Aspergillosis: Interactions of *Aspergillus fumigatus* and Human Airway Cells

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AUTHOR'S DECLARATION

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any university or similar institution.

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Abstract

Aspergillus fumigatus is a filamentous fungus that colonises the lungs of immunosuppressed patients and causes aspergillosis. Despite recent medical advances, infectious diseases caused by opportunistic pathogens such as *A. fumigatus* are still one of the main causes of morbidity and mortality among immunosuppressed patients.

Gliotoxin is a mycotoxin and a secondary metabolite of *A. fumigatus*. This toxin possesses immusuppresive activity affecting cells of immune system. This research shows that human adenocarcinoma alveolar epithelial cells (A549) are highly susceptible to gliotoxin- induced cell death. Wild type strains of *A. fumigatus* cause death in these cells whereas mutant strains (gliotoxin deficient) do not. Gliotoxin induced cell death is mostly via apoptosis programmed cell death rather than necrosis. Long term incubation of A549 cells with gliotoxin-deficient strain causes morphology changes (swelling/partial damage) in these cells. This research proposes that gliotoxin induced death/ inflammation of pneumocytes by programmed apoptosis may contribute to the pathogenesis of aspergillosis, particularly invasive aspergillosis. In addition, other produced metabolites (e.g. proteases or other toxins) also contribute (to a lesser extent) to the lung tissue damage in the absence of gliotoxin.

One of the key risk factors in developing aspergillosis is corticosteroid therapy. This is due to the effect of glucocorticoids on suppressing body's defence mechanism. As hydrocortisone is present during *Aspergillus* infection, understanding the ability of the fungus to perceive this host-factor enhances the understanding of *A. fumigatus* interaction with the host within the host environment. Previous reports have shown that physiological and pharmacological concentrations of hydrocortisone enhance the growth-rate of *A. fumigatus in vitro*. The present work demonstrates the effect of hydrocortisone on cultures of *A. fumigatus* at both morphological and molecular levels. Hydrocortisone causes an early onset of sporulation and seems to play a role in regulation of haemostasis and stress response in this fungus. Expression of stress-related proteins (hsp70, catalase), proteases (e.g. serine protease, metalloprotease), and carbohydrate metabolism-related proteins increased after supplementation of A. *fumigatus* cultures with hydrocortisone. Stimulation studies investigating the effect of secondary metabolites of *A. fumigatus* on A549 cells with and without pre-treatment of the fungal cultures with hydrocortisone indicated subtle increases in the number of dead cells after hydrocortisone pre-treatment. This study shows further that hydrocortisone treatment of the lung alveolar cells enhances the binding of *A. fumigatus* spores to these cells. The obtained data suggests that presence of hydrocortisone may contribute to the survival of this pathogen within the host environment.

This study provides additional knowledge at molecular and cellular level where complications exist as a result of infection by *A. fumigatus* fungus and during treatment with glucocorticoids.

“The most beautiful experience we can have is the mysterious - the fundamental emotion which stands at the cradle of true art and true science.”

Albert Einstein (1879 - 1955)

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List of Abbreviations

11-β HSD	11-beta hydroxy steroid dehydrogenase
2D-GE	two-dimensional gel electrophoresis
A549	The human alveolar adenocarcinoma cell line
ABPA	Allergic Bronchopulmonary Aspergillosis
ACTH	adernocortocotropin hormone
AHL	acyl-homoserine lactone
AMB	amphotericin B
AMI	antibody mediated immunity
APS	ammonium persulfate
BH3	BCL-2 family with BCL-2 Homology domain 3
BLAST	Basic Local Alignment Search Tool
CCPA	chronic cavitary pulmonary aspergillosis
Cdc2 /CDK2	Cell Division Cycle 2 / Cyclin-Dependent Kinase 1
cDNA	Complementary DNA
CDP-choline	cytidine-diphospho-cholin pathway
CHAPS	3- ((3-Cholamidopropyl)dimethylammonio)-1-Propanesulfonic Acid
CRH	corticotropin releasing hormone
CT	computed tomography
Cyt c	cytochrome c
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	deoxyribose Nucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline
DTT	Dithiothreitol
EC50	effective concentration
ECM	extracellular matrix
EDTA	ethylenedaminetetraacetic acid
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
G-	Gram-negative
G+	Gram- positive
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
GR	glucocorticoid receptors
GRE	glucocorticoid response elements
HMEC-1	Human microvascular endothelial cell line
HPA System	hypothalamic-pituitary-adrenocortical
HPAECs	human pulmonary artery endothelial cells
HPLC	High- Performance liquid chromatography
hsps	heat-shock proteins
ICH	Immunocompromised host
IEF	Isoelectric focusing
IgE	Immunoglobulin E

IgG	Immunoglobulin G
IL-1, -6, -12	interleukin-1, -6, -12
INF-γ	interferon-gamma
IPA	Invasive pulmonary aspergillosis
IPG	immobilised pH gradient gel strip
L-Phe	L-phenylalanine
L-Ser	L-serine
MAPK	mitogen-activated protein kinase system
MEA	Malt Extract Agar
MHC-II	Myosin Heavy Chain Gene II
mRNA	Messenger RNA
MS	Mass Spectroscopy
NFκB	transcription factor nuclear factor κB
NRPS	NonRibosomal Peptide Synthesis
OD	Optical Density
PAF	platelet activating factor
PAMPs	Pathogen Associated Molecular Patterns
PARP	poly-ADP-ribose-polymerase
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PRRs	Pattern Recognition Receptors
PS	phosphatidylserine
qRT PCR	quantitative real time PCR
QS	Quorum sensing
REST	Relative Expression Software Tool
RNA	Ribonucleic Acid
ROS	reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute medium
SDA	Sabouraud Dextrose Agar
SP-D	surfactant protein D
T- , B-cell	T and B-lymphocytes
TBE	tris base boric acid EDTA
TEMED	tetramethylethylenediamine
TFA	Trifluoroacetic acid
Th-1	T-helper -1
TLC	Thin Layer Chromatography
TLR-2, -4	Toll-like receptors
TNF/TRAIL	Tumor Necrosis Factor/TNF Alpha Related Apoptosis-Inducing Ligand
TNF-α	Tumour Necrosis Factor -alpha
ΔCq	Delta quantification cycle
ΔΔCq	Delta Delta quantification cycle

Chapter I: Introduction and Literature Review

1 Introduction and Literature Review

1.1 Host-pathogen interactions

Early research on host-pathogen relationships during an infection were mainly focused on pathogen characteristics (Smith, 1913). Two types of pathogenicity were considered: offensive or aggressive, for microbes with the ability to produce toxins and damage the host; and defensive or passive, that reflected microbial characteristics such as capsule production allowing them to endure the host environment (Casadevall and Pirofski, 1999). Nowadays, infectious disease is defined as a "clinical manifestation of damage that results from host-pathogen interactions" (Casadevall and Pirofski, 2000). For a pathogen to become virulent it should possess some characteristics. This include the ability to penetrate the protective barriers of the host, ability to survive the defence mechanisms of the host (whether cellular or humoral), ability to find a favourable environment for growth, and ability to produce substances or conditions that cause physiological or pathological damage to the host. The damage may be mediated by either the pathogen (pathogen factors that induce cell apoptosis or necrosis) or the host (unusual immune response e.g. in rheumatic fever) or both (Casadevall and Pirofski, 1999). The extent of this however is different depending on the type of pathogen. In the setting of opportunistic pathogens (i.e. pathogens that cause disease mainly in the presence of weak immune response), the damage caused during infection can be both host or pathogen mediated. For instance during the infection with *Pneumocystis Jiroveci (carinii)* (the fungus that causes pneumonia in immunosuppressed patients) the morbidity/mortality due to infection can be significantly reduced by the use of immunosuppressant agents like glucocorticoids. This means that during this infection damage is mainly a consequence of the host inflammatory responses. In contrast, in *Candida albicans*, or *Aspergillus fumigatus* (both opportunistic fungal pathogens), secretion of metabolites and formation of hyphae are invasive and cause tissue damage in immunosuppressed individuals. This indicates that in these pathogens the host damage is primarily pathogen mediated. However, it should be

mentioned that in some types of aspergillosis (e.g. allergic bronchopulmonary aspergillosis, described later); the damage can be both pathogen and host mediated as this type of infection is caused in patients with hypersensitivity (e.g. Asthmatic patients) (Steinbach, 2008).

The host pathogen relationship has been the subject of many studies. All aim at investigating mechanisms of various infections caused by pathogenic organisms. Once a pathogen breaches the initial physical and chemical barriers of the host, it causes injuries and induces immune response and inducible effector molecules (e.g. antimicrobial peptides, stress response proteins) (Tzou *et al.*, 2002). Given this, it is not surprising that majority of the studies have focused on host immune responses as part of the host pathogen interactions (George *et al.*, 2003). For instance during infection with *Pseudomonas aeroginosa* (which causes pneumonia and sepsis, particularly in patients with cyctic fibrosis), interaction of this bacteria/its biofilm with polymorphonuclear leukocytes (innate immune response) has been investigated in several studies (Jesaitis *et al.*, 2003; Brinkmann *et al.*, 2004; van Gennip *et al.*, 2012). Similar studies have also been carried out in other pathogens including human fungal pathogen *A. fumigatus* (Philippe *et al.*, 2003; Bruns, 2010; McCormick *et al.*, 2010).

Nonetheless, other environmental factors of the host (i.e. the microenvironment that pathogens encounter) including host proteins and hormones have also come into consideration in other recent studies. In particular, effect of these factors on altering the behaviour (e.g. virulence gene expression, resistance) of pathogens inside the host has been investigated (Sperandio *et al.*, 2003; Hughes and Sperandio, 2008; Kim *et al.*, 2010).

