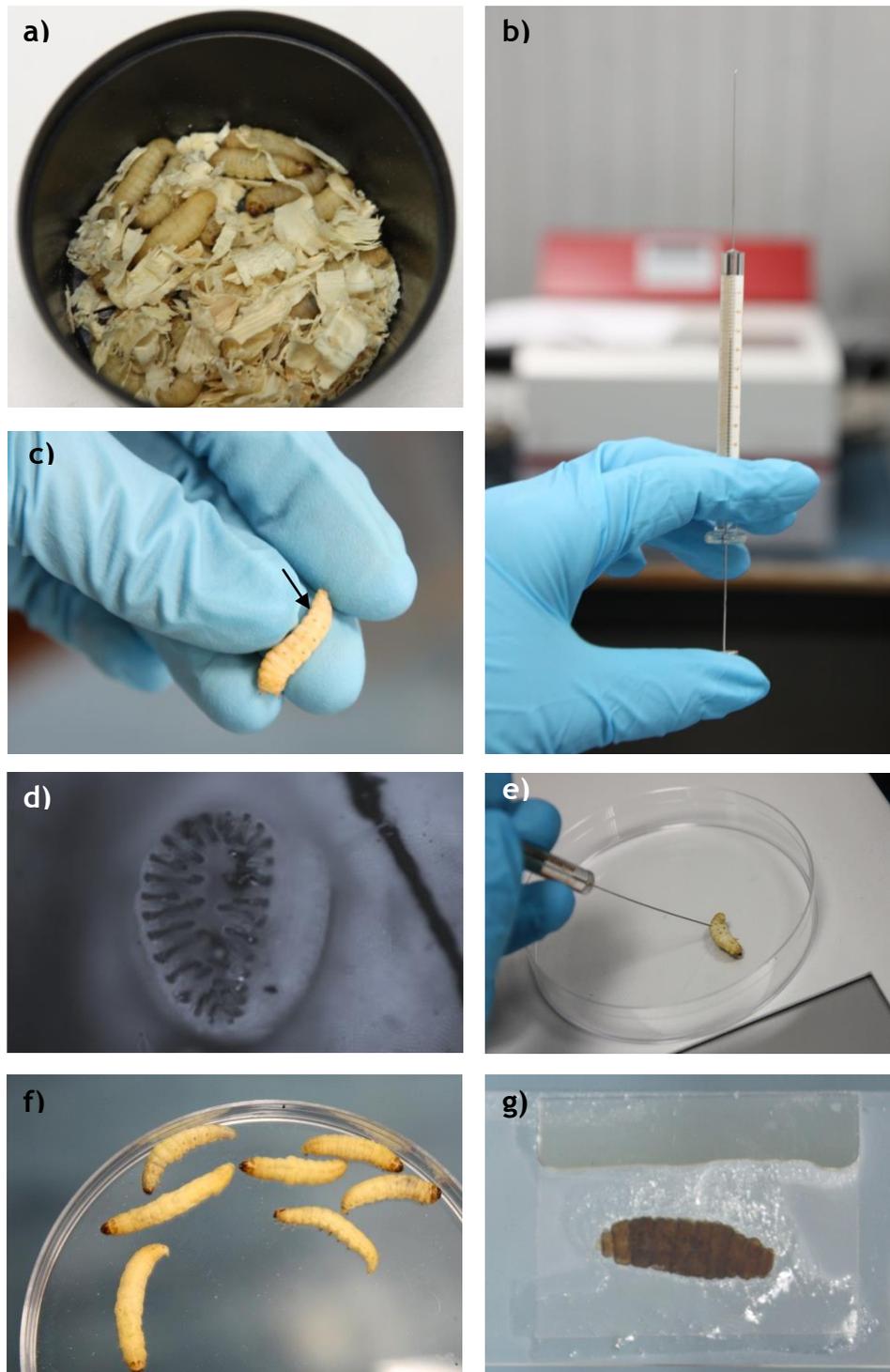
*A. fumigatus* Af293 and a transformant expressing green fluorescent protein (Af-GFP) was used for this study, as described previously (Wasylnka and Moore, 2002). All the isolates were stored as previously described in section 2.2.1. All *A. fumigatus* strains were grown on Sabouraud dextrose agar at 37°C for 72 h. Conidia were then harvested as described in section 2.2.2. Conidia were then counted on a Neubauer haemocytometer and adjusted to a standardised suspension in PBS. All procedures were carried out in a class 2 biological safety cabinet (MDH Intermed).

### 5.2.2 *Galleria mellonella*

The *Galleria* wax worms were ordered from Livefoodsdirect.co.uk (Figure 5.1 a). The larvae (2cm in length and weigh 0.2-0.3mg) were supplied in a plastic box and stored in refrigerator until use.

### 5.2.3 *G. mellonella* infection

To optimise the inoculum concentration for subsequent survival assays, larvae were inoculated with standardised Af293 conidia dorsolaterally by injecting 10µl aliquots of the serial dilutions ( $10^4$  -  $10^7$  conida/larvae) into the haemocoel, through the last pro-leg using a Hamilton syringe (Figure 5.1 b-e). Ten larvae were injected per dilution, and at least four different serial dilutions were inoculated. Larvae were incubated in petri dishes at 37°C (Figure 5.1 f), and the number of dead larvae was scored 1 to 10 days after infection. A larva was considered dead when it displayed no movement in response to touch and was melanised.



**Figure 5.1 - *Galleria mellonella* infection.** a) *G. mellonella* larvae stock in wood shavings, b) Hamilton syringe, c-d) pictures showing the proleg region of larva, e) inoculation of larva using Hamilton syringe at proleg region, f) inoculated larvae in petri dish ready for incubation, g) section of post infected larva.

### 5.2.3.1 Quality control

For each experiment a negative and positive control set were included to ensure that neither the injection procedure nor the drugs was responsible for any mortality observed.

Negative control set consists of:

- (i) Group of 10-20 uninfected, unmanipulated larvae
- (ii) Group of 10-20 larvae pierced into the last pro-leg with the Hamilton syringe
- (iii) Group of 10-20 larvae injected with 10 $\mu$ l of PBS into the last pro-leg

A positive control set consists of:

- (i) Group of 10-20 infected only, untreated larvae
- (ii) Group of 10-20 larvae injected with only drug, at identical time points to test larvae.

### 5.2.3.2 Phase dependant killing of *G. mellonella*

The virulence of different phases of *A. fumigatus* multicellular development was assessed using a *G. mellonella* survival assay. *A. fumigatus* ( $10^3$  conidia/ml) was grown up to 0, 8, 12 and 24 h in RPMI which provided conidia, germlings, initial hyphae and matured hyphae. Then 10 $\mu$ l of each inoculum was injected into larvae as described above. The infected larvae were incubated at 37°C and the survival was recorded over the period of 7 days.

### 5.2.4 Tissue sectioning

Larvae sectioning was performed to observe fungal cell morphology post-infection. In brief, larvae were infected with AfGFP conidia ( $10^6$  conidia/larvae) as described above. The infected larvae were fixed with formalin and snap-frozen with liquid nitrogen. Crude thin sections of the frozen tissue was then made by using a surgical scalpel blade, and placed on glass slides for microscopic imaging (Figure 5.1 g).

### 5.2.5 Effect of antifungal agents on *G. mellonella* survival

An optimised *G. mellonella* killing assay was used to test the efficacy of different antifungal agents against *A. fumigatus* infection. Larvae were infected with  $10^6$  conidia/larvaeas described above. After 8 h or 24 h of post infection larvae were treated with voriconazole at a concentration of 6 mg/kg. Survival was recorded over the period of seven days and larvae samples were collected for molecular analysis.

### 5.2.6 Nucleic acid extraction

Total nucleic acid and RNA was extracted from experimental larvae for assessing the fungal burden and gene expression studies. Larvae were snap-frozen using liquid nitrogen at each defined time point. These were ground up using a mortar and pestle, and then collected in a 2ml screw cap tube. Total nucleic acid was then extracted using BioRobot<sup>®</sup> EZ1 (Qiagen), as per manufacture's instruction. For RNA extraction, ground samples were transferred into 2mL screw cap tubes (Stratech) with 1mL of Trizol<sup>®</sup>. The cells were then mechanically disrupted using 200 $\mu$ L (0.5mm diameter) of silicon glass beads with a mini Bead Beater<sup>™</sup> (Biospec Products). The RNA was then extracted as described in section 2.2.9. RNA concentration was then assessed using a NanoDrop<sup>™</sup> spectrophotometer ND-1000 and stored at -80°C until needed for cDNA synthesis.

### 5.2.7 Real time PCR analysis

At first, cDNA was synthesised from RNA with high capacity RNA to cDNA master mix (Applied Biosystems) using a MyCycler PCR machine (Bio-Rad) and stored at -20°C for expression analysis as described in chapter 2.

#### 5.2.7.1 Expression of gallerimycin

The expression of a gallerimycin was assessed as a biomarker of *A. fumigatus* infection in *G. mellonella*. The primer sequences for gallerimycin are show in Table 5.1. The PCR cycling conditions for these primers are as follow: 94°C denaturation for 5 min, 35 cycles of 94°C denaturation for 60 s, 55°C for 90 s, 68°C extension for 90 s and final extension at 68°C for 10 min.

The amplified PCR products were then visualised by electrophoresis through a 1% (w/v) agarose gel (Invitrogen), prepared with 1 × Tris acetate buffer (Fisher Scientific). The agarose solution was heated in a microwave oven until it was molten, cooled to a safe handling temperature and 1.5µl of ethidium bromide (10mg/ml) added and mixed. This was then poured into a casting tray and allowed to solidify at room temperature. The PCR products were mixed with appropriate loading dye (approximately 5µl) on a Parafilm® sheet and 10µl of each sample loaded into the wells. An aliquot (5µl) of 100bp DNA hyperladder (Bioline, London, UK) was loaded into a separate well to estimate the PCR product size. The tank was connected to the electric source and run at 50V for 50min. The gel was observed under the UV light excitation and the image digitally photographed (Bio-Rad Gel Doc 2000, Bio-Rad Life Sciences).

#### 5.2.7.2 Colony forming equivalents

The colony forming equivalents (CFE) of *A. fumigatus* (CFE/100mg) were determined using an 18S rRNA real-time PCR as described previously (McCulloch et al., 2012). The primers and probe sequences targeting 18S rRNA are shown in Table 5.1. The PCR cycling conditions for this primers were as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. All PCR reactions were carried out in a total volume of 25µL. The standard total nucleic acid extracted from serially titrated *A. fumigatus* conidia was run in conjunction with each set of samples to quantify the *Aspergillus* burden.