The main focus of this research project is to study interactions of *Aspergillus fumigatus* (opportunistic fungal pathogen) and human lung epithelial cells, through the produced metabolites and the environmental factors, during invasive aspergillosis.

1.2 An introduction to epidemiology and socioeconomic factors associated with aspergillosis

Despite all advances in medicine particularly over the past decades, infectious diseases are emerging as one of the main causes of morbidity and mortality in patients. Among them, are invasive and chronic fungal infections that have turned into a difficult clinical challenge; with *Aspergillus fumigatus* one of the leaders causing aspergillosis. Aspergillosis is the name for a series of infectious diseases caused mainly by *A. fumigatus*.

In earlier decades, around 1939, scientists considered aspergillosis in humans as a very rare infection with little clinical importance (Latgé, 1999; Steinbach, 2009). Advances in medical treatments including increased use of more aggressive therapies such as chemotherapy in cancer patients and organ transplantations in patients suffering from organ dysfunction has increased survival of patients in general. However these patients live in an immunosuppressed state. Additionally, the expanded use of aggressive treatments for a variety of diseases such as inflammatory and interstitial lung diseases and rheumatic diseases has added to the number of patients in an immunosuppressed state (Steinbach, 2009; Kauffman and Nicolasora, 2010; Henriet *et al.*, 2013). *Aspergillus* is a ubiquitous fungus in nature, with airborne conidia, found in soil, water, air (including hospital air), decaying matter, dust, and food. Considering the ubiquitous nature of this fungus, lack of efficient innate and acquired immunity in the immunosuppressed patients makes it easy for *Aspergillus* to enter (mostly via inhalation of the conidia) and survive in the human body. Study of the epidemiology of aspergillosis over the past decades shows a notable steady increase in the number of aspergillosis cases in the autopsy of cancer patients in hospitals and among living patients suffering from leukaemia as well as transplant patients. It is reported that 10-15% of solid organ or allogenic hematopoietic stem cell transplants and of leukemic patients develop this

infection, leading to 45 to 90% mortality rate as a result of aspergillosis (Lin *et al.*, 2001).

Treating aspergillosis started by the use of Amphotericin B, developed in 1950's, which is still used as an effective antifungal agent. However, due to problems associated with side effects of this medicine, there has been an increase in the development and use of other drugs such as azols. Combined drug therapy has also improved patients' response to the disease. However, there is a need for better options to eliminate the hindering factors such as drug resistance and lack of highly sensitive, specialised, and standardized diagnostic tests for this infection(Steinbach, 2009; Garbati *et al.*, 2012). Therefore, an extensive research in various areas of aspergillosis is on-going and the need to understand the mechanism of this infection and comprehending the host pathogen relationship is essential.

This chapter focuses on different aspects related to aspergillosis and *Aspergillus fumigatus* in light of the main theme of this research project which is on the interplay of *Aspergillus fumigatus* and human airway epithelium during invasive aspergillosis.

1.3 An overview of *Aspergillus* species

Aspergillus species are Ascomycetous fungi belonging to the subkingdom of Dikarya where the majority of the known fungal species (>95,000) belong. The Dikarya subkingdom contains the phyla Ascomycota and Basidiomycota (Spatafora and Robbertse, 2010). Ascomycota are reported as the largest phylum of fungi with more than 63,000 spices (Kirk *et al.*, 2008). They include three subphyla: Taphrinomycotina, Saccharomycotina, and Pezizomycotina. The latter is the one referred to as filamentous fungi in fungal biology and includes 10 classes, 55 orders, and more than 60,000 species including some well-studied organisms such as *Neurospora, Penicilium* and *Aspergillus* (Spatafora and Robbertse, 2010).

Aspergillus is a filamentous fungus. A main characteristic for its identification is the presence of hypha. Most cellular activities such as colonization, secretion of hydrolytic enzymes, nutrient assimilation, recognition of environmental signals and morphogenesis are through hyphal growth. Hyphal cytoplasm contains most of the organelles and subcellular inclusions found in other heterotrophic eukaryotic organisms, such as nuclei, mitochondria and vacuoles. (Bennett *et al.*, 2010; Roberson *et al.*, 2010). Similar to plants, fungi, including *Aspergillus* species, possess polar growth and cell wall; although physiologically they are more similar to animals than plants (Bennett *et al.*, 2010).

The cell wall of *Aspergillus* species is a dynamic structure with its composition changing during different stages of fungal growth. At conidial stage, the cell wall consisted of two layers: an inner layer and a pigmented outer layer. At the surface of the outer layer of conidia are rodlet hydrophobins that give them their hydrophobicity. Once conidia start swelling and germinating, the cell wall structure loses its hydrophobic layer and a layer of β-1, 3 glucan emerges on the cell surface, (in pathogenic fungi e.g. *A. fumigatus*, this layer is recognized by dectin-1 receptor and/or monoclonal antibodies of the host). During this stage a new inner layer is

also formed in the cell wall of the new hyphae (Mouyana and Fontaine, 2009). Cell wall of *Aspergillus* species mainly consist of polysaccharides; a network of glucan and chitin micro-fibrils that protect fungal cells from osmotic pressure. It also contains mannoproteins (e.g. galactomannam or mannan linked to proteins) that are important for cell wall porosity, antigenicity, and fungal virulence. The main backbone of the fungal cell wall is the β-1,3 glucan polysaccharide (a polymer of glucose) with β-1,6 links at branches. Other polysaccharides such as chitin (a polymer of N-acetylglucosamine), galactomannam, β-1,3/1,4 glucan, and galactosaminogalactan (a polymer of N-acetyl-galactosamine and galactopyranose) are cross-linked to the main β-glucan structure at side chains. Fungal cell wall plays an important role in fungal development, survival, and proliferation (Mouyana and Fontaine, 2009; Osherov and Yarden, 2010).

1.3.1 Morphology

In terms of morphology, under microscope, *Aspergillus* species have a long stalk called conidiophore that usually rises directly from submerged hyphae, or as short branches from aerial hyphae, and ends in a swollen part called vesicle (Figure 1.1.A). On the surface of the vesicle are a number of cells called phialides. It is reported that continual mitotic division in the nucleus of phialides generates a chain of asexual spores called conidia which are hydrophobic and easily dispersed by air (Baker and Bennett, 2008; Samson *et al.*, 2009). On agar plate (e.g. potato dextrose agar (PDA), Czapek agar (CZA), Sabouraud dextrose agar (SDA), and malt extract agar (MEA)) *Aspergillus* species usually produce coloured colonies (depending on the species, regardless of the media used the colour is different). *Aspergillus fumigatus* for example, generates blue-green colonies (Figure 1.1.B) on SDA, CZA, and MEA plates. Agar colonies are comprised of a compact network of conidiophores interacted with aerial hyphae (Samson *et al.*, 2009).

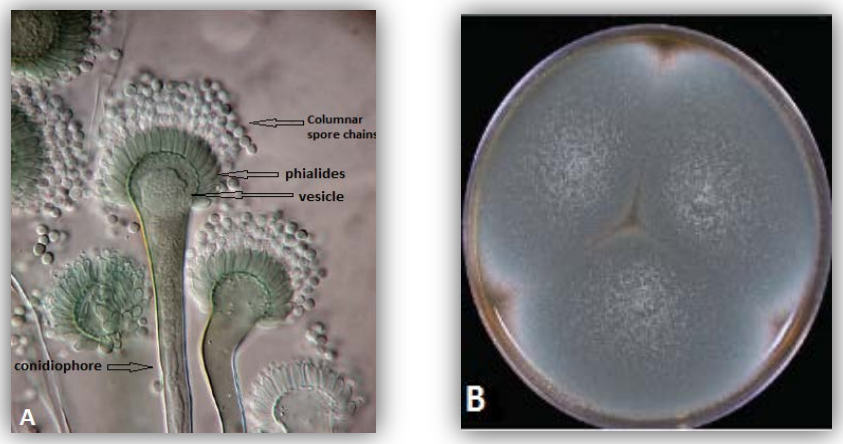


Figure 1.1

A- Structural morphology of *Aspergillus fumigatus* under microscope**.** Conidiophores are relatively short, smooth-walled and approximately 300µm in length. Conidiophore structure ends in round shaped vesicle with a diameter of approximately 20-30µm. From the surface of the vesicle head phialides arise which are responsible for generating chains of spores that appear columnar until dispersed. Conidia of *A. fumigatus* are very small in size (2-3 µm diameters) and are easily aerosolized and dispersed. (Picture adopted from (Samson *et al.*, 2007) and modified. **B-** *A. fumigatus* colony growing on agar plate, picture adopted from (Samson *et al.*, 2007).

1.3.2 Secondary metabolites

Aspergillus species are ubiquitous in the environment due to their ability to grow on a variety of substrates in different environmental niches under various temperatures moisture contrnts, and having high sporulation capacity (Latgé, 2001). They are able to secrete various acids and enzymes into their surrounding environment which break down polymeric molecules and aid their absorption into the cell (Bennett *et al.*, 2010). Moreover, as a result of their metabolic capacity they are capable of secreting several low molecular weight biologically active chemical compounds called secondary metabolites. Secondary metabolites are known to be involved in the ecological signalling of *Aspergillus* species (Baker and Bennett, 2008).

Secondary metabolites are usually produced after the primary growth-phase has ended. Their absence does not result in significant changes in the standard metabolism or any observable phenotypic changes in the producing organism. Nonetheless, these compounds are considered important in the general biology of fungi and confer selective advantages to the producing fungi (Cramer *et al.*, 2009; Garvey and Keller, 2010). In pathogenic fungi for instance, they can play a role as virulence factor. One main characteristic of these compounds is that the genes encoding them are mostly clustered together in the genome (Keller *et al.*, 2005; Garvey and Keller, 2010). Secondary metabolites include variety of compounds such as antibiotics, mycotoxins, immunosuppressant agents, and cholesterol lowering agents (Baker and Bennett, 2008). Based on the enzymes involved in their biosynthesis, secondary metabolites are classified into different classes of polyketides (e.g. aflatoxin produced by *A. flavus* and lovastatin synthesised by *A. terreus*), non-ribosomal peptides (e.g. gliotoxin produced by *A. fumigatus* and siderophore ferricrocin by *A. nidulans*), terpenes (e.g. aristolochenes by *A. terreus*), and indole alkaloids (e.g. fumigaclavines and fumitremorgens both produced by *A. fumigatus*) (Keller *et al.*, 2005; Garvey and Keller, 2010).

Both *A. terreus and A. fumigatus* are known to secrete a variety of toxins and enzymes during their growth, many of which seem to have a putative role in virulence of these fungi. *A. terreus* produces a range of metabolites such as terreic acid (anti-tumour activity), tremorgenic mycotoxins (effect on the central nervous system), gliotoxin (a sulphur containing antibiotic with immunosuppressive activity), citreoviridin (an inhibitor of mitochondrial ATPase), citrinin (nephrotoxin), butyrolactone I (inhibitor of eukaryotic cyclin-dependent kinases) (Linnett, 1978; Pahl *et al.*, 1996a; Schimmel *et al.*, 1998; Pugliese *et al.*, 1999). *A. fumigatus* also produces variety of toxins and enzymes including ribonucleases (responsible for host cell-death), haemolyisn (causes lysis of red blood cell), fumitremorgin and verruculogen (tremorgenic mycotoxins that cause cell cycle arrest and tremor), fumigaclavine and festuclavine (toxic/ tremorgen, present on the conidia), fumagillin (angiogenesis inhibitor, anticancer agent), helvolic acid (steroidal antibiotic) and gliotoxin (toxic, immunosuppressive activity), serine protease, aspartic protease, metalloprotease, and dipeptidylpeptidases (enhancing lung matrix colonization and degrading humoral factors), catalases and superoxide dismutases (antioxidant), and phospholipases (epithelial damage)(Latgé, 2001; Cramer *et al.*, 2009).

Among different enzymes secreted by *A. fumigatus*, proteolytic enzymes have been studied extensively as potential virulence factors. These enzymes consist of two main groups of endo- (cleave peptide bonds internally in polypeptides) and exo- proteases (cleave peptide bonds at N or C terminal of polypeptides) and are classified on the basis of their catalytic mechanism and their active site (Monod *et al.*, 2005; Monod *et al.*, 2009). There are 252,204 identified proteases in *A. fumigatus* for example aspartic endopeptidase, metalloproteases, carboxypeptidase, peptidase, aminopeptidase, dipeptidyl peptidase, serine protease, and autophagic serine protease Alp (Monod *et al.*, 2009; Rawlings *et al.*, 2012). It has been suggested that proteolytic activity plays a role in tissue invasion during invasive aspergillosis. Several *in vivo* and *in vitro* experiments

have generated supporting evidence for this statement. For instance, proteases have been shown, by enzyme immunoassay, to be secreted *in vivo* via demonstrating protease specific antibodies in the sera of patients (Reichard *et al.*, 1990b). In other studies sera from patients with Aspergillosis contained specific antibodies for Mep (metalloprotease), Pep (prolidase), and DppV (dopeptidyle peptidase) (Monod *et al.*, 1993; Moutaouakil *et al.*, 1993b; Beauvais *et al.*, 1997). In a study, Kogan and colleagues showed the ability of serine protease Alp to disrupt the actin fibre cytoskeleton of lung pneumocytes *in vitro* (Kogan *et al.*, 2004). It has been suggested that host factors may play a role in induction of protease production during aspergillosis (Monod *et al.*, 2009).

Sequence analysis of the *Aspergillus* genome including *A. fumigatus* has revealed a large number of secondary metabolite gene clusters in these fungi. Among them gliotoxin synthesised by *A. fumigatus* and butyrolactone I produced mainly by *A. terreus* have been subject of some experimental analysis in this research project and are discussed in here.

1.3.2.1 Gliotoxin

Amongst secondary metabolites secreted by different strains of *Aspergillus,* predominantly *A. fumigatus,* gliotoxin has been the subject of many investigations for its role as a putative virulence factor. Gliotoxin is a mycotoxin, a secondary metabolite with a small molecular size (Mw=326Da). It is a non-ribosomal dipeptide from the family of epipolythidioxopiperazine. Gliotoxin is a heterocyclic compound, an alkaloid, containing nitrogen and an intramolecular disulphide bridge (Figure 1.2). Gliotoxin is believed to be a redox active toxin acting through production of reactive oxygen species and formation of mixed disulphides with proteins having accessible thiol groups (Garvey and Keller, 2010). Various reports indicate the immunosuppressive activity of gliotoxin including inhibition of superoxide release, inhibition of the migration of leucocytes, apoptosis in macrophages, inhibition of cytokine

release by leukocytes, and mast cell degranulation, inhibition of the of the NF-κB activation, inhibition of neutrophil and macrophage oxidative killing and production of reactive oxygen species by macrophages (Kamei and Watanabe, 2005; Bok *et al.*, 2006; Kupfahl *et al.*, 2006; Kwon-Chung and Sugui, 2009).

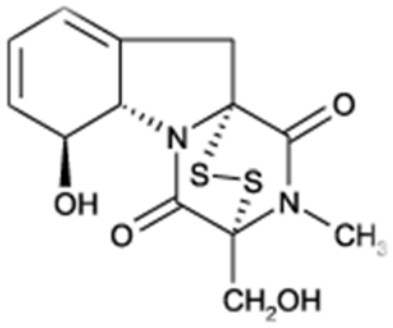


Figure 1.2
Gliotoxin, A non-ribosomal dipeptide secreted by *A. fumigatus* and a member of epipolythidioxopiperazine family of compounds. Picture adopted from Fermentek biotechnology (FERMENTEK, 2013).

Gliotoxin is among the non-ribosomal class of secondary metabolites in *Aspergillus* and its gene expression is proposed to be regulated by a putative gliotoxin biosynthetic gene cluster containing 13 genes (Figure 1.3) including the pathway specific zinc finger (Zn_2-Cys_6 zinc finger structure) transcription factor *gliZ*, the non-ribosomal peptide synthase (NRPS) *gliP*, glutathione-S-transferse (*gliG*), cytochrome P450 monooxygenase (*gliC*), and gliotoxin oxidoreductase responsible for disulphide bridge formation (*gliT*) (Bok *et al.*, 2006; Garvey and Keller, 2010; Scharf *et al.*, 2012). Transcription of the genes in gliotoxin gene cluster is reported to be affected by various factors such as culture medium composition, pH, temperature, aeration and agitation of the culture (Cramer *et al.*, 2006; Scharf *et al.*, 2012). Among all the genes involved in the gliotoxin biosynthesis pathway *gliP* and *gliZ* have been reported to play critical roles in gliotoxin biosynthesis and their deletion has resulted in the loss

of gliotoxin production in *A. fumigatus* clinical isolates (Bok *et al.*, 2006; Cramer *et al.*, 2006).

gliP is the largest gene in gliotoxin gene cluster encoding a multi-domain enzyme NRPS which is responsible for catalysing the enzymatic reaction resulting in formation of dipeptide and cyclization (the diketopiperazine frame), from L-Phe and L-Ser amino acids, in the first step of the gliotoxin biosynthesis pathway (Garvey and Keller, 2010; Scharf *et al.*, 2012). Each domain in this enzyme contains several regions that allow recognition, activation and covalent binding of the domain specific amino acids and eventually leads to the formation of the peptide bonds between the joined amino acids (Keller *et al.*, 2005).

gliZ, is the transcription factor with a zinc finger structure and regulation of the gliotoxin gene cluster is reported to be controlled by this gene (Bok *et al.*, 2006). As mentioned earlier the expression of the secondary metabolite gene clusters is affected by environmental conditions such as pH, carbon and nitrogen sources. These types of transcription factors (Zn_2-Cys_6 zinc finger structure), in secondary metabolite pathways, positively or negatively regulate the gene expression. This is done by responding to the environmental signals via their zinc finger structure, and eventually regulate the response of the secondary metabolite pathways with regards to the demands of general cellular metabolism (Keller *et al.*, 2005). They encode DNA-binding proteins that are able to recognize and bind to palindromic sequences in the promoter of the biosynthetic genes (Keller *et al.*, 2005).

As a final point, with regards to the pathogenic strains of *Aspergillus* such as *A. fumigatus* and their pathobiology, there are accumulating reports indicating the role of gliotoxin in supporting the colonization of this fungus in the host through inactivation of the host immune system and other effects such as inhibition of angiogenesis. More research, however, is required to clarify the role of this secondary metabolite in invasive aspergillosis.

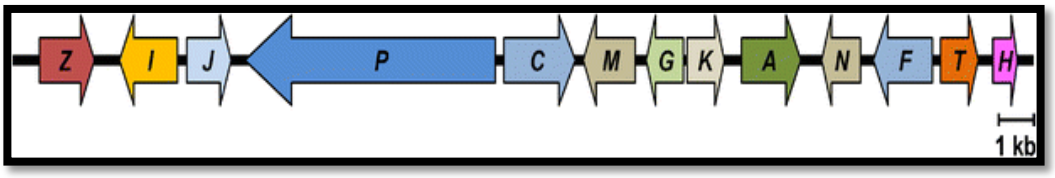


Figure 1.3
The putative gliotoxin biosynthesis gene cluster. It is consisted of 13 genes including: the regulatory zinc finger transcription factor (*gliZ*), 1-aminocyclopropane-1carboxylate synthase (*gliI*), dipeptidase (*gliJ*), non-ribosomal peptide synthase (*glip*), cytochrome p450 monooxygenases (*gliC* and *gliF*), O-methyltransferase (*gliM*), glutathione-S-transferase (*gliG*), hypothetical proteins (*glik* and *gliH*), transporter (*gliA*), methyltrasnferase (*gliN*), gliotoxin oxidoreductase(*gliT*). The figure is taken from (Scharf *et al.*, 2012).