#### 5.2.7.3 Gene expression study

Expression of selected resistance genes was assessed using quantitative RT-PCR using SYBR® GreenER™ (Invitrogen). Three independent samples from each time point for control and voriconazole treatment (6 mg/kg) groups were analysed in duplicate using MxProP Quantitative PCR machine and MxProP 3000 software (Stratagene). The primer details and PCR cycling conditions for *MDR1-4* and *ChitA-E* genes were described in previous chapters (drug efflux - Chapter 2, chitinase - Chapter 4). The primer sequences for all other genes are shown in

Table 5.1. The PCR conditions for these primers were as follows 5 min at 95°C (initial denature), 30s at 95°C (denaturation), 30s at 50-65°C (annealing), 30s at 72°C (extension) for 40 cycles, followed by a final extension at 72°C for 10 min. All PCR reactions were carried out in a total volume of 25µL. All the gene expression levels were normalised to *TEF1* housekeeping gene according to the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

**Table 5.1 - List of oligonucleotide primers used for real time PCR analysis.**

Genes	Primers	
Gallerimycin	F	5'-GAAGATCGCTTTCATAGTCGC-3'
	R	5'-TACTCCTGCAGTTAGCAATGC-3'
β actin	F	5'-GGGACGATATGGAGAAGATCTG-3'
	R	5'-CACGCTCTGTGAGGATCTTC-3'
18s rRNA	F	5'-GCCGCGGTAATTCCAGCTCCAATA-3'
	R	5'-GAGCAAAGGCCTGCTTTGAACA-3'
	P	5'-CGCACGGGTCCGCCTCACCGCGAGTACTGCGTGCG-3'
<i>Fks1</i>	F	5'-GCTGCGCCCAAGTCGCCAAATC-3'
	R	5'-GAACAACAAGTGGGGCAATG-3'
HSP90	F	5'-TGACCAAGGCTGATTTGATC-3'
	R	5'-CAACAAGGTAAGCAGAGTAG-3'
Tef1	F	5'-CCATGTGTGTCGAGTCCTTC-3'
	R	5'-GAACGTACAGCAACAGTCTGG-3'

### 5.2.8 Statistical analysis

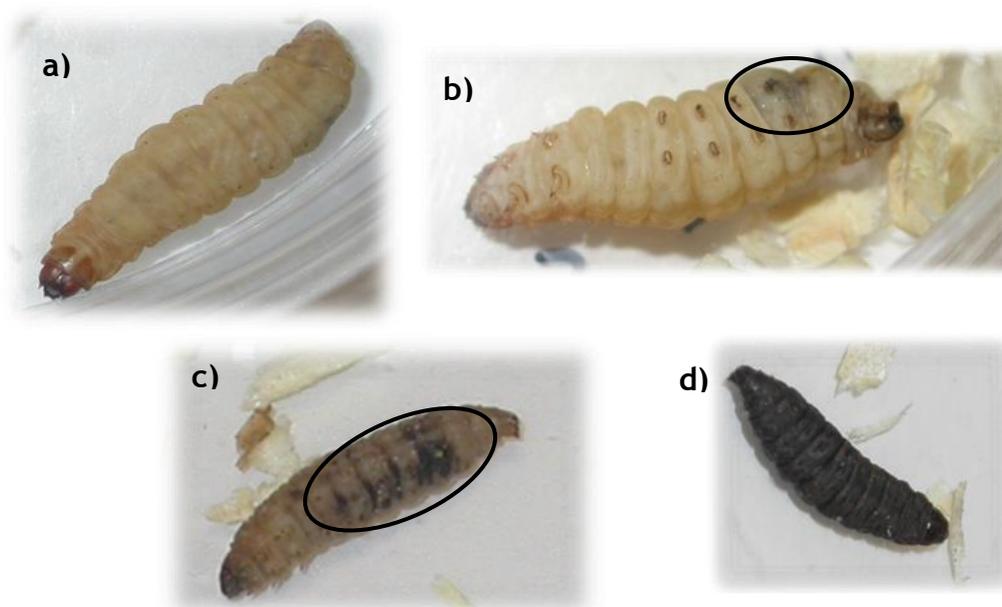
Proportional data (fold change) were transformed to natural logarithm values before statistical tests were undertaken. Analysis of variance (ANOVA) and *post hoc* Bonferroni test was used to investigate independent sample data across a number of groups. Alternately when only two groups were under comparison a Student t-test was utilized. The Log-rank (Mantel-Cox) test was used to analyse the *G. mellonella* survival curves. SPSS (Version 11) was used for these analysis and GraphPad Prism (Version 4) for the production of the figures.

## 5.3 Results

### 5.3.1 *G. mellonella* infection

#### 5.3.1.1 Melanisation

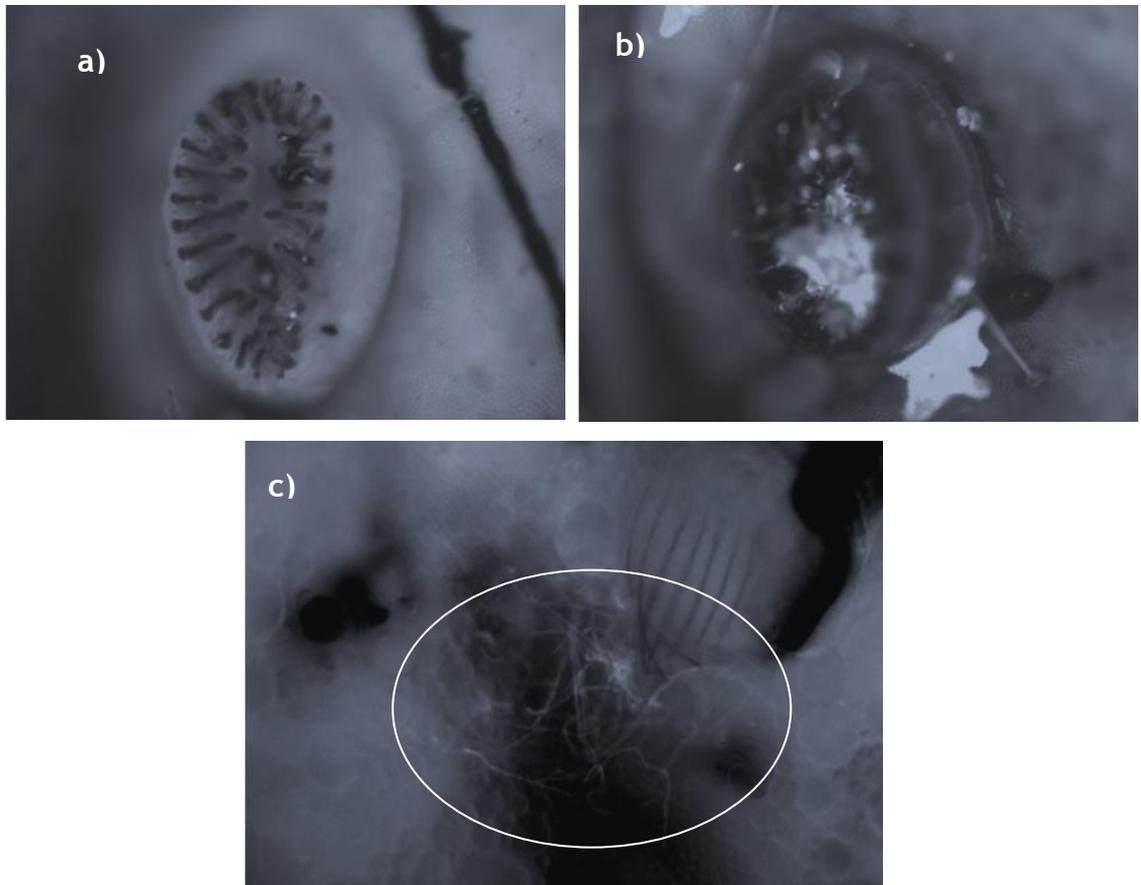
Melanisation immune response in *G. mellonella* was used to monitor the pathogenicity of *A. fumigatus* (Figure 5.2a). With high inoculum concentrations ( $10^6$  or  $10^7$  conidia/larvae) melanisation was observed near the proleg region on the same day of infection (Figure 5.2b). When the infection progressed over time a gradual increase in melanisation was observed (Figure 5.2c), and full melanisation observed between day 1 to 3 (Figure 5.2d). The full melanisation indicates the death of larvae which was confirmed by lack of movement of larvae in response to touch.



**Figure 5.2 - Melanisation in *G. mellonella* larvae.** *G. mellonella* larvae were inoculated with  $10^6$  conidia/larvae and incubated for 1-3 days at  $37^{\circ}\text{C}$ . Melanisation in larvae were visually monitored everyday and imaged using digital camera. Images in this figure shows (a) Uninfected larva with no melanisation, (b) larva infected with  $10^6$  conidia showing melanisation near proleg region (circled) on same day post-infection, (c) increased melanisation (circled) on day 1 post-infection with  $10^6$  conidia, (d) dead larva displaying full melanisation on day 2 post-infection.

### 5.3.1.2 Filamentation

*A. fumigatus* conidia inoculated into the hemocoel are retained by the fat body and other internal structures. In order to analyse the *A. fumigatus* growth in *G. mellonella* model tissue sections of infected larvae were observed by light microscopy. The site of inoculation (proleg region) of uninfected vehicle control larvae (Figure 5.3a) and infected larvae (with  $10^6$  conidia of AfGFP strain) were observed 8 h post infection. The 8 h micrograph demonstrated no visual signs of infection or melanisation with PBS controls, and in infected larvae the initial spread of infection with little melanisation was observed (Figure 5.3b). The tissue sections of 24 h post-infected larvae were also imaged, which displayed filamentous growth of *A. fumigatus* in association with *G. mellonella* internal structures (Figure 5.3c).

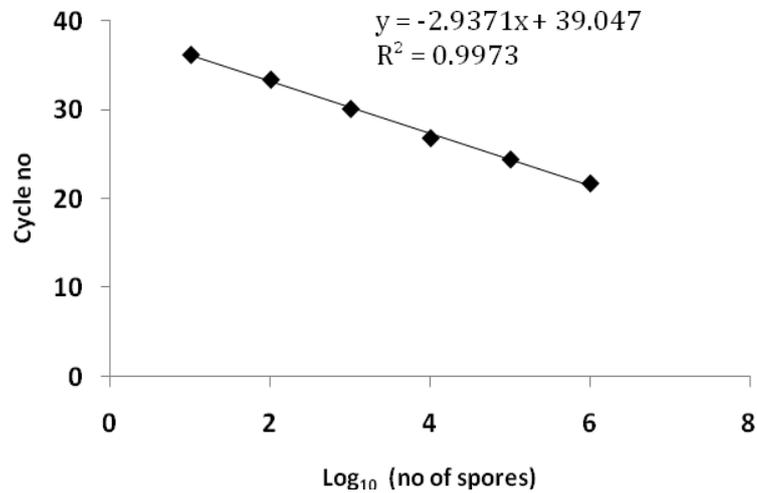


**Figure 5.3 - Filamentous growth of *A. fumigatus* in a *G. mellonella* infection model.** *G. mellonella* larvae were inoculated with  $10^6$  conidia/larvae and incubated for 24 h at 37°C. Proleg region and larvae sections were imaged after 8 h and 24 h post infection respectively under fluorescence microscope. Images in this figure are the representative 4X magnification micrographs from each group showing proleg region of (a) uninfected larva, (b) infected larva and (c) a tissue section showing filamentous growth (indicated by white circle) of *A. fumigatus* with *G. mellonella* internal structures (20x Magnification).