1.3.2.2 Butyrolactone I

Butyrolactone I (Figure 1.4), (α-oxo-β-(*p*-hydroxyphenyl)-ϒ-(*p*-hydroxy-m-3, 3-dimethylallyl-benzyl)-ϒ-methoxycarbomy-ϒ-butyrolactone), is a fungal secondary metabolite secreted by *Aspergillus* species, mainly by *A. terreus* strains. Similar to other fungal secondary metabolites, Butyrolactone I also has a low molecular weight (MW=424 Da). Butyrolactone I is an inhibitor of the cell cycle by effecting the Cdc2 and CDK2 kinases, hence in earlier studies of this compound, it has been suggested to use Butyrolactone I to study the functions of Cdc2 and CDK2 kinases (Suzuki *et al.*, 1999). It is also reported that Butyrolactone I acts as a self-regulating factor in some bacteria by influencing some important functions such as antibiotic production, biofilm formation, virulence factor production, bioluminescence, and conjugal plasmid transfer. Similarly, studies of the effect of this compound on *A. terreus* have revealed that this compound is inducing morphological changes as well as enhancing the production of some other secondary metabolites in the culture of this fungus. (Schimmel *et al.*, 1998) .

Hence a role in the cell to cell communication has been suggested for this compound (Schimmel *et al.*, 1998; Raina, 2008).

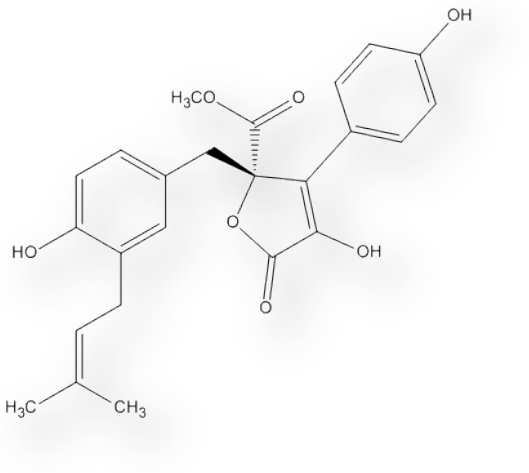


Figure 1.4
Molecular structure of Butyrolactone I, a lactone containing molecule with a molecular weight of 424Da, produced by *A. terreus* strains. Picture adopted from (BioVision)

1.3.3 Aspergillus infection

Several *Aspergillus* species have been identified as industrially important due to their ability to produce valuable primary and secondary metabolites, *A. niger* for instance is used for the production of citric acid and gluconic acid, *A. terreus* for the production of both itaconic acid and lovastatin, and *A. oryzae* is used for the production of kojic acid. However, some strains of *Aspergillus* are recognized as important human pathogens (Bennett, 2010). As mentioned earlier *Aspergillus* species are ubiquitous in nature. Ability of *Aspergillus* species, specially pathogenic strains, to grow under various environmental conditions with numerous stress factors such as nutritional limitations, high temperature and low pH conditions, has enabled this fungus to develop many metabolic and physiological properties that help it to adapt easily to the mammalian host (Latgé, 1999).

Moreover their capability to generate large number of conidia helps these fungi to proliferate readily. The generated conidia are very small in size (2-3μm) and are covered with hydrophobic proteins (Rod A and Rod B) and pigment. These properties help them to easily aerosolise and consequently be inhaled by human and animals and reach the lung. The outcome of this encounter, though, depends on the immunological status of the host; Figure 1.5 shows the normal life cycle of *Aspergillus* in the environment and its introduction into the host (Rhodes and Askew, 2010).

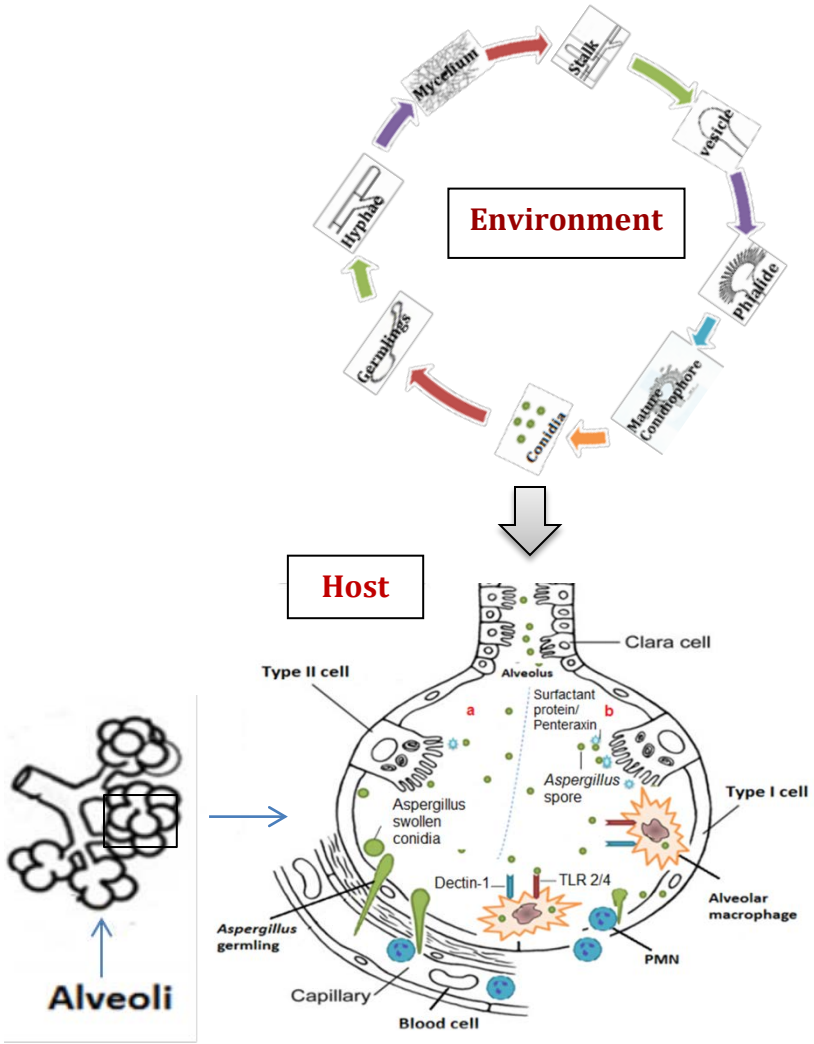


Figure 1.5

Life cycle and pathogenesis of *Aspergillus fumigatus*. In the environment, *A. fumigatus* proliferates by liberating asexual conidia into the atmosphere. Once conidia face a suitable environment they start their life cycle by germination followed by formation of hyphae and development of a mass of hyphae or mycelium. In response to environmental stimuli some hyphal strands begin an asexual development containing a series of morphological changes which starts with formation of aerial hyphae or stalk from a foot cell. Stalk tip will then swell and form vesicle (conidiophore vesicle). Once the vesicle formed a single layer of cells called phialides form on its surface. Chains of conidia subsequently develop from phialides. Conidia are very small in size (2-3µm), hence after inhalation they easily reach the alveoli. In healthy individuals (b) *A. fumigatus* conidia are effectively cleared by alveolar macrophages but in an immunosuppressed host (a) they geminate into invasive hyphae that penetrate lung epithelial cells and eventually reach the vasculature and migrate to other body sites. Diagram adapted from (Rhodes and Askew, 2010) with major modifications.

1.4 Aspergillosis

Aspergillus infection results in a range of diseases, depending on the status of the host. Figure 1.6 demonstrates various clinical conditions/infections caused in different hosts by this fungi (Soubani and Chandrasekar, 2002). Similar to other diseases, *Aspergillus* infection emerges as a consequence of damage to the host. The cause of damage however can either be the fungus or the host in response to the fungus. The first explanation of human aspergillosis was reported by Virchow in 1856 reviewed in (Steinbach, 2008). Since then many others have expressed the incidence of *Aspergillus* infection in human, other animals and birds. Aspergillosis has now emerged as the most frequently reported infection by opportunistic filamentous fungi (Rhodes and Askew, 2010). Majority of Aspergillosis cases, around 80%, are pulmonary aspergillosis and are predominantly caused by *Aspergillus fumigatus*. However, there are evidences of other *Aspergillus* species involved in causing infection. *Aspergillus flavus* for example is reported as the second leading cause of aspergillosis with around 15-20% of cases of aspergillosis (Hedayati *et al.*, 2007; Krishnan *et al.*, 2009). *Aspergillus terreus* is also among the frequent causes of aspergillosis after the two strains mentioned earlier with reported *in-vitro* resistance to antifungal treatments such as amphotericin B (Iwen *et al.*, 1998; Walsh *et al.*, 2003; Nucci and Marr, 2005). Some other strains of *Aspergillus* are also reported among clinical isolates from patients with Aspergillosis including *Aspergillus niger*, *Aspergillus ustus*, *Aspergillus lentulus*, *Aspergillus nidulance,* and *Aspergillus glaucus*. The latter strains have been reported also to demonstrate resistance to antifungals; nonetheless, they are among the rare and unusual causes of infection (Verweij *et al.*, 1999; Walsh *et al.*, 2008; Person *et al.*, 2010). The most common types of infection caused by *A. fumigatus* are Allergic bronchopulmonary aspergillosis (ABPA), Aspergilloma, and Invasive pulmonary aspergillosis (IPA).