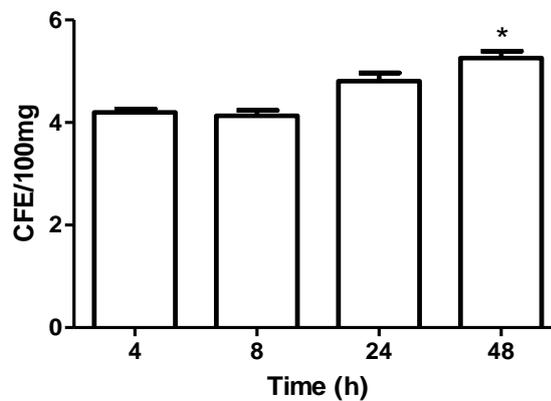
### 5.3.1.3 Fungal burdens - colony equivalents

An 18S rRNA real-time PCR analysis was performed with nucleic acid extracted from  $10^1$ - $10^6$  conidia to standardise the assay. A linearity of qPCR assay is shown in Figure 5.4a. The  $R^2$  value of 0.99 indicates a good linearity between Ct value and conidial count. The bar graph in Figure 5.4b illustrates the tissue burden in *G. mellonella* over a period of infectivity. CFE data was calculated using the standard curve equation. Overall, the trend of the graph shows an increase in tissue burden over the period of infection progress. The CFE value of 4.2 from 100mg of 4 h post infected larvae sample significantly increased to 5.3 with 48 h post infected sample ( $p < 0.05$ , ANOVA, Bonferroni post test for multiple comparisons).

a)



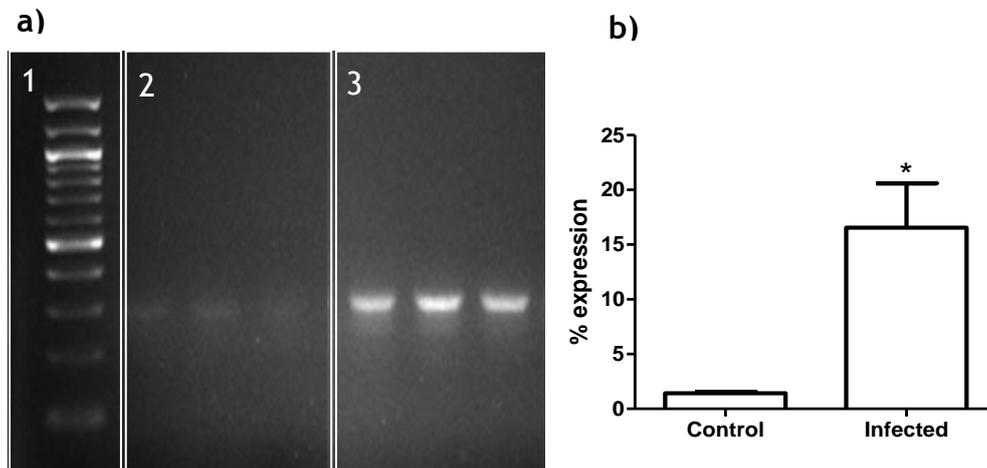
b)



**Figure 5.4 - Assessment of fungal burden by 18s rRNA real time PCR analysis.** Total nucleic acid was extracted from serially diluted *A. fumigatus* conidia ( $10^1$  -  $10^6$  conidia/mL) for assay standardisation and from 4, 8, 24 and 48 h post infected larvae for assessing the colony forming equivalents (CFE). The 18S rRNA real time PCR analysis was performed and (a) standard graph was plotted using the Ct values (y-axis) and no of spores (x-axis). (b) The bar graph in the figure shows the fungal burden in *G. mellonella* over the period of infection progress. The error bar in the graph indicates the standard deviation in CFE data among the replicates. \* $p < 0.05$ , ANOVA with a Bonferroni post test for multiple comparisons.

### 5.3.1.4 Expression of gallerimycin

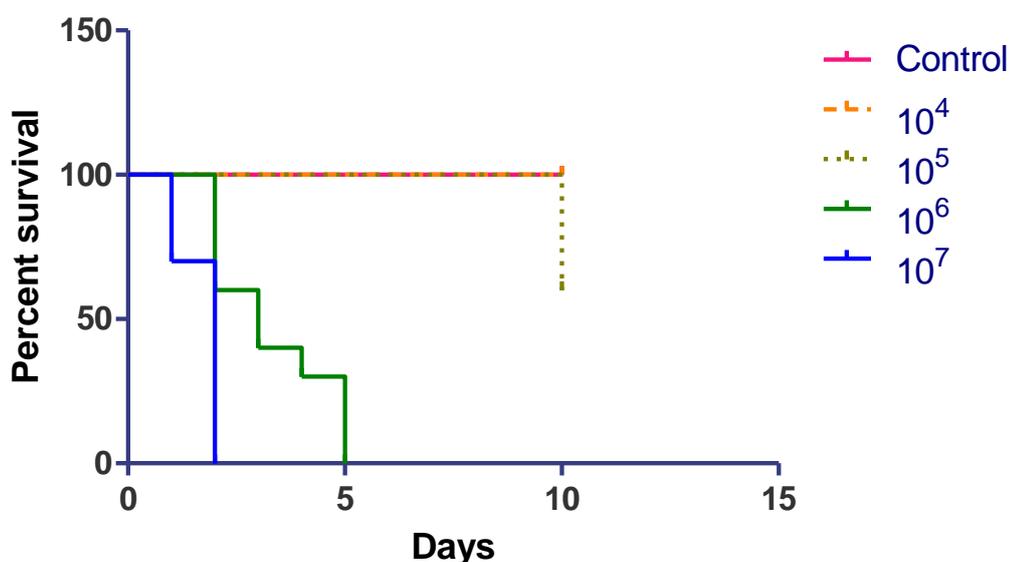
The expression of the gallerimycin gene in *G. mellonella* larvae was investigated as an indicator of *A. fumigatus* infection. RNA extracts from *G. mellonella* tissue samples were reverse transcribed and gallerimycin gene was amplified by PCR process. Gel electrograph in Figure 5.5 shows the expression of gallerimycin (300bp) in uninfected controls (Figure 5.5a-Box 2) and infected larvae (Figure 5.5a-Box 3). Real-time PCR analysis showed the expression of the gallerimycin gene was significantly increased from 1.4% in uninfected controls to 16% in *A. fumigatus* infected larvae (Figure 5.5b) (\*  $p < 0.05$ ).



**Figure 5.5 - *A. fumigatus* infection induces the expression of gallerimycin.** RNA extracted from control and infected larvae were used as the template for cDNA synthesis and subsequent PCR reactions products were visualised. a) gel image showing a bright band with infected larvae samples (box 3, lane 5-7), bands of uninfected controls (box 2, lane 2-4) and a DNA ladder (box 1, lane 1), b) bar graph showing the % expression of gallerimycin in uninfected and infected larvae compared to a house keeping gene  $\beta$ -actin. \* $p < 0.05$ , ANOVA, Bonferroni post test for multiple comparisons.

#### 5.4 A. *fumigatus* kills *G. mellonella* in a dose dependant manner

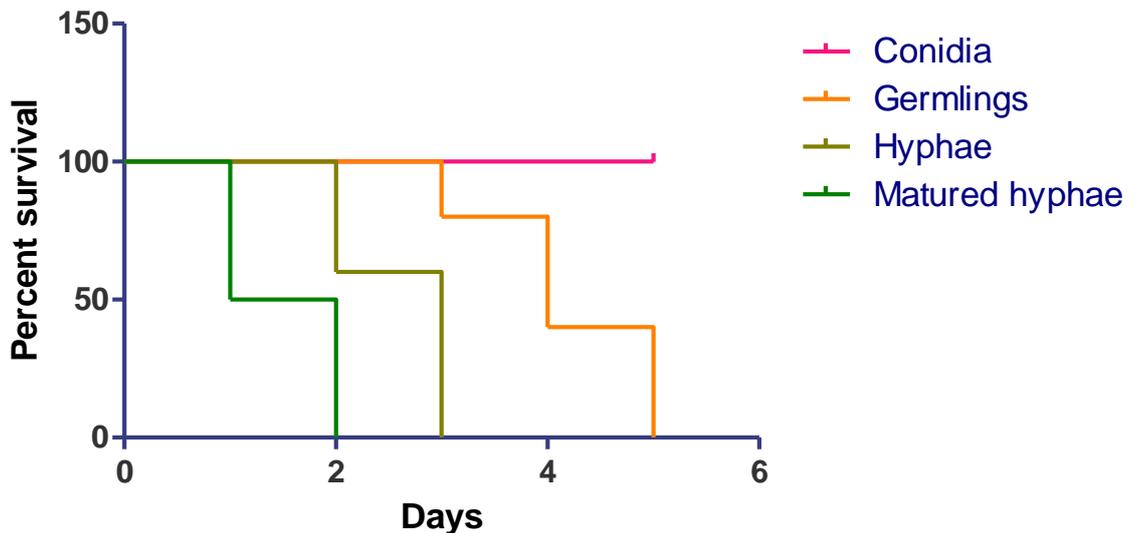
The susceptibility of *G. mellonella* to different inoculum concentrations was assessed by injecting  $10^7$ ,  $10^6$ ,  $10^5$  and  $10^4$  conidia/larva, and mortality recorded up to 10 days post-infection. Injection of a higher inoculum of  $10^7$  conidia/larva resulted in 100% lethality within 1 to 2 days, with  $10^6$ /larva causing a delay in killing (2-3 days), but still with a 90-100% lethality recorded in all individual experiments (Figure 5.6). Doses below  $10^6$  conidia/larva caused further delay in killing (> 5 days) and reduced lethality ( $p < 0.0001$ , Log-rank (Mantel-Cox) test). No deaths were recorded when larvae were injected with vehicle alone. Thus, for subsequent experimental assays  $1 \times 10^6$  to  $1 \times 10^7$  conidia/larva were used as the inoculating dose to study antifungal drug treatment. This dose dependence was statistically significant and is shown for *A. fumigatus* Af293 in figure 5.6.



**Figure 5.6 - Dose dependant *G. mellonella* survival.** *G. mellonella* larvae were infected with serially ten-fold diluted *A. fumigatus* conidia ( $10^4$ - $10^7$  conidia/larva) and the survival was monitored over the period of 10 days. The survival curve in the figure shows the percentage survival of larvae with respect to different inoculum concentrations (0,  $10^4$ ,  $10^5$ ,  $10^6$  and  $10^7$ ). The survival curves of different inoculum dosages are significantly different  $p < 0.0001$ , Log-rank (Mantel- cox) test.

#### 5.4.1 *A. fumigatus* growth phase dependant killing of *G. mellonella*

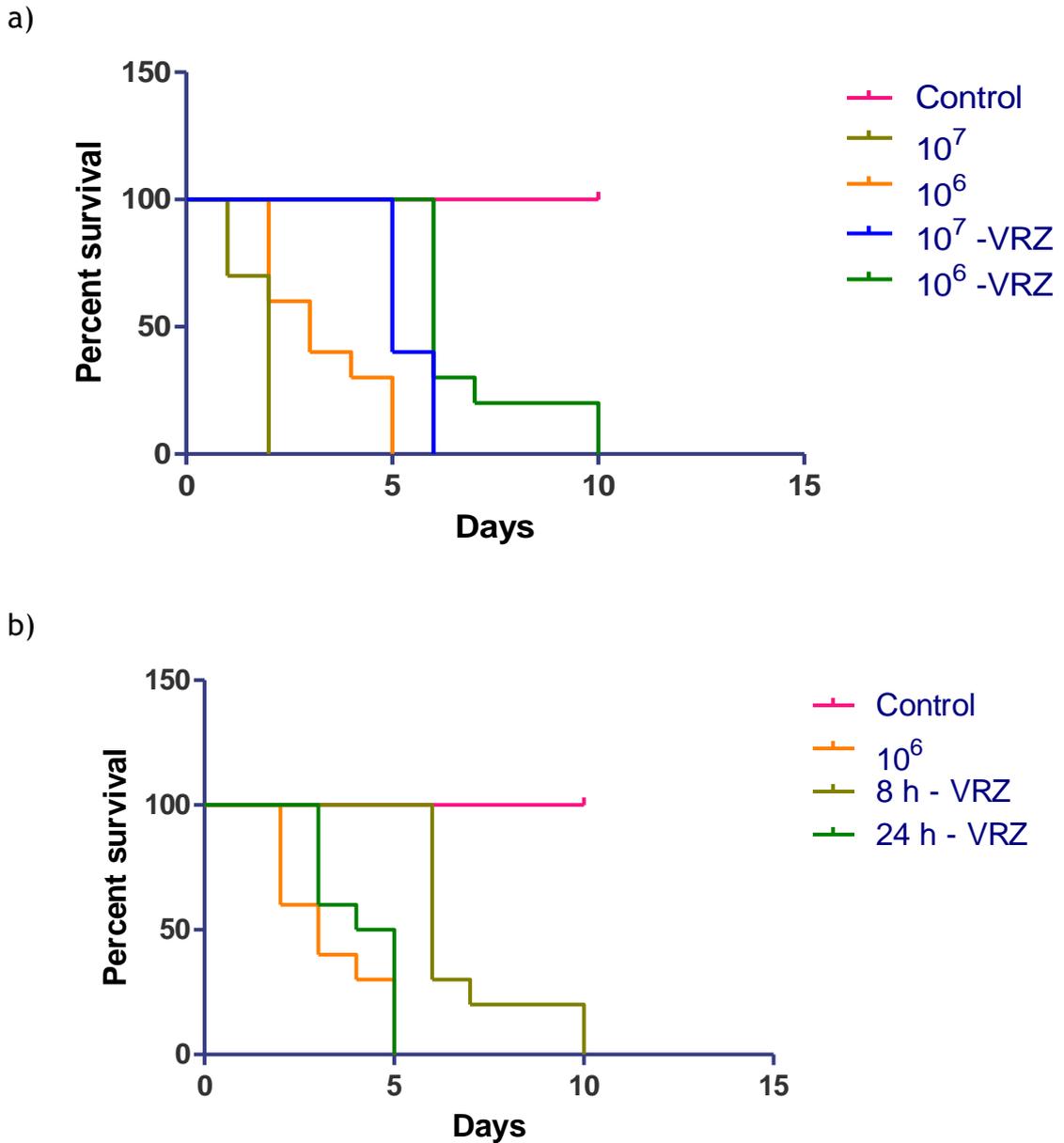
To assess the effect of *A. fumigatus* multicellular growth on *G. mellonella* survival, larvae were infected with conidia (0 h), germlings (8 h), initial hyphae (12 h) and matured hyphae (24 h), which coincides with selected time points in previous chapters. The survival curve in Figure-5.7 illustrates the percentage and days survival of larvae infected with different phases of *A. fumigatus* growth. This data shows a growth phase dependant killing of larvae. Injection of matured hyphae killed the larvae quicker in less than 2 days ( $p < 0.0001$ ). With germlings and initial hyphae the survival was 6 and 4 days respectively. It is significantly lower than the resting conidia controls ( $P < 0.0001$ ).