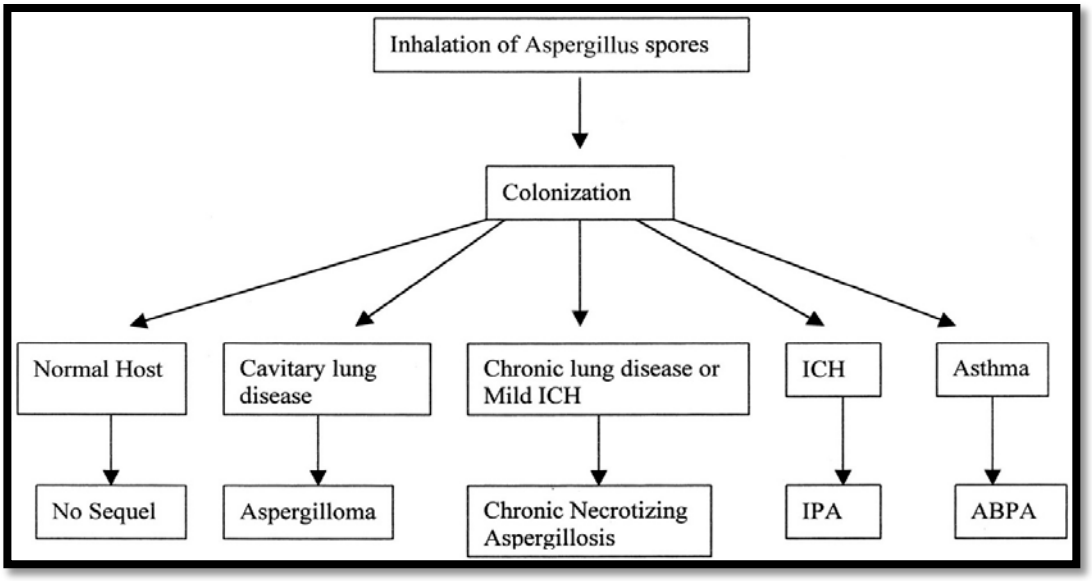


Figure 1.6
The spectrum of clinical conditions resulting from inhalation of *Aspergillus fumigatus* spores. ICH: Immunocompromised host; IPA: Invasive pulmonary aspergillosis; ABPA: Allergic bronchopulmonary aspergillosis. Figure adopted from (Soubani and Chandrasekar, 2002).

1.4.1 Immune mediated disease: Allergic bronchopulmonary aspergillosis (ABPA) and allergic rhinitis

Both allergic bronchopulmonary aspergillosis (ABPA) and allergic rhinitis are types of infection that result from excessive immunological response in the host and for this reason they are classified among the hypersensitivity disorders caused predominantly by *A. fumigatus*. Throughout these infections the damage to the host is caused as a result of responses from the host immune system to *Aspergillus* antigens or antigens on metabolites released by this fungus (Tillie-Leblond and Tonnel, 2005; Rhodes and Askew, 2010). Most of the patients who develop ABPA have asthma or cystic fibrosis. A common characteristic of these diseases is compromised mucociliary clearance ability (Moss, 2009). These patients are mostly immunocompetent with no impaired activity of polymorphonulcear leucocytes and alveolar macrophages (Tillie-Leblond and Tonnel, 2005). After inhalation of the conidia, the fungus germinates to hyphae

in the mucus or attaches to the altered epithelium of the respiratory tract. However these germinated conidia are unable to penetrate the lung tissue (Moss, 2009; Rhodes and Askew, 2010). Hence the presence of fugal antigens in the mucus is recognised by immune system. This is then followed by immune responses (antibody and cell mediated response) and if continued over time it will result in formation of granuloma (i.e. collection/accumulation of macrophages) which if undiagnosed will result in damage to the lung tissue and respiration difficulties. (Soubani and Chandrasekar, 2002; Rhodes and Askew, 2010).

Allergic rhinitis is also a hypersensitivity reaction that is initiated by exposure to *Aspergillus* conidia and results in hay fever like symptoms (Rhodes and Askew, 2010).

1.4.1.1 Diagnosis of ABPA

Clinical, radiographic, and laboratory methods are employed to diagnose ABPA, however it is not always successful due to the lack of standardised methods (Rhodes and Askew, 2010). Presence of eosinophils and Immunoglobulin E (IgE) in large numbers are considered as characteristics of ABPA diagnosis (Tillie-Leblond and Tonnel, 2005; Denning, 2009). Culturing sputum plugs and serological assays like skin testing or *in vitro* immunoassays of *A. fumigatus* extracts have been used for diagnosis of ABPA for a long time, however efficiency is restricted due to inconsistency of the antigenic content and lack of standardization. Another method is the measurement of IgG, IgE and IgA antibodies in response to *A. fumigatus* which is also a diagnostic difficulty especially in the case of IgG detection, because of the lack of standardized *Aspergillus* antigens and limited sensitivity and specificity. During acute stages of illness, ABPA can be described by pulmonary infiltrate on chest radiographs as well. Chest computed tomography (CT) is also used as a more sensitive

method. However it is not completely specific for ABPA due to underlying lung complications specially in cystic fibrosis patients(Moss, 2009).

1.4.1.2 Treatment of ABPA

Early treatment trials of ABPA using antifungals (polyene/azole) have not shown any improvements in the patient's conditions compared to treatment with steroids alone. Therefore, subsequent trials consisted of using antifungals, mainly itraconazol (an oral antifungal), together with corticosteroids which resulted in an improvement of patient conditions in most cases with a more efficient effect of antifungals (Rhodes and Askew, 2010). However, these patients are considered to be followed closely for there is a risk of recurrent ABPA (Patterson and Strek, 2010; Proesmans *et al.*, 2010).

1.4.2 Chronic aspergillosis: Aspergilloma

Fungal ball or Aspergilloma is a chronic type of aspergillosis that involves colonization of *A. fumigatus* in pre-existing lung cavities which are formed because of different pulmonary disorders such as tuberculosis, pulmonary sarcoidosis, or cancer (specially lung carcinoma) (Soubani and Chandrasekar, 2002; Rhodes and Askew, 2010). The disease was initially described, for the first time, by Bennett in 1842. He reported the presence of cryptogamic plants (plants that reproduce using spores) that are able to germinate in live human and animal tissues (Bennett, 1842; Denning, 2009). The *Aspergillus* colony lives in the pulmonary cavity either freely or attached to the cavity wall. This type of infection generally occurs without any specific symptoms initially, until the cavity expands over time and erodes into blood vessels, in which case it is called chronic cavitary pulmonary aspergillosis (CCPA) and result in symptoms such as blood expectoration and blood contained sputum from bronchi or lungs (haemoptysis). It is however reported that at this stage (CCPA), 50% of patients radiography does not show a visible aspergilloma (Denning, 2009; Rhodes and Askew, 2010).

Chronic invasive *Aspergillus* rhinosinusitis, also called sinus aspergilloma, is another type of chronic aspergillosis that involves formation of a fungal ball or aspergilloma in the sinus. This type of infection is not very common and is frequently caused by *A. flavus* rather than other species.

Other types of chronic Aspergillosis have also been reported such as chronic fibrosing pulmonary aspergillosis, *Aspergillus* bronchitis, and *Aspergillus* otitis. There are no specific clinical signs of chronic pulmonary aspergillosis, but patients normally suffer from weight loss, chronic cough, hemoptysis, fatigue, and shortness of breath.

1.4.2.1 Diagnosis of Aspergilloma

Radiological examinations such as chest radiograph and chest computed tomography (CT) are among the main diagnostic techniques used for aspergilloma, which reveals one or more than one cavity with (in nearly all aspergilloma cases) or without (in many of the CCPA cases) the presence of fungal ball (especially in CCPA). Other clinical diagnostic methods include bronchoscopy, sputum culture, and serological assays (e.g. demonstration of precipitating *Aspergillus* antibodies in serum). The serological and microbiological tests, however, tend to fail to diagnose the infection in many of cases (Kreymborg *et al.*, 2006; Denning, 2009).

1.4.2.2 Treatment of Aspergilloma

Surgery is often the main treatment method for Aspergilloma due to insufficient drug penetration into the lung cavities (Rhodes and Askew, 2010). But patients are recommended to be treated pre or post operation with antifungals (Denning, 2009)

1.4.3 Invasive disease

The first report of invasive aspergillosis in human was in 1953 (reviewed in Baker and Bennett, 2008), since then there has been a dramatic increase in the number of patients suffering from the disease (Baker and Bennett, 2008). Currently invasive aspergillosis is the most common and the main cause of death due to *Aspergillus* infection with an overall mortality rate of 40-90% in immunocompromised patients (Latgé, 1999; Muñoz *et al.*, 2006; Pfeiffer *et al.*, 2006; Zaas and Alexander, 2009). The incidence of invasive aspergillosis increases with increase in the levels of immune deficiency. Disease usually begins as pulmonary infection of immunosuppressed hosts, specially patients with prolonged neutropenia, receiving high doses of corticosteroid therapy, suffering from hematological malignancies (e.g. leukaemia), and following hematopoietic stem cell or solid organ transplantation (Herbrecht *et al.*, 2002; Marr *et al.*, 2002; Upton *et al.*, 2007; Wingard *et al.*, 2008; Zaas and Alexander, 2009; Rhodes and Askew, 2010). Other risk factors for developing invasive aspergillosis including broad spectrum antibiotic therapy, hereditary conditions such as chronic granulomatous diseases, cytomegalovirous and AIDS infection have also been reported (Rhodes and Askew, 2010). In immunocompetent people after inhalation of *A. fumigatus* conidia, it will be efficiently ingested by bronchoalveolar macrophages and if conidia germinate into hyphae polymorphoneuclear leukocytes (PMNs) destroy the hyphae. On the contrary, in immuncompromised patients the deficient innate immunity is not able to destroy the inhaled conidia or prevent them from germinating. As a result conidia can easily germinate and penetrate into the lung epithelium and reach the endothelial cells. Upon encounter with a blood vessel the hyphae can penetrate the vessel walls and access the vasculature (Figure 1.5). This process is called angioinvasion. Once in the blood vessel, pieces of fungi can also be seperated and circulate throughout the circulatory system. While in circulatin the infection can spread into any other organ, often brain and skin. This phase is called the disseminated stage of the disease and is associated with very high

mortality rates of up to 80-90% (Muñoz *et al.*, 2006; Upton *et al.*, 2007; Wingard *et al.*, 2008; Rhodes and Askew, 2010).

1.4.3.1 Diagnosis of invasive aspergillosis

Invasive aspergillosis is difficult to diagnose since the clinical and radiological signs are non-specific (Muñoz *et al.*, 2006; Pfeiffer *et al.*, 2006; Rhodes and Askew, 2010). Diagnosis usually involves a combination of clinical findings in patients with risk factors including culture and histology of the infected tissue or specimens (sputum and/or bronchalveolar lavage), radiographic examination and computed tomography, PCR methods, and serological assays for detection of *Aspergillus* antibodies and antigens. Galactomannan, is a polysaccharide in the *A. fumigatus* cell wall, released by this fungus. Detection of the circulating galactomannan in patients is considered as a relatively specific marker of invasive aspergillosis (Pfeiffer *et al.*, 2006; Rhodes and Askew, 2010). Similarly (1-3)-β-glucan is another cell wall component of *Aspergillus* used in the diagnosis of invasive aspergillosis. Employing PCR methods for detection of genomic fungal DNA is a relatively recent diagnostic method and helps timely detection of *A. fumigatus* infection. However, despite all these tools, diagnosis of invasive aspergillosis remains a challenge and is not always accurate and timely, especially in early stages, due to non-specific symptoms and false negative test outcomes. For example many cases of invasive aspergillosis without a positive culture remain undiagnosed (Pfeiffer *et al.*, 2006). Overall, despite all advances in diagnostic and therapeutic techniques the mortality rate in invasive aspergillosis patients is high, particularly in patients with prolonged recovery of immune system (Zaas and Alexander, 2009).

1.4.3.2 Treatment of invasive aspergillosis

Antifungal administration is the main treatment of invasive aspergillosis. An early/timely initiation of antifungal therapy plays and important role in the survival rate of patients suffering from this infection. Three classes of antifungals have been approved for the treatment of invasive aspergillosis including polyenes, azoles, and echinocandins (Walsh *et al.*, 2008; Rhodes and Askew, 2010).

Polyenes include amphotericin B (AMB) family and its lipid associated formulations such as liposomal amphotericin B. AMB is a naturally occurring antifungal initially isolated from *Streptomyces nodosus* in 1950s (Groll and Walsh, 2009). This group of antifungals work by binding to ergosterol in the cell membrane of the *Aspergillus spp.* The result of this binding is the generation of trans-membrane channels causing efflux/leakage of protons and other monovalent cations from the cell, changes in membrane potential and eventually resulting in cell-death (Groll and Walsh, 2009; Rhodes and Askew, 2010). The amphotericin family of drugs have been used for a long time in the prevention and treatment of invasive aspergillosis due to their broad spectrum, potent dose dependent activity on animal models (that has been shown in various studies), and abundant clinical documentations on their efficacy. However, their usage and effectiveness is limited because of their nephrotoxicity, even in lipid containing formulations (which are developed as a less toxic alternative to AMB). Their toxicity is mostly due to the binding of the drug to mammalian cholesterol in the membrane (Groll and Walsh, 2009; Kontoyiannis, 2009; Rhodes and Askew, 2010).

The azole family of antifungals are widely used for treatment of aspergillosis. They act by inhibiting the fungal cytochrome P450 dependent enzyme, 14-alpha-lanosterol demethylase in the ergosterol biosynthetic pathway. This

enzyme is important for ergosterol biosynthesis (Herbrecht and Nivoix, 2009; Rhodes and Askew, 2010). Azoles include two groups of imidazoles and triazoles. Triazoles have higher affinity to fungal CYP450 compared to the mammalian one and therefore are mostly used for treatment of fungal infections (Herbrecht and Nivoix, 2009). Examples of frequently used triazoles include Voriconazole, Itraconazole, and Posaconazole. Voriconazol among others seems to be the drug of choice and has a major role in treatment of invasive aspergillosis and its utility in immunocompromised patients suffering from invasive aspergillosis has been promising (Herbrecht *et al.*, 2002; Herbrecht and Nivoix, 2009). Itraconazole and posaconazole are used for prophylaxis (preventive medicine) against invasive aspergillosis in high risk patients (e.g. patients with hematologic malignancies, solid organ transplant patients, and hematopoietic stem cell transplant patients) or follow up therapy in patients with invasive aspergillosis (Herbrecht and Nivoix, 2009; Rhodes and Askew, 2010).

Echinocandins are the third class of antifungals used for treating invasive aspergillosis. Principally they act by inhibiting the β-1,3-D-glucan synthesis enzyme, an important enzyme in the cell wall biosynthesis that catalyses the polymerization of glucose into β-1,3-D-glucan in *Aspergillus* cell wall. Inhibition of this enzyme results in decrease of cell wall glucan and osmotic shock which eventually leads to cell lysis (Maertens and Maertens, 2009; Rhodes and Askew, 2010). This group of antifungals act on the cell wall of the fungi, a target that is absent in human cells. Therefore, this group tends to be more specific to fungus and have less side effect/toxicity in human cells (Maertens and Maertens, 2009). Echinocandins used for therapy of fungal infections including aspergillosis are Caspofungin, Micafungin, and Anidulafungin (Rhodes and Askew, 2010). Despite all above mentioned facts about this group of drugs, research has shown that echinocandins are unable to completely inhibit the growth of *Aspergillus,* and are known to act fungistatically rather than fungicidally (Maertens and Maertens, 2009). Among all three types of

echinocandins, caspofungins has been subject of many clinical studies and are suggested to be used as alternative treatment and/or for empirical therapy of invasive aspergillosis especially when patients are intolerant to other standard treatments for invasive aspergillosis. With regards to the other two types of echinocondins, clinical data is still being accumulated and further research is required to unravel their effectiveness in the treatment of invasive aspergillosis (Maertens and Maertens, 2009; Rhodes and Askew, 2010).

1.5 Interactions of *A. fumigatus* and Host defence mechanisms

As mentioned earlier, development and severity of aspergillosis depends on the efficiency of the immune system. In immunocompromised patients *Aspergillus* conidia can easily germinate and develop into hyphae which are invasive and cause diseases. However, in immunocompetent individuals, one of the main targets of the immune system is detection and destruction of the conidia and prevention of its transition to hyphae form. Mechanical barrier is usually the first line of defence against *Aspergillus* spores, which is provided through the ciliary action of the lung epithelium, responsible for removal of aerosol particles. However, many reports have shown that this mechanism of defence might not be very efficient as *A. fumigatus* is able to produce metabolites such as gliotoxin and proteases (e.g alkaline protease) that inhibit or slow down the ciliary action and cause damage to human respiratory ciliated epithelium (Amitani *et al.*, 1995; Kupfahl *et al.*, 2008; Lydyard *et al.*, 2010).

1.5.1 Innate immunity

Innate immunity is another mechanism of early response to *Aspergillus* conidia/hyphae in immunocompetent individuals. Once conidia reached the lung alveoli, they (conidia) are recognized by bronchoalveolar macrophages which are the resident phagocyte cells in the lung. Macrophages detect the conidia through Pathogen Associated Molecular Patterns (PAMPs) via their

Toll-like receptors (TLR-2, TLR-4) and try to eliminate them by phagocytosis (Lydyard *et al.*, 2010). [Toll like receptors, are a group of proteins generally referred to as Pattern Recognition Receptors (PRRs) that play an important role in the innate immune system. They are able to recognise conserved molecular patterns that are generally shared by a large group of microorganisms and are distinct from the host. These molecules are referred to as PAMPs (Akira et al., 2001)]. Conidia of *A. fumigatus* are covered by a layer of hydrophobic proteins (Latgé, 2001). Furthermore, the extracellular matrix/ cell wall of *A. fumigatus* consists of galactomannan, α1,3 glucans, monosaccharides and polyols, melanin (hydrophobic pigments that are negatively charged and have high molecular weight) and some proteins which all play a role in both recognition of this fungus by immune system and its survival against body's immune system (Beauvais *et al.*, 2007). For instance, binding of soluble receptors in the lung such as surfactant protein D (SP-D) and pentraxin-3 to galactomannan in the cell wall of conidia enhances phagocytosis by bronchoalveolar macrophages (Crouch, 2000; Lydyard *et al.*, 2010). On the other hand, the hydrophobic protein layer (also called the rodlet layer) on the conidia surface protects it from immune system by covering the galactomannam and β-glucan polymers. A study by Paris and colleagues showed that rodletless mutants were more sensitive to killing by alveolar macrophages compared to the wild type strains (Paris *et al.*, 2003).

Digestion of the conidia by macrophages is via oxidative (i.e. NADPH oxidase) dependent killing and non-oxidative killing. Oxidative killing is mainly through generation of reactive oxygen species (ROS) which are toxic to fungal cells. However, this mechanism is not always successful as *A. fumigatus* is able to resist the oxidative damage by producing oxidoreductase enzymes and blocking phagocytosis (Nosanchuk and Casadevall, 2003; Bottone, 2006; Heinekamp *et al.*, 2013). In addition, the melanin content of conidia is a very effective hunter of free radicals, and possesses electron transfer properties which help to transfer the generated electron from free radical species. The mechanism by

which melanin blocks phagocytosis is not very clear, however it seems to be through alteration of surface hydrophobicity and cell charge (Nosanchuk and Casadevall, 2003).