**Figure 5.7 - Survival of *G. mellonella* with different phases of *A. fumigatus* growth.** *G. mellonella* larvae were infected with resting conidia (0 h), germlings (8 h), initial hyphae (12 h) and matured hyphae (24 h) and incubated at 37°C. The survival was recorded over the period of 5 days. The survival curve in this figure shows the percentage survival of larvae over 5 day period. The survival curves with inoculation of different phases of *A. fumigatus* growth are significantly different to each other  $p < 0.0001$ , Log-rank (Mantel-Cox) test.

#### **5.4.2 Azole treatment prolongs the survival of infected *G. mellonella***

To assess whether *G. mellonella* can be used as an infection model to test the efficacy of azoles against *A. fumigatus* infection, larvae were infected with  $10^6$  or  $10^7$  conidia/larva and infected larvae were treated with voriconazole (6 mg/kg). Treated larvae 8 h post-infection significantly increased the time of survival of larvae (Figure 5.8a), ( $p < 0.0001$ , Log-rank [Mantel-Cox] test) compared with the untreated controls. Treatment with voriconazole 24 h post-infection resulted in reduced length of survival compared to treatment at 8 h post infection. Injection with voriconazole (6 mg/kg) alone was not toxic and had no effect on the survival of larvae.



**Figure 5.8 - Voriconazole prolongs the survival of *A. fumigatus* infected larvae.** *G. mellonella* larvae were infected with  $10^6$  or  $10^7$  conidia/larva. After 8 h or 24 h post infection the larvae were treated with voriconazole at a concentration of 6mg/kg. Survival of larvae was recorded over the period of 10 days. (a) Survival curves of untreated infected controls ( $10^6$ ,  $10^7$  conidia/larvae), voriconazole treated (VRZ [6 mg/kg]) larvae and uninfected controls, (b) survival curves of untreated infected controls ( $10^6$  conidia/larvae), voriconazole treatment after 8 h post infection (8 h-VRZ [6 mg/kg]), voriconazole treatment after 24 post infection (24 h-VRZ [6 mg/kg]). The survival curves of untreated controls were compared with voriconazole treated larvae using Log-rank (Mantel-Cox) test and they are significantly different from each other ( $p < 0.0001$ ).

### 5.4.3 The expression profile of antifungal resistance genes

The *in vivo* expression of selected antifungal resistance genes was assessed in the *G. mellonella* infection model. The expression over the period of Day 1, 2 and 3 post-infection was assessed by real time PCR analysis. All the gene expression levels were normalised to *TEF1* housekeeping gene using the  $2^{-\Delta\Delta Ct}$  methodology previously described (Livak and Schmittgen, 2001). Table 5.2 shows the percentage expression of each gene on day 1-3  $\pm$  standard deviation. Out of four efflux genes tested *AfuMDR2* was highly expressed at a level of 39% on day 2, 51% on day 3 and 33% on day 4, whereas *AfuMDR3* was expressed in relatively lower levels of 0.02% on day 2, 0.03% on day 3 and 0.36% on day 4. The *AfuMDR1* and *AfuMDR4* genes showed a pattern of up-regulation over different phases of biofilm formation (Table 5.2 a).

The qRT-PCR results for *ChitA* and *C* show a gradual increase in gene expression over the period of time (D1<D2<D3 and D1< D2-3) (Table 5.2 c). *CHITA* gene expressed at a level of around 2% on day 2, 3.5% of day 3 and 13.8% on day 4. *CHITC* was expressed at 4.6, 8.6 and 9.5% on day 2, 3 and 4 respectively. But the expression of other chitinase genes *ChitB* and *D* were undetectable on day 2 and 3. The expression level of *ChitE* shows no significant difference over time (Table 5.5 c). The expression of  $\beta$ -glucan synthase gene *FKS1* and stress response protein HSP90 gene were found to be the same at all three time points and no significant difference was found (Table 5.2b).

**Table 5.2 - Expression profile of *A. fumigatus* resistance genes in *G. mellonella* infection model. The numerical values indicate the percentage expression compared to *Tef1* housekeeping gene  $\pm$  standard deviation.**

a) Efflux pump genes

	<i>MDR1</i>	<i>MDR2</i>	<i>MDR3</i>	<i>MDR4</i>
<b>Day2</b>	0.47 $\pm$ 0.12	38.61 $\pm$ 6.76	0.02 $\pm$ 0.01	13.09 $\pm$ 1.34
<b>Day3</b>	0.77 $\pm$ 0.53	51.34 $\pm$ 11.44	0.03 $\pm$ 0.01	28.86 $\pm$ 2.59
<b>Day4</b>	1.65 $\pm$ 0.46	33.22 $\pm$ 7.74	0.36 $\pm$ 0.59	27.18 $\pm$ 6.83

b)  $\beta$  - glucan synthase and heat shock protein genes

	<i>FKS1</i>	<i>HSP90</i>
<b>Day2</b>	32.42 $\pm$ 4.74	3.75 $\pm$ 1.21
<b>Day3</b>	43.13 $\pm$ 31.72	1.09 $\pm$ 0.10
<b>Day4</b>	45.35 $\pm$ 22.00	3.29 $\pm$ 1.05

c) Chitinase genes

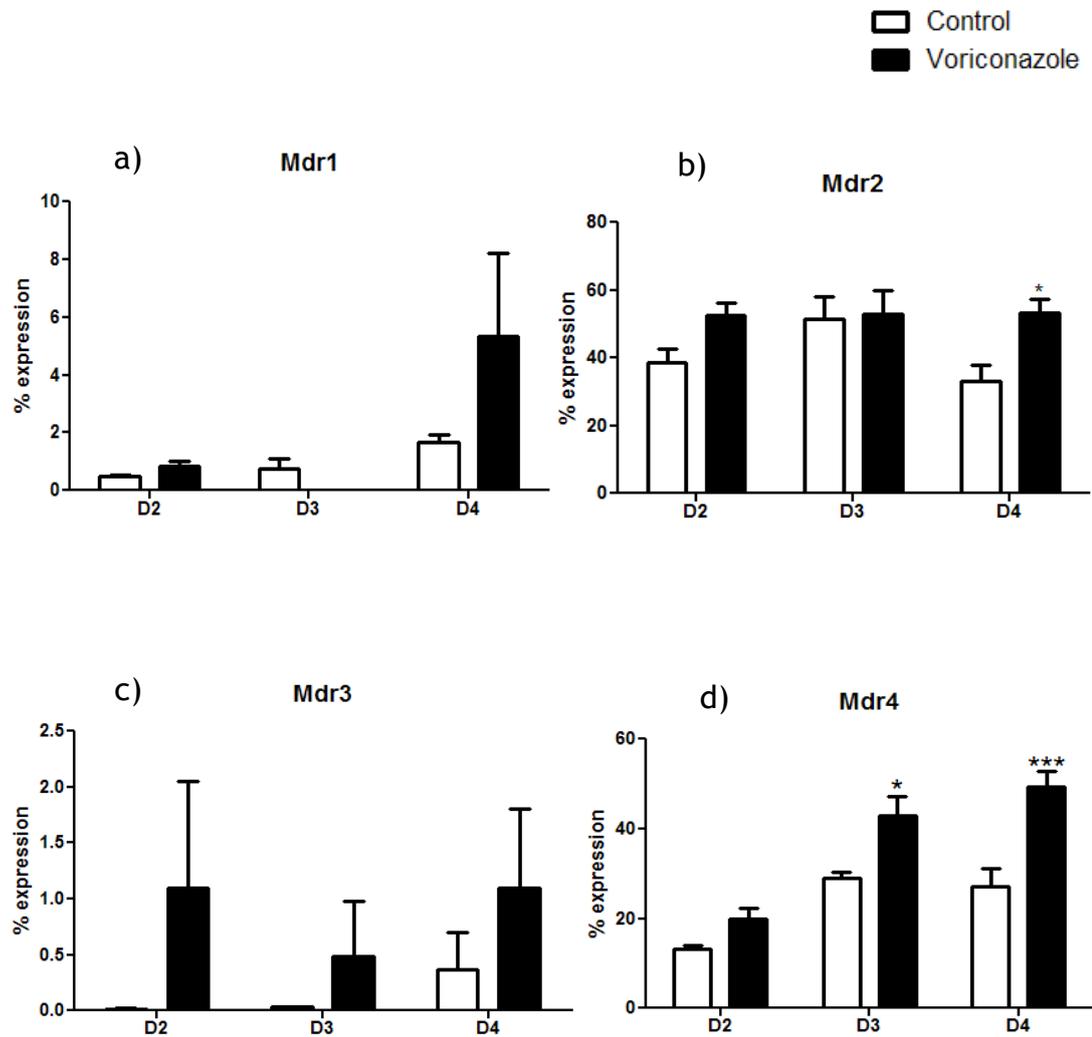
	<i>ChitA</i>	<i>ChitB</i>	<i>ChitC</i>	<i>ChitD</i>	<i>ChitE</i>
<b>Day2</b>	2.31 $\pm$ 1.00	0.06 $\pm$ 0.03	4.59 $\pm$ 4.94	0.00 $\pm$ 0.00	0.07 $\pm$ 0.02
<b>Day3</b>	3.47 $\pm$ 1.44	0.00 $\pm$ 0.00	8.64 $\pm$ 3.58	0.00 $\pm$ 0.00	0.16 $\pm$ 0.06
<b>Day4</b>	13.79 $\pm$ 2.95	0.88 $\pm$ 1.44	9.47 $\pm$ 4.41	0.02 $\pm$ 0.03	0.30 $\pm$ 0.11

#### **5.4.4 Azole treatment affects the expression of antifungal resistance genes**

The *in vivo* expression of each gene was also assessed with voriconazole treatment. The expression over the period of Day 2, 3 and 4 post-treatment was assessed by real time PCR analysis. The results were analysed by calculating fold change in expression compared to untreated controls for each time point.