The non-oxidative killing of macrophages involves swelling of the conidia inside macrophages or in the bronchoalveolar space that alters the cell wall composition and exposes fungal β-glucan. This in turn facilitates their detection via lectin-1, a mammalian β-glucan receptor, and elicits further fungicidal responses against conidia (Lydyard *et al.*, 2010). Moreover, generation of toxic molecules such as lectoferrin, defensins and other microbial peptides also contribute to the killing of conidia by macrophages and polymorphonulcear leucocytes (PMN) (Playfair, 1995; Nosanchuk and Casadevall, 2003; Lydyard *et al.*, 2010).

Unsuccessful removal/clearance of the conidia by macrophages results in the germination of conidia into hyphae. At this stage, neutrophils bind to the surface of the hyphae and try to eliminate them by different mechanisms including respiratory burst, production of ROS, release of lysozyme, and production of polysaccharide hydrolases (Latgé, 2001; Bottone, 2006; Lydyard *et al.*, 2010). At this stage platelets (as part of innate immunity) also play a role in the body defence against aspergillosis by adhering to the cell wall of the hyphae and participating in the hyphal damage (Lydyard *et al.*, 2010).

In immunodeficient patients various factors such as chemotherapy-induced neutropenia, corticosteroid treatment, and monoclonal antibody treatment, result in neutrophil dysfunction, prolonged neutropenia, and thrombocytopenia which in turn helps development of hyphae and eventually invasive aspergillosis (Lydyard *et al.*, 2010).

Another defence mechanism of *A. fumigatus* against innate immunity is that at hyphae level this fungus produces catalase and superoxide dismutase enzymes, which help to protect the fungus from hydrogen peroxide and oxygen radicals of phagocytes (Beauvais *et al.*, 2007; Lydyard *et al.*, 2010). Moreover, steroids

in the fungus membrane can supress phagocytosis by preventing the phagolysosomal fusion (Bottone, 2006). Gliotoxin has also been shown in many studies to cause cell-death in macrophages and neutrophils. Alkaline proteases produced by *A. fumigatus* have been reported also to degrade complement proteins such as C3, C4, and C5 (Cramer *et al.*, 2011).

1.5.2 Adaptive immunity

Adaptive immunity against aspergillosis involves response from both T and B-lymphocytes and secretion of cytokines and antibody generation. The latter is however, not fully understood. The protective T-cell response to *A. fuumigatus* is mainly through Th-1 (T-helper -1) via their cell surface receptor, CD4. Once CD4+ Th-1 cells recognize the antigen (*A. fumigatus*) they are able to recruit neutrophils and enhance neutrophil mediated killing through releasing various cytokines such as INF-γ, interleukin-1 (IL-1), IL-6, IL-12, and IL-18, granulocyte colony stimulating factor (G-CSF), and granulocyte macrophage colony stimulating factor (GM-CSF) which all result in increase in the neutrophil and monocyte mediated killing of *Aspergillus* hyphae. Neutrophils in turn release different cytokines such as IL-8 which facilitates release of antimicrobial peptides at the site of infection (Lydyard *et al.*, 2010; Cramer *et al.*, 2011).

On the other hand, the T-cell immunity against aspergillosis is not always protective, especially in the allergic bronchopulmonary aspergillosis (ABPA), where immune response involves recruitment of Th2 cells and production of IL-4, IL-5, and IL-10 and antibodies. Generation of cytokines and antibodies are in turn involved in impairing of neutrophil mediated antifungal activity by suppressing oxidative killing of neutrophils. This eventually leads to the development of hypersensitivity, inflammation, tissue damage and enhances the susceptibility of patients to *Aspergillus* infection as well as further development of *A. fumigatus* at the site of infection (Cenci *et al.*, 1997; Cramer *et al.*, 2011). Particularly IL-4, among others, has been reported to be associated

with susceptibility against *Aspergillus* invasion (Cenci *et al.*, 1997; Cenci *et al.*, 1999).

The antibody mediated immunity (AMI) against *A. fumigatus* infection is a relatively new field of research and not fully understood yet (Lydyard *et al.*, 2010; Casadevall and Pirofski, 2012). Investigation of AMI during aspergillosis, started by production of protective monoclonal antibodies against *Aspergillus* β-glucan and cell wall glycoproteins. However, soon after, research revealed that monoclonal antibodies in fungi can mediate different responses i.e. the responses to these antibodies were varied and not always protective. This was partly due to the genetic variation in different hosts. Despite all the controversy in the effects of antibodies during fungal infections, AMI still considered as an attractive option for immune therapy such as vaccine development especially because there are evidences of the possibility of antibodies generating long term immunity against *A. fumigatus* (Montagnoli *et al.*, 2003; Casadevall and Pirofski, 2012). Mechanism of protective immunity by antibody against fungi has been suggested to be via direct growth inhibition, mediation of innate immunity responses, and neutralization of fungal metabolites such as toxins and enzymes, in order to prevent the damage they cause to the host tissues (Casadevall and Pirofski, 2012).

Some antibody responses to fungal infection can be harmful to the host. For instance, IgE antibody produced during allergic bronchopulmonary aspergillosis has been associated with increase in both type I and type II hypersensitivity. This has been linked to Th-2 cell response and generation of IL-4 which stimulates B-cells to produce IgE antibody along with IL-5 mediated eosinophil, resulting in tissue damage and inflammation that ultimately facilitates fungal invasion (Lydyard *et al.*, 2010). Th-2 response has also been reported to increase susceptibility to *Aspergillus* infection by inhibiting Th-1 responses (Rivera and Pamer, 2010). During Aspergilloma (chronic aspergillosis), the antibody response against *Aspergillus* cell wall carbohydrates

and glycoproteins involves specific IgM and IgG production, as their levels has been shown to be elevated during this infection (Lydyard *et al.*, 2010).

1.6 Hydrocortisone signalling: Immunosuppressive therapy

Hydrocortisone (cortisol) is a naturally occurring glucocorticoid steroid hormone produced mainly by adrenal glands, under the influence of hypothalamic-pituitary-adrenocortical (HPA) axis activity in the body. Steroid hormones are cholesterol derived regulatory molecules that have been described as the marker of body's adaptation to stress. They are also involved in regulation of carbohydrate metabolism (Stocco, 2003; Dedovic and Duchesne, 2012). Only 5% of the secreted cortisol is freely circulating in the body and is biologically active, the rest (95%) of body's secreted cortisol is bound to proteins such as albumin and transcortin and is mainly removed by hepatic metabolism (Gonzalez-Alvarez *et al.*, 2012). It is suggested that binding of cortisol to these proteins (also called cortisol binding globulins) is likely to have a buffering activity preventing extensive fluctuations in cortisol levels in the body (Adcock, 2003).

Glucocorticoids play an important role in regulation of the body's homeostasis by effecting metabolic actions such as carbohydrate, lipid and protein metabolism. In human and animals, glucocorticoids stimulate processes that lead to release of both amino acids and free fatty acids from muscles and adipose tissue respectively, and are involved in deamination of amino acids and gluconeogenesis in the liver. Hence as a results of these activities glucocorticoid hormones are able to raise blood sugar levels (McMahon *et al.*, 1988; Brillon *et al.*, 1995; Bamberger *et al.*, 1996; Larson *et al.*, 2003; Christiansen *et al.*, 2007). However excess levels of cortisol in the body can lead to harmful effects such immune suppression, hypertension, osteoporosis, and depression (Dedovic and Duchesne, 2012; Ray and Leikle, 2012).

In human/animals glucocorticoids have their physiological activity by binding to and activating glucocorticoid receptors (GR), available in different target

tissues, and modulating (i.e. activating and repressing) gene expression (Figure 1.7). GRs are comprised of various domains responsible for hormone binding, transcriptional activation, DNA binding, and nuclear localization (Larson *et al.*, 2003).

Steroid hormones diffuse through the cell membrane, due to their lipophilic nature, and reach the cytoplasm to interact with the GR. GRs are located mainly in the cytoplasm of the cells, kept in an inactive complex by heat-shock proteins (Figure 1.7). After binding to their glucocorticoid ligand such as cortisol, GRs are activated as a result of conformational changes and release of heat-shock proteins. These conformational changes also allow the (GR) translocation into the nucleus, dimerization of the GR monomers, binding to glucocorticoid response elements (GRE) of DNA, and finally interaction of GR dimers with various transcriptional regulatory proteins which ultimately results in activation of gene expression (Figure 1.7). Mechanisms of gene suppression by GRs are yet to be completely understood (Larson *et al.*, 2003; Page *et al.*, 2006; Gonzalez-Alvarez *et al.*, 2012). Among the activated genes by GRs are the ones encoding for enzymes involved in gluconeogenesis such as the rate limiting enzyme phsphoenolepyrovate carboxykinase, glucose-6- phosphatase, serine dehydrogenase, and tyrosine aminotransferase. Among the supressed genes by glucocorticoids are an array of pro-inflammatory cytokines such as Interlukine-1α (IL-1α), IL-1β, IL-3, IL-5, IL-6, IL-8, IL-12, tumour necrosis factor α (TNF-α), granulocyte colony stimulating factor, and macrophage colony stimulating factor (Larson *et al.*, 2003). The mechanism of this suppression is known to be due to stress (physical or psychological). Stress has physiological effects in the immune system. Research has shown that stress increases the production of cytokines such as IL-6, TNFα, IL-1β, and C-reactive proteins. Increased production of cytokines in turn affects the HPA system by stimulating hypothalamus and pituitary glands to secret corticotropin releasing hormone (CRH) and adernocortocotropin hormone (ACTH) respectively which eventually leads to the production of cortisol by the adrenal cortex. The

secreted cortisol has a supressing effect on the immune system by affecting the function of immune system cells such as leukocytes and supressing the production of inflammatory cytokines such as IL-1, IL-6, IL-8, IL-12, TNF-α, interferon-γ etc. (Dedovic and Duchesne, 2012).