##### **5.4.4.1 Efflux genes**

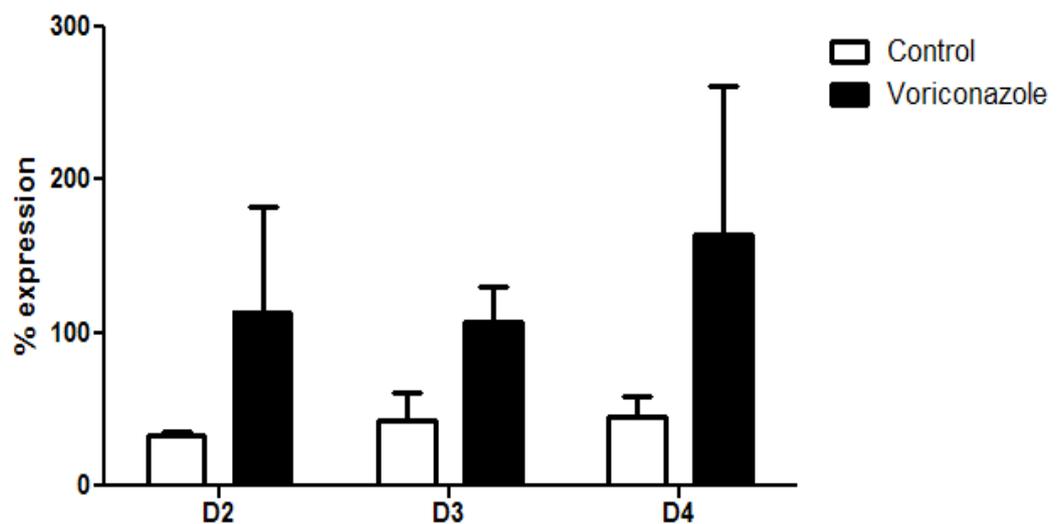
With voriconazole treatment, expression of *AfuMDR4* was shown to be significantly up-regulated according to ANOVA after day 2 post treatment (Figure 5.9d). A significant up-regulation was observed at day 2 and 3 compared to untreated controls ( $p < 0.05$  and  $p < 0.001$ , respectively). *AfuMDR2* showed a significant up regulation on day 3 and no significant differences on day 1 and 2 (Figure 5.9b). The expression levels of *AfuMDR1* and 3 were lower in all three time points compared to other efflux genes and were not significantly different from untreated controls (Figure 5.9a, c).



**Figure 5.9 - Effect of voriconazole on expression of *A. fumigatus* efflux genes in *G. mellonella* infection model.** Following RNA processing from larvae samples, quantitative RT-PCR was performed on each cDNA population using *TEF1* as a housekeeping gene to calculate the relative expression of *AfuMDR* genes (a) *MDR1*, (b) *MDR2*, (c) *MDR2*, (d) *MDR4* according to the  $2^{-\Delta\Delta CT}$  method. Three untreated larvae from each time point were used as a control for comparison to 6 mg/kg voriconazole treatment (n=3 at D1, D2 and D3). The error bars represent the standard deviation of the means. \*p<0.05, \*\*\*p<0.001, two-way ANOVA, Bonferroni post-test.

#### 5.4.4.2 *Fks1* gene

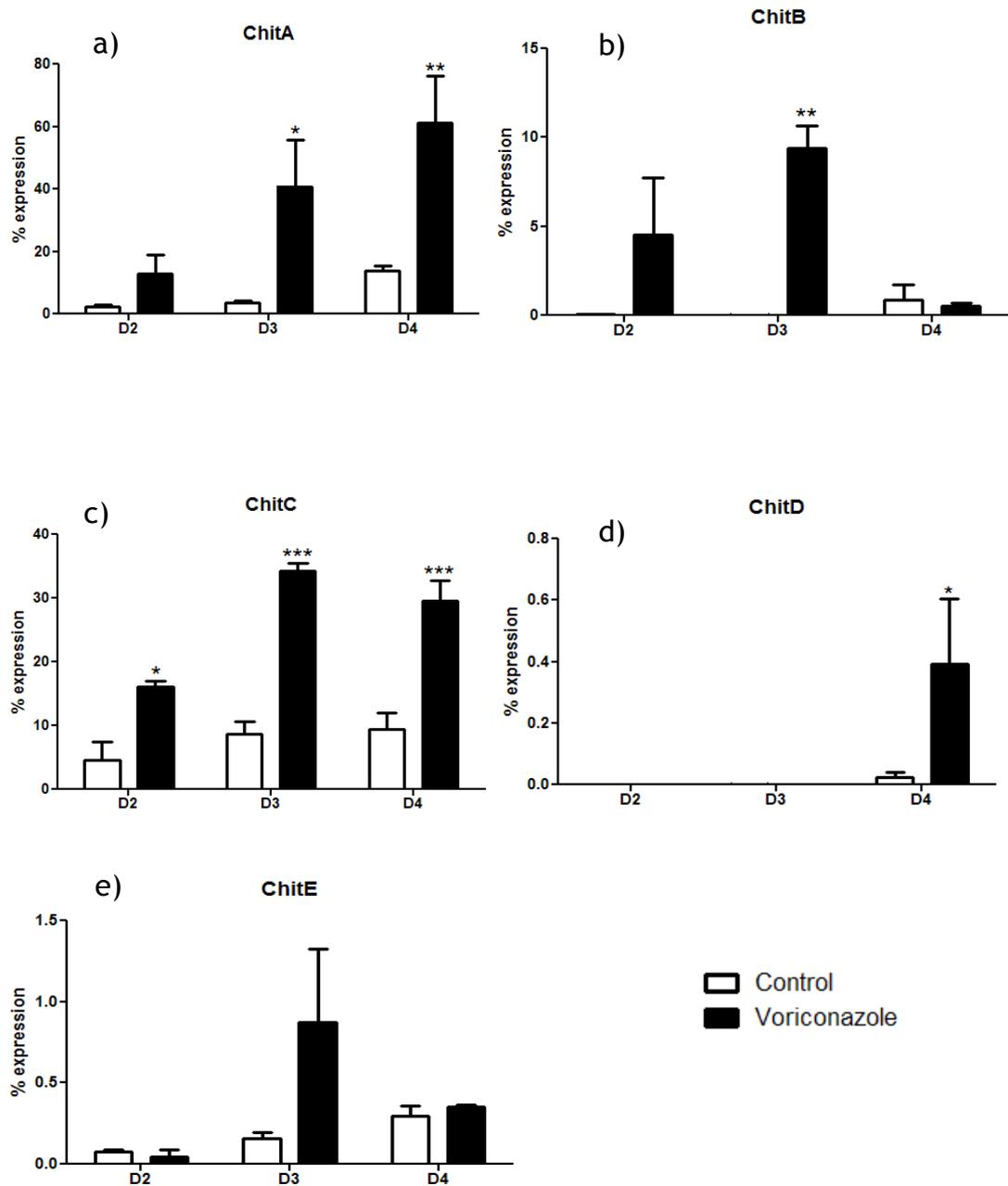
The percentage expression of glucan synthase gene *FKS1* was also calculated by comparing with *Tef1* housekeeping gene. Figure 5.10 details the percentage expression of *FKS1* gene in untreated and voriconazole treated larvae samples at day2, day 3 and day 4 of post treatment. It shows that the level of expression is not significantly different over time or with voriconazole treatment.



**Figure 5.10 - Expression of *A. fumigatus FKS1* gene in *G. mellonella* infection model.** Following RNA processing from larvae samples, quantitative RT-PCR was performed on each cDNA population using *Tef1* as a housekeeping gene to calculate the relative expression of *FKS1* gene according to the  $2^{-\Delta\Delta CT}$  method. Three untreated larvae from each time point were used as a control for comparison to 6 mg/kg VRZ treatment (n=3 at D1, D2 and D3). The error bars represent the standard deviation of the means.

#### 5.4.4.3 Chitinase genes

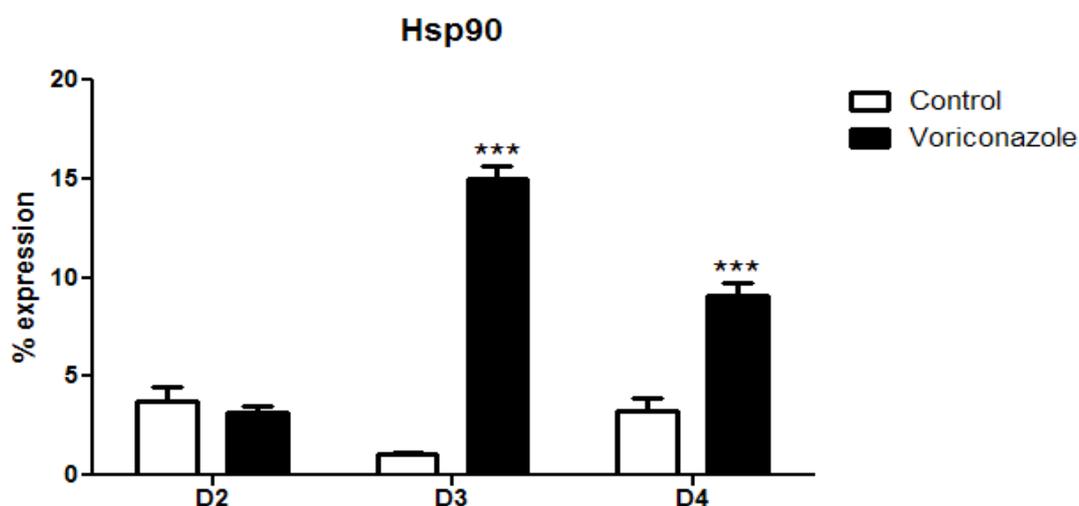
The relative changes in expression for each of the selected 5 chitinase genes with and without antifungal treatment are shown in Figure 5.11. The RT-PCR results for *ChitA* and *C* show a gradual increase in gene expression over the period of time (D1<D2<D3 and D1< D2-3) and further upregulation with VRZ treatment (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001). A significant up regulation with VRZ treatment was reported with *ChitB* expression on day 3 and *ChitD* on day 4 compared to untreated controls. The expression level of *ChitE* was also assessed and no significant difference over time or with VRZ treatment was noticed.



**Figure 5.11 - Voriconazole treatment affects the expression of chitinase genes.** Following RNA processing from larvae samples, quantitative RT-PCR was performed on each cDNA population using *TEF1* as a housekeeping gene to calculate the relative expression of Chitinase genes (a) *ChitA*, (b) *ChitB*, (c) *ChitC*, (d) *ChitD* and (e) *ChitE* according to the  $2^{-\Delta\Delta CT}$  method. Three untreated larvae from each time point were used as a control for comparison to 6 mg/kg VRZ treatment (n=3 at D1, D2 and D3). The error bars represent the standard deviation of the means. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, two-way ANOVA, Bonferroni post-test.

#### 5.4.4.4 HSP90 gene

The expression of stress related molecular chaperone *HSP90* gene was assessed with untreated and VRZ treated larvae samples over three days of post treatment. The graph shows the percentage expression of *HSP90* in relation to *Tef1* housekeeping gene. The expression was not significantly different over the period of time with untreated controls but up-regulation was noticed with voriconazole treatment group. On day 3 and day 4 post treatment group the expression was significantly up regulated compared to untreated controls ( $p < 0.001$ ).



**Figure 5.12 - Azole treatment up regulates the expression of *HSP90* gene.** Following RNA processing from larvae samples, quantitative RT-PCR was performed on each cDNA population using *Tef1* as a housekeeping gene to calculate the relative expression of *HSP90* gene according to the  $2^{-\Delta\Delta CT}$  method. Three untreated larvae from each time point were used as a control for comparison to 6 mg/kg VRZ treatment ( $n=3$  at D1, D2 and D3). The error bars represent the standard deviation of the means. \*\*\* $p < 0.001$ . Two-way ANOVA, Bonferroni post-test.

## 5.5 Discussion

Infection models are essential tools for studying fungal pathogenesis. Mammalian models such as mice or rats are considered the 'gold standard' for studying *in vivo* infections caused by pathogenic fungus such as *A. fumigatus*, but are associated with animal welfare and bioethical issues. Due to these and regulatory issues, it is now more challenging to perform LD<sub>50</sub> experiments with mice. In addition, insect models such as *D. melanogaster* and *C. elegans* are somewhat more difficult to work with, and *A. fumigatus* in these models does not appear to give consistent results. Therefore, this study aimed to investigate *A. fumigatus* infection and adaptive resistance in a *G. mellonella* model. The data reported from these studies was comparable with other *in vitro* and *in vivo* infection models.

Data from this study show that *G. mellonella* is a sensitive and consistent infection model for *A. fumigatus* and possessed several advantages over other mammalian infection models. The progression of *A. fumigatus* infection in the *G. mellonella* model is comparatively easy to monitor. Infecting *G. mellonella* larvae with fungal cells induces innate immune responses such as melanisation and production of antifungal peptides (Singh, 2000, Askew, 2008). However, these responses were not sufficient to achieve insect protection from infection. It can therefore be used as an indicator, or biomarker, of *A. fumigatus* virulence (Askew, 2008). Gradual increase of melanisation over time indicates the progress of disease and host pathogen interaction. Increased expression of gallerimycin, an antifungal peptide, in infected larvae is another indicator of infection. The fungal burden assessed by 18S rRNA real time PCR shows an increase in colony forming equivalents (CFU) in a time dependent manner, which confirms the infection progress in this model. This result is similar to the pattern of increase in fungal burden over time published previously with a rat inhalation model (Gibbons et al., 2011).

The results from this chapter demonstrate that *G. mellonella* is susceptible to *A. fumigatus* infection in a dose-dependent manner. This correlates with data observed with other fungal pathogens, such as *C. albicans* and *C. neoformans* (Walsh et al., 2008, Cornet et al., 2002). In this study, it was shown that the dose of  $1 \times 10^6$  conidia/larva is suitable for virulence studies of *A. fumigatus* strains Af293. This inoculum is in line with the inoculum used for studying other pathogenic yeasts (Stevens et al., 2003, Walsh et al., 2008). The treatment of infected larvae with antifungal drugs such as azoles prolongs the survival of *G. mellonella* larvae. Consequently, the results of this chapter confirm previous findings indicating that this model can be used as an *in vivo* system to evaluate toxicity and efficacy of new antimicrobial agents (Grow et al., 2002, Cornet et al., 2002, Kunst et al., 2006, Marr et al., 2002)

There are several studies that have investigated morphological and multicellular development of fungi in relation with their pathogenicity (Mowat et al., 2008a, Chatzimichalis et al., 1998, Greenberg et al., 2002). The results from this study show *A. fumigatus* proliferates gradually at the site of inoculation, forming multicellular hyphae and invading the tissue. These findings resemble histological findings for human patients (Greenberger, 2002, Ricketti et al., 1984) and experimentally infected mice (Lai et al., 2002, Saxena et al., 2003). In addition, *G. mellonella* killing assay testing the virulence of different phases of *A. fumigatus* proliferation demonstrate multicellular hyphal cells are more lethal compared to germlings and resting conidia. A study by Mesa-Arango et al (2012) demonstrated that haemocytes of *G. mellonella* are capable of phagocytosing *A. fumigatus* conidia less than 3.0  $\mu\text{m}$  in diameter, but that multicellular hyphae are greater than this and are too large to be engulfed (Geller et al., 1999). The data from this study showed 8 h post-infection larvae are easier to treat compared to 24 h hyphal infection. This result agrees with the previously published *in vitro* data showing *A. fumigatus* growth phase dependant susceptibility to antifungal drugs (Mowat et al., 2008a).

To investigate the phase dependant antifungal resistance previous studies assessed the differential expression of various *A. fumigatus* genes over the period of multicellular development in both *in vitro* and *in vivo* models. Transcriptional profiling of various genes associated with biofilm adaptive resistance, including *AfuMDR1-4* and *ChitA-E*, were performed using *in vitro* biofilm model in previous chapters. To further investigate the expression of other resistance genes in an *in vivo* model, samples were collected from infected larvae at different time points and real time PCR undertaken. Up-regulation of *AfuMDR1* and *4* gene expression was observed in a time dependant manner. Comparing this data with the azole treated group demonstrated that *AfuMDR4* expression was up regulated. This is similar to the *in vitro* and *in vivo* data presented in chapter 2. Ferreira and colleagues (2004) also observed azole-constitutive and induced efflux pump expression in *A. fumigatus* following treatment with itraconazole (da Silva Ferreira et al., 2004). Similarly, the expression of *CaMDR1* expression in a catheter based *C. albicans* biofilm model has been reported, but at a lower constitutive level (Nett et al., 2009). Moreover, the same group assessed the expression of efflux genes *CaMDR1*, *CaCDR1* and *CaCDR2* in their *in vivo* murine model following fluconazole treatment. This study reported induction of *CaCDR1* and *CaCDR2* expression with fluconazole treatment, but no change in the expression of *CaMDR1* (Lepak et al., 2006). Out of four efflux genes assessed in this study *AfuMDR2* was the one expressed in high level and *AfuMDR3* was expressed in low level and no significant difference was found between different time points. These levels of expression are similar to the *in vitro* data published in chapter 2.

The next resistance gene assessed with this model is *FKS1* gene, encoding a subunit of the  $\beta$ -1, 3-D-glucan synthase enzyme. The mutation or over expression of this gene was reported to be involved in multidrug resistance in *A. fumigatus* (Skov et al., 2005, Zeaske et al., 1988). The  $\beta$ -1-3 glucan is one of the predominant constituents of biofilm matrix, which was shown to be elevated over the period of *A. fumigatus* maturation (Nett et al., 2007). In this study the expression of *FKS1* gene was not significantly different over the period of time and with azole treatment a general trend of up regulation was found. However,

due to higher standard deviation in the expression level within the treatment group it was not statistically significant. A study by Nett et al (2009) assessed the time course expression of *FKS1* gene in *C. albicans* using an *in vivo* venous catheter biofilm model and reported differential expression with 24 h compared to 12 h (Nett et al., 2009). No such notable time course expression studies were done with *A. fumigatus* for comparison.

The expression of chitinase genes involved in the cell wall biosynthesis and autolysis pathway were also studied with this *G. mellonella* model. All tested class III chitinase genes *ChitA-E* were found to be up-regulated in a time dependant manner but the level of expression is quite variable among these genes. Azole treatment induces the expression chitinase genes especially *ChitA* and *C*. These expression patterns were somewhat similar to the *in vitro* data shown in Chapter 4 but they are not exactly same. The expression of *ChitB* and *D* on day 2 and 3 from this model were not in the detectable level but they were expressed in an *in vitro* model. A study by Alcazar-Fuoli also detected the expression of all of these genes *in vitro* in resting conidia, swollen conidia and germlings except for *ChitC* gene (Patterson et al., 2000). The difference in expression of these genes with this model may be because of interference of host immune response at early stage of *A. fumigatus* proliferation.

Overall this study demonstrates that *G. mellonella* is a consistent model for studying *A. fumigatus* infections and antifungal efficacy testing. To study the virulence gene expression in *A. fumigatus* this model will give a better idea about the host dependant differential expression of genes. However due to lack of data from other mammalian models for correlation analysis further studies are needed to give conclusive statement about suitability of this model for molecular studies.

## **Chapter 6**

### **General Discussion**

## 6.1 Introduction

In recent decades, fungal pathogens have emerged as a predominant cause of human disease, especially in immuno-compromised individuals. *Candida* and *Aspergillus* species together account for ~70% of all invasive fungal infections, with *C. albicans* and *A. fumigatus* prevailing as the leading causal agents of opportunistic mycoses (Pfaller and Diekema, 2010). In patients with pulmonary disorders such as asthma or CF, *A. fumigatus* infection can cause ABPA leading to severe complications, but has also been recently described in the context of biofilms, or multicellular communities (Ramage et al., 2009b). For these fungal species, there are numerous factors that contribute to the pathogenicity and recalcitrance of resulting infections to antifungal treatment, including the ability to evolve and maintain resistance to conventional antifungal therapy (Cowen and Steinbach, 2008).

Azoles are the most commonly used antifungal therapy and resistance to these has been increasingly observed in *Candida* and more recently *Aspergillus*. The fungistatic nature of the azoles towards fungal cells culminates in strong directional selection on the surviving population to evolve drug resistance (Anderson, 2005, Cowen, 2008). The mortality of patients with multi-azole resistant invasive aspergillosis was 88%, compared with 30-50% in those infected with azole sensitive strains (Howard et al., 2009). Time from diagnosis to resolution of the infection or death in invasive forms of fungal disease is typically a few weeks, whereas in allergic or chronic diseases patients may take antifungal medication for many years. In both cases drug resistance is highly problematic. In a recent survey of azole resistant *A. fumigatus* in a UK referral clinic a striking rise in drug resistance was observed (Howard et al., 2009, Bueid et al., 2010). Until 2008 all observed resistance was attributable to mutations in the *cyp51A* gene, however between 2008 and 2010 resistant isolates containing no mutations in this gene or promoter became more prevalent. In 2010, 51% of Manchester isolates that were resistant to azoles were non-*cyp51A*, indicating that other mechanisms of resistance were responsible (Bueid et al., 2010).

Antifungal resistance is both complex and multifaceted. It can be inducible in response to a compound, or an irreversible genetic change resulting from prolonged exposure. Specifically, these include alterations or overexpression of target molecules, active extrusion through efflux pumps, limited diffusion, tolerance, and cell density, which are all characterized mechanisms utilized by fungi to combat the effects of antifungal treatment (Niimi et al., 2010). Planktonic cells generally rely on irreversible genetic changes to maintain a resistant phenotype, but which comes with a cost to overall fitness (Huang and Kao, 2012, Ben-Ami and Kontoyiannis, 2012). Whereas, biofilms are able to persist due to their physical presence and the density of the microbial community, which provides an inducible or 'responsive' resistant phenotype, irrespective of defined genetic alterations.

The overarching hypothesis of this study was that the multicellular biofilm phenotype provided *A. fumigatus* with the ability to respond to environment pressures without the need for any defined mutations. Therefore, the objectives were to investigate and identify adaptive resistance mechanisms associated with these multicellular forms.

## **6.2 Adaptive resistance mechanisms in *Aspergillus fumigatus***

*C. albicans* has been described to respond to antifungal drug pressures in an adaptive way (Walker et al., 2010). Recently, it has been demonstrated in a clinical isolate of *C. albicans* that in addition to a defined *FKS1* hotspot mutation, chitin remodelling was also contributing to echinocandin resistance (Walker et al., 2010). This demonstrated that dual resistance mechanisms can and do occur clinically. The implications of these studies suggest that pathogenic fungi have the capacity to adapt to their environment, whether in a human host or elsewhere, and resist specific environment selection pressures.

The basis of this study was the previous reports from our group that *A. fumigatus* exhibited a phase-specific resistance phenotype to azoles, polyenes, and echinocandins as multicellular development progressed (Mowat et al., 2008a). This observation suggested that, like in *C. albicans*, adaptation during

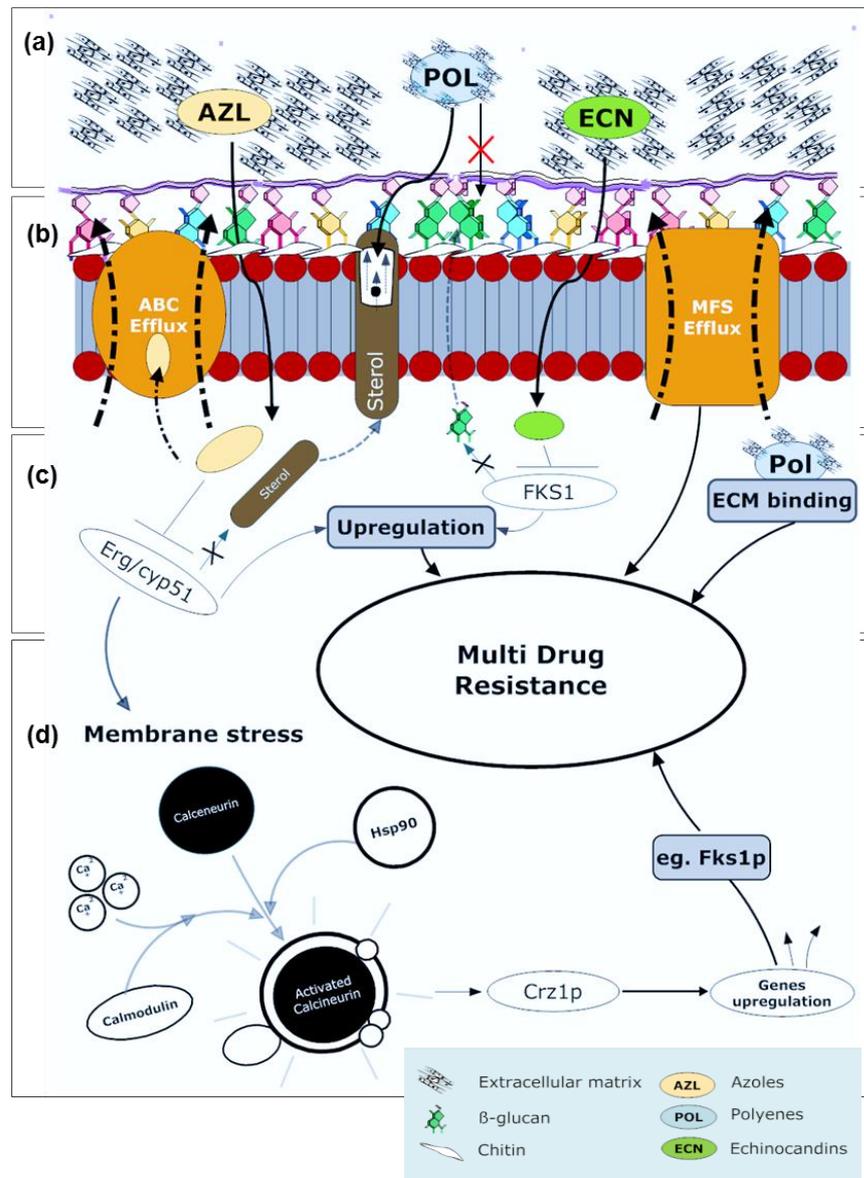
biofilm growth endowed *A. fumigatus* with an increasingly resistant phenotype. The unexplained azole resistance phenotypes described as non-*cyp51A* mechanisms from studies in Manchester gave further evidence that adaptive resistance mechanisms may be important in *A. fumigatus* (Howard et al., 2009, Bueid et al., 2010). In fact, it has been recently reported from work in a collaboration with our group and Manchester that the efflux pump *Cdr1b* was significantly associated with non-*cyp51A* azole resistance (Marcin et al., 2013), despite previous reports that there is little evidence to suggest that these play an active role in clinical resistance (Cannon et al., 2009).

Sequence analysis suggests that *A. fumigatus* has 278 different MFS and 49 ABC transporters (Nierman et al., 2005). *A. fumigatus* MDR pumps have previously been shown to be associated with increased resistance to itraconazole (da Silva Ferreira et al., 2004, Nascimento et al., 2003). In this study it was hypothesized that efflux-mediated mechanisms may therefore be an important mechanism of resistance during biofilm maturation. It was shown that efflux pump activity was an important factor during biofilm maturation, which was demonstrated both at a biochemical and molecular level *in vitro* and *in vivo*, and that exposure to azole stimulated efflux pump expression (Rajendran et al., 2011b). Interestingly, expression of *Mdr4* transcripts suggested that efflux pump expression was biphasic, showing maximal expression during the early to intermediate phases of growth. Collectively, this data in addition to the available literature support the hypothesis that efflux pumps are an important, but not exclusive, determinant of fungal biofilm resistance to azoles (Cannon et al., 2009, Morschhauser, 2010). Their primary role may be for homeostasis within complex environments to protect themselves from acute toxicity (Piddock, 2006), but within clinical environments exposure to azoles drugs may enhance the levels of efflux pump expression, therefore either contributing towards or inducing clinical resistance (Bueid et al., 2010) (Marcin et al., 2013). However, it is likely that they play a greater protective (resistance) role in the early phases of biofilm growth until the production of ECM, one of the primary mechanisms of biofilm resistance.

ECM has been shown to act as a drug ‘sponge’ for azoles in *C. albicans* biofilms, thereby preventing the ability of the molecule to reach the cell and elicit an effect (Nett et al., 2010b). This has been shown to be mediated by the *FKS1* beta-1,3-glucan synthase enzyme, which is inducible in the biofilm phenotype (Taff et al., 2012), suggesting this is another adaptive resistance mechanism. The presence of ECM during *A. fumigatus* biofilm maturation has been observed *in vitro* (Mowat et al., 2008c) and within *in vivo* models (Loussert et al., 2010). Degradation of ECM using glucanases has been reported to improve antifungal sensitivity in *C. albicans* biofilm (Nett et al., 2007), whereas in *A. fumigatus* biofilm sensitivity was unchanged, suggesting that other components play a greater role in biofilm integrity and resistance (Beauvais et al., 2007a). A key contributing component of the ECM in *C. albicans* was shown to be eDNA (Martins et al., 2010). It was therefore hypothesized that this may be a novel adaptive antifungal resistance mechanism in *A. fumigatus*. Given that eDNA has been shown to be an important component in microbial biofilm ECM *per se* (Montanaro et al., 2011), this suggested that this may be conserved and possibly an active microbial biofilm process. Detailed analysis showed this to be the case, where it was shown to be architecturally important and also impacted antifungal sensitivity. Interestingly, chitinase activity correlated with the release of eDNA, which is a marker of autolysis and cell wall biosynthesis (Selvaggini et al., 2004). Autolytic events may therefore play an active role in environmental adaptation, which are controlled centrally through cell stress response pathways, such as the calcineurin signalling pathway via heat shock proteins such as HSP90.

Given that HSP90 has a high degree of connectivity in diverse signalling cascades, it could also affect biofilm drug resistance in a multitude of other ways, such as by regulating remodeling of the cell wall and cell membrane (Mukherjee et al., 2003, Nett et al., 2007), signalling cascades important for ECM production (Nett et al., 2010b, Nobile et al., 2009), or the function of contact-dependent signalling molecules that initiate responses to surfaces (Kumamoto, 2005). The data presented herein supports this hypothesis, as HSP90 was also shown to affect both eDNA release and antifungal sensitivity, though the precise pathway in which this is controlled has yet to be determined. Recent

studies in *C. albicans* have shown that cell wall remodeling and the release of ECM are important determinants of antifungal resistance that do not impact cell fitness *per se* (Nett et al., 2011b, Walker et al., 2010). Collectively, the series of investigations presented herein have identified several novel mechanisms associated with an adaptive resistance phenotype in multicellular *A. fumigatus* biofilms. It was clear from these studies that a level of organization between the mechanisms was evident, with efflux mediated resistance acting in the early phases of growth until the production of ECM. These, along with stress responses pathways during maturation, simultaneously provided protection from antifungal agents. The interaction of these pathways is presented in Figure 6.1.



**Figure 6.1 - Molecular mechanisms of fungal biofilm resistance.** Antifungal drug resistance in fungal biofilms is both complex and multifactorial. The diagram illustrates the mechanisms of different class of antifungal agent action (azoles [AZL], polyenes [POL], and echinocandins [ECN]) and resistance: (a) the layer of ECM containing eDNA present in the biofilm shields the cells from antifungal agents by binding and reducing penetration; (b) the membrane transporter system ABC and MFS efflux pumps extrude antifungal molecules and reduces its intracellular concentration; (c) mutation in *ERG11*, *cyp51*, and *FKS1* genes alters the drug target leading to cross-resistance; (d) antifungal treatment induces stress responses, such as the calcineurin signalling pathway, which is activated, and coping responses occur through upregulation of various signal transducers.

### 6.3 Bench to bedside: translational perspective

Our new insights into how *A. fumigatus* responds and adapts to its niche and antifungal exposure provides a unique opportunity to devise new chemotherapeutic strategies of control, whether as pharmacological targets or as augmenting conventional antifungal agents

#### 6.3.1 Pharmacological inhibitors

Throughout the studies described within this thesis, the use of inhibitors has been shown to improve antifungal activity, even against the most tenacious of biofilm structures (Figure 6.2). This work establishes that inhibition of HSP90 with geldanamycin (17-AAG), that has advanced in clinical trials for the treatment of cancer (Usmani et al., 2009, Kim et al., 2009) transforms antifungals from ineffective to highly efficacious in an *in vitro* *A. fumigatus* biofilm model. HSP90 inhibitors also exhibit potent activity against malaria and *Trypanosome* infections, thus extending their spectrum of activity to the protozoan parasites *Plasmodium falciparum* and *Trypanosoma evansi* (Pallavi et al., 2010, Shahinas et al., 2010). The development of HSP90 as a therapeutic target for infectious disease may benefit from the plethora of structurally diverse HSP90 inhibitors that have been developed, many of which are in advanced phase clinical trials, with substantial promise due to the depletion of a myriad of oncoproteins upon inhibition of HSP90 (Trepel et al., 2010). Given the importance of HSP90 in chaperoning key regulators of cellular signalling in all eukaryotes the challenge of advancing HSP90 as a target for infectious disease lies in avoiding host toxicity issues. Indeed, although well tolerated in the mammalian host individually or in combination therapies (Trepel et al., 2010), HSP90 inhibitors have toxicity in the context of an acute disseminated fungal infection (Cowen et al., 2009). This toxicity may be due to HSP90's role in regulating host immune and stress responses during infection. Toxicity was not observed in the studies of biofilm infections in the mammalian model, perhaps owing to both the localized infection and drug delivery, suggesting that this therapeutic strategy could rapidly translate from the laboratory bench to the patients' bedside. In the broader context, the challenge for further development

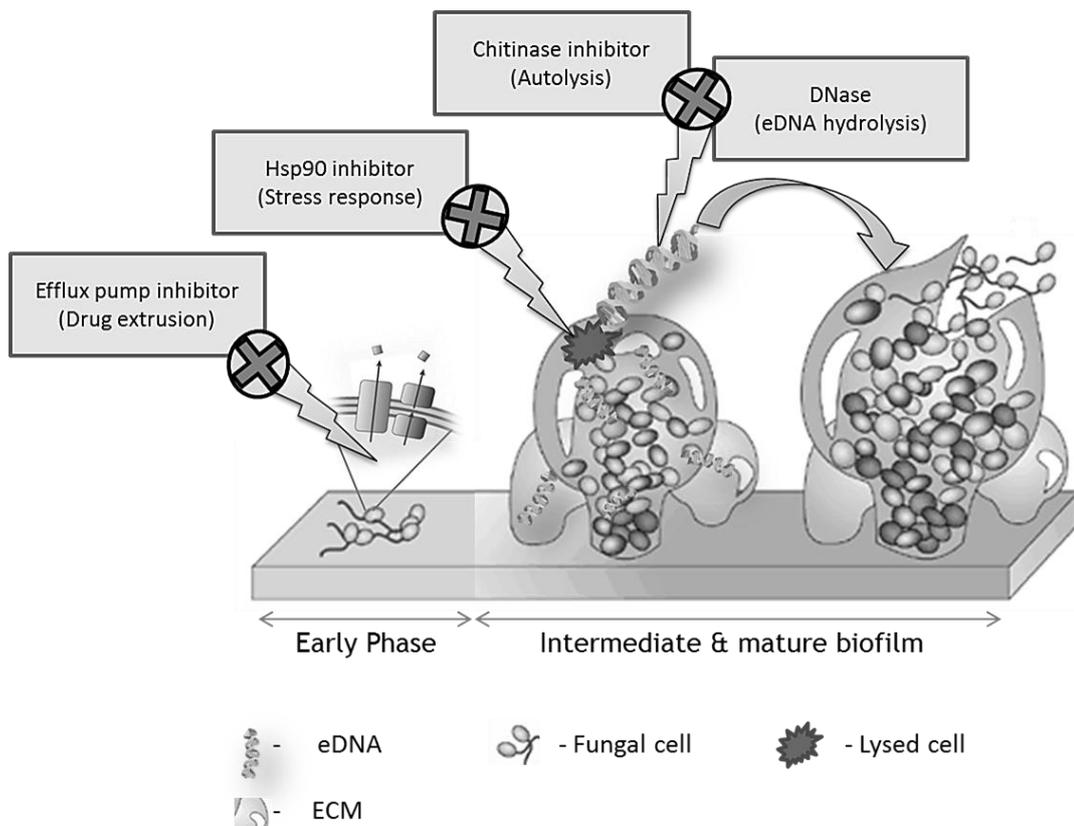
of HSP90 as a therapeutic target for infectious disease lies in developing pathogen-selective inhibitors or drugs that target pathogen-specific components of the HSP90 circuitry governing drug resistance and virulence.

In addition to HSP90 inhibitor, this study also showed the potential of chitinase and efflux pump inhibitors in enhancing the antifungal drug efficacy. Several studies are currently being conducted testing co-administration of natural and manmade chemical components to act as inhibitors for chitinases and efflux mediated extrusion of antibiotics. A recent study screened a library of marketed drug molecules for chitinase inhibition and published that methylxanthine drugs are chitinase inhibitors (Rao et al., 2005). Clinically, methylxanthine drugs are used as bronchodilators to stimulate the cardiovascular and respiratory systems in COPD patients. Further studies are needed to test the potential of this component in combination with antifungal drugs to treat fungal infections. The use of efflux inhibitors are also in serious investigation for their potential to use in combination therapies with existing antimicrobial drugs. As shown in this study some of the inhibitors are used to determine the efflux prevalence in clinical isolates but none of them are yet in clinical use. The problem using this inhibitor in humans is the efflux transporters are also part of human cells distributed along particular portions of the renal proximal tubule, intestine, liver, blood-brain barrier, and other portions of the brain. Therefore a fungal specific inhibitor needs to be developed for combination therapy.

### 6.3.2 Biofilm disruption agents

Recently, the combined use of drugs (Espinel-Ingroff, 2009), or drugs with other agents such as enzymes (Kaplan, 2009), has received considerable attention. ECM degrading enzymes present broad-spectrum activity that is unlikely to induce antimicrobial resistance. The combined use of ECM degrading agents and antifungals has been previously tested *in vitro*. Here in this study it was shown that *A. fumigatus* biofilm susceptibility to polyenes and echinocandins was increased by addition of a hydrolytic enzyme DNase. The synergy between these components showed the therapeutic potential of DNase against fungal biofilm infections. DNase is a hydrolytic enzyme that cleaves phosphodiester linkage in

DNA, used in this study exhibited significant anti-biofilm activity (Figure 6.2). Clinically, the DNase Pulmozyme® is currently used as a therapeutic agent for cystic fibrosis treatment as an adjunct to antibiotic treatment (Frederiksen et al., 2006). The dornase alfa (Pulmozyme®) is a recombinant human DNase-I used to selectively hydrolyse DNA present in mucus or sputum in CF lungs. Its activity against bacterial and fungal DNA in biofilms remains unclear. Since this protein therapeutic agent is in use for CF treatment in humans, a nontoxic broad spectrum DNase can be developed as a potential component to clear both mucus and disrupt biofilms to enhance the efficacy of antifungals. Other mucolytic agents such as ambroxol and N-acetylcysteine (NAC) used in respiratory diseases has also been shown to offer anti-biofilm activity against bacterial and fungal biofilms (Pulcrano et al., 2012, Aslam and Darouiche, 2011). Alginate lyase, another similar compound has been reported to be effective against *A. fumigatus* biofilms (Bugli et al., 2012, Papi et al., 2012). A cross species interactions between CF pathogens *Pseudomonas* and *Aspergillus* has been recently published by our group. The data from this study showed the importance of quorum sensing molecules at inhibiting biofilm formation of fungi. The molecules such as homoserine lactone (HSL), decanol, dodecanol and farnesol have been shown to affect the biofilm formation (Hogan et al., 2004, Mowat et al., 2010). Given the antibiofilm nature of these compounds, future studies will answer whether they can be used to potentiate antifungal treatment. However, limitations to these chemotherapeutic approaches include the expense and increased risk of dispersing cells to distal sites of colonisation (Kaplan, 2009).



**Figure 6.2 - Novel antifungals.** The pharmacological agents such as inhibitors of efflux pumps, HSP90 and chitinase activity interfere with different resistance mechanisms and enhance antifungal drug efficacy. In addition a hydrolytic enzyme DNase degrades the matrix eDNA, which affects the biofilm formation, biofilm stability and in consequence helps antifungals to reach their target.

#### 6.4 Disease modeling and future perspective

The majority of the studies presented in the preceding chapters used a plastic substrate in order to investigate antifungal resistance mechanisms. However, whether this relates to how the organism responds within a host is difficult to ascertain. Experiments in a chamber model were performed to evaluate efflux pump expression, but these were cumbersome and expensive experiments, and the validity of a diffusion chamber to invasive *A. fumigatus* infection can be argued. Therefore, alternative approaches to studying this pathogen are required, particularly if evaluating novel antimicrobial strategies as eluded to in the preceding section. Whilst mammalian rodent models are considered the

'gold standard' for studying fungal infections, ethical concerns, labour, time and cost all impact their utility. Therefore, cheaper models such as the invertebrate *G. mellonella* model have received greater scrutiny. In this study *G. mellonella* was used, which was shown to be easy to maintain and was unrestricted in experimental design. Previous studies have used the *G. mellonella* infection model for antifungal efficacy testing (Mesa-Arango et al., 2012, Cowen et al., 2009, Rowan et al., 2009). A caveat to its use however is that this invertebrate only has an innate immune system, which its use for detailed virulence studies. However, for the purpose of this investigation it was used primarily as a vehicle to study the effects of antifungal treatment on *A. fumigatus* infection and to study adaptive resistance responses *in vivo*. The model was shown to be effective for comparing antifungal treatments, and provided harvestable *A. fumigatus* cells in order to undertake transcriptional profiling. These latter data showed that key adaptive resistance gene transcripts could be detected during infection and in response to antifungal treatment.

Limitations to this model with respect to a 'biofilm' phenotype are obvious, i.e. do they actually form a biofilm *in vivo*? Compared to the *in vitro* models, there was little similarity with mature biofilms, whereas the early and intermediate forms were more similar due to the long hyphae observed. These differences may account for the lack of obvious gene expression patterns, but nevertheless, key transcripts were observed. In fact, inoculation with mature cells led to enhanced pathogenicity and lack of response to antifungal treatment. A further limitation of the study was that the pharmacological agents discussed above were not investigated as combinational agents along with conventional antifungal agents due to lack of time.

Given the antifungal resistance in fungal biofilm is complex and multifactorial, more than one resistance factors may be involved in the respective biofilm phase susceptibility. The efflux activity and HSP90 were shown to be associated with early phase azole resistance but the mechanism of resistance with intermediate and matured *A. fumigatus* growth is not clear. Therefore further study is needed to investigate the combination of these resistance factors in matured fungal biofilm. The inhibition of more than one resistance factors by

using combination of inhibitors may help to answer this question. In addition, as previously mentioned there are number of other efflux pumps and signaling pathways present in fungal cells and their contributions in antifungal resistance is yet to be investigated. Due to limitations of techniques, cost and time associated with this study it was only possible to investigate a small number of them. The use of other techniques such as microarray and small interfering RNA (siRNA) and screening of mutant libraries using *G. mellonella* model may be useful to investigate more resistance genes in the future. It also helps to advance our knowledge to make a cocktail of inhibitors along with existing antifungal drugs to treat fungal infections. Future studies in this area will help to test the hypothesis that supplementary agents could improve the treatment of *A. fumigatus*.

The majority of patients with invasive fungal infections experience treatment failure because of clinical resistance, which is a concept critical to the outcome of a fungal infection. The early diagnosis of invasive fungal infections itself is complicated in a clinical setting in this situation identification of resistance isolates and their corresponding resistance mechanism is more complex and there are no definitive diagnostic criteria for this. Alarmingly, the clinical relevance of antifungal resistance and tolerance has been demonstrated infrequently. Recent studies by our group suggest the use of molecular methods for early diagnosis of invasive fungal infections (McCulloch et al., 2012, McCulloch et al., 2009). The current study showed the expression profile of various resistance genes over the period of *A. fumigatus* multicellular development and use of inhibitors to identify different resistance factors. These data suggest that transcriptional qPCR targets may be an appropriate diagnostic approach. The expression profile of other resistance genes needs to be investigated before molecular assays can be developed to provide rapid diagnosis of drug resistance. The available methodology to perform *in vitro* susceptibility testing has limitations (Ghannoum et al., 1996). In general, the correlation between MICs and the clinical outcome is controversial and still under investigation. The exposure of azole drug at a sub MIC level was shown to be up regulating the expression of efflux pumps therefore either contributing towards

or inducing clinical resistance. Therefore, it is important to be aware of the importance of adequate dosing. The data from this study suggest the use of inhibitors in antifungal sensitivity testing along with qPCR assay will be helpful to know more about resistant isolates before treatment.

## 6.5 Summary

To summarise, the key findings of the research work presented in this thesis are as follows

- Efflux pump activity in *A. fumigatus*
  - Associated with susceptibility of the strain.
  - Up-regulated over the period of biofilm formation.
  - Azole treatment further up-regulates the efflux activity.
  
- Extracellular DNA in *A. fumigatus*
  - *A. fumigatus* release eDNA over the period of multicellular development.
  - It contributes to biofilm formation and stability.
  - Associated with phase dependant antifungal resistance.
  - The chitinase regulated autolysis process is one of the mechanisms of eDNA release in *A. fumigatus*.
  - The molecular chaperone HSP90 regulates eDNA release and biofilm resistance.
  
- *G. mellonella* infection model was developed in this study to evaluate the adaptive antifungal resistance in *A. fumigatus*.

Overall these findings help us to develop our understanding of the different adaptive resistance mechanisms associated with *A. fumigatus* biofilms. This will contribute to the development of improved novel treatment regimens for invasive fungal infections.

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