Control of the normal level of cortisol in the body is regulated by the action of two enzymes: 11-beta hydroxy steroid dehydrogenase (11-β HSD) type II and 11-βHSD type I. The type II enzyme is responsible for one-directional conversion of cortisol into cortisone. Cortisone is an inactive form of cortisol lacking the physiological abilities of the cortisol. Type I enzyme on the other hand is in charge of the opposite reaction; conversion of inactive cortisone into cortisol and vice versa. Inactivation of the two enzymes results in raised cortisol levels in the body (Ray and Leikle, 2012).

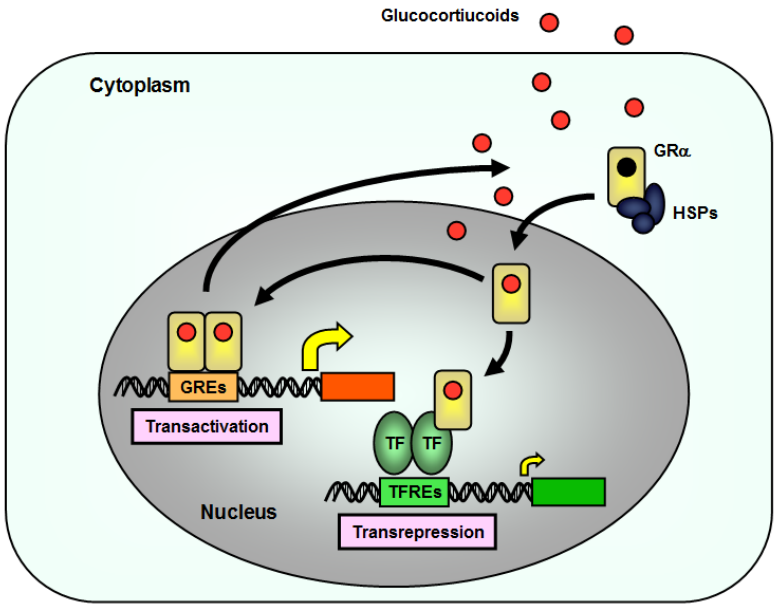


Figure 1.7
Mechanism of action of glucocorticoids at the cellular level is via their interaction with glucocorticoid receptors (GR). Glucocorticoid receptors are hormone activated transcription factor that (directly or indirectly) regulates the expression of certain genes in response to glucocorticoid ligands. Figure adopted from (Kino *et al.*, 2010).

Most of the reports on glucocorticoid receptors suggest the presence of these receptors in the cytoplasm and their translocation to the nucleolus only after binding to their specific ligand and generation of ligand-receptor complex, as described earlier. However, it has been reported recently that there are two types of cortisol receptors in the target tissues: Type I or mineralocorticoid receptors, and type II or glucocorticoid receptors. These receptors are transcription factors that once bound with their ligand can regulate the gene expression. The receptors are reportedly membrane-bound (non-genomic) and genomic (Dedovic and Duchesne, 2012).

1.6.1 Glucocorticoid therapy

As mentioned earlier glucocorticoid hormones such as cortisol are able to supress the immune system when in excess. Hence, in medicine, in order to supress the provoked immune system, steroid hormones such hydrocortisones are administered into the body in pharmacological doses (Dedovic and Duchesne, 2012). Glucocorticoid drugs are partial or selective agonists of natural steroid hormones chemically modified in order to increase their immunosuppressive activity and reduce the side effects. Apart from immunosuppressive effects, glucocorticoids are also used in medicine for their anti-inflammatory and cytostatic effects (Larson *et al.*, 2003; Ray and Leikle, 2012). Steroid hormones have been used as therapeutic agents in anti-inflammatory therapy since 1949 (Lukens, 1954). They are used for the treatment of disease such as adrenal insufficiencies, neuropsychiatric disorders, blood and kidney disease, and a variety of inflammatory diseases such as asthma, and allergies. Due to their immune suppressive activity they are also used for the treatment of diseases like rheumatoid arthritis, inflammatory bowel disease as well as reducing the likelihood of implant rejection in organ transplantation (Lukens, 1954; Larson *et al.*, 2003).

As mentioned earlier the anti-inflammatory effects of steroid hormones are through binding to their specific receptor and up-regulation of anti-inflammatory proteins and down regulation of pro-inflammatory proteins. Their administration into the body varies according the type and severity of the disease, and the affected organ (Ray and Leikle, 2012). Despite all the benefits of this type of therapy, their administration is associated with different side effects such hypertension, arthritis, and above all predisposition to various fungal and bacterial infections (Lionakis and Kontoyiannis, 2003; Ray and Leikle, 2012). The mechanism of suppression of immune system by glucocorticoid therapy is understood through different means: a) induction of the production of lipocortin-1 polypeptide, which in turn inhibits the expression of phospholipase A2 enzyme. Phospholipase A2 plays an important role in the production of inflammatory mediators such as prostaglandins, platelet activating factor (PAF), and leukotriene. Besides, glucocorticoids are able to inhibit the transcription factors responsible for cytokine production (e.g. IL-5, TNF-α) in inflammatory cells via interacting with glucocorticoid response elements in those cells (Figure 1.6). b) They are able to modulate the monocyte activity in order to reduce the production of cytokines generated by monocytes such as IL-1 as well as supressing their bactericidal/fungicidal activity (Page *et al.*, 2006). c) They down-regulate interleukin-1 induced expression of IL-6 and IL-8 through inhibiting NFκB (transcription factor nuclear factor κB) (Lionakis and Kontoyiannis, 2003). d) Similarly they are able to reduce oxidative burst and superoxide release by neutrophils (Lydyard *et al.*, 2010). Glucocorticoids decrease the number of alveolar dendric cells and hamper their antigen presenting ability by down regulation of expression of MHC-II molecules on the surface of these cells (Lionakis and Kontoyiannis, 2003).

Hydrocortisone, beclometasone, dexamethasone, and prednisolone are examples of steroids used in medicine. Some glucocorticoids (e.g. Prednisolone) are known to inhibit the activity of 11-βHSD type 2 enzyme resulting in

increased levels of cortisol, and cortisol in turn supresses the function of immune system (Ray and Leikle, 2012). It has been reported that not all glucocorticoids have equal anti-inflammatory effects even if administered in equivalent pharmacological doses (Fauci, 1976; Lionakis and Kontoyiannis, 2003). For example when hydrocortisone, dexamethasone and prednisolone are administered in equal pharmacological doses, dexamethasone causes greater defect in lymphocyte-mediated cytotoxicity than the other two steroids (Fauci, 1976). In another study methylprednisolone, although less potent, demonstrated more inhibitory effect of lymphocyte proliferation and cytotoxicity compared to dexamethasone with an intermediate potency similar to hydrocortisone (Langhoff *et al.*, 1987). The observed differences between the effects of various glucocorticoids could be due to the differences in interactions of these steroids with the glucocorticoid receptor complex and with NFκB, gene mutations and polymorphism in patients (Lionakis and Kontoyiannis, 2003). However, a point to consider is that although undergoing immunosuppressive therapy, not all patients develop similar responses to glucocorticoids; the potency of various glucocorticoids is also variable in different patients. Yet, altogether these patients are still at high risk of developing invasive fungal infections.

1.6.2 Glucocorticoid effects on other organisms

In 1981 Loose and colleagues reported, for the first time, the presence of a glucocorticoid binding protein in the cytoplasm of human fungal pathogen *Candida albicans*, suggesting that the evolutionary basis of steroid hormone receptors might be going back to the unicellular eukaryotes (Loose *et al.*, 1981). Other studies haven't identified the presence of the receptor, but have reported the evidences of the effect of steroid therapy; for instance hydrocortisone treatment, on the growth of primary organisms. A study by Tsumura and collaborates has reported the effect of hydrocortisone succinate on the growth

of *Chlamydia pneumonia*, an intracellular bacterial pathogen. They have reported an increase in the number of inclusion bodies *in vitro* as a result of hydrocortisone treatment (Tsumura *et al.*, 1996). Likewise, Ng and collaborator have reported an enhanced growth rate of *A. fumigatus* and *A. flavus* when hydrocortisone sodium succinate was present in the culture medium, they have therefore suggested the possibility of the presence of a glucocorticoid receptor in this organism (Ng *et al.*, 1994).

1.7 Models for studying aspergillosis

Various animals (both invertebrate and vertebrate models), tissues and cell lines have been used to study different aspects of aspergillosis. *Aspergillus* isogenic strains including wild type, mutant, and complemented mutant have been used to infect different study models in order to understand the mechanism of virulence of this fungus (Rhodes and Askew, 2010). Examples of invertebrate models are *Drosophila melanogaster* (fruit fly), *Dictyostelium discoideum*, and *Galleria mellonella* (wax moth larva). These models have been used, due to their cost effectiveness, availability of mutant strains, and above all the similarities of the innate immune system of these insects to mammals. Hence they have been employed to study the role of immune response in developing invasive aspergillosis as well as the effect of anti-fungal therapy (Lionakis *et al.*, 2005; Renwick *et al.*, 2006; Kavanagh and Fallon, 2010; Rhodes and Askew, 2010). Mice/murine models are the most commonly used vertebrate models of aspergillosis particularly because of the reproducibility and homogeneity of the infection caused in this model (Rhodes and Askew, 2010). Similar to human, normal mice are resistant to developing *Aspergillus* infection, hence they are used to study the effect of immunosuppressive therapy in developing the infection. They are also used as inoculum delivery models. They are also used due to their different genetic backgrounds and immune response, in order to gain insights in to different immune responses

seen in humans (Eisenstein *et al.*, 1990; Cenci *et al.*, 1998; Sheppard *et al.*, 2004; Balloy *et al.*, 2005; Bonnett *et al.*, 2006). Other animal models include rats, rabbits and guinea pigs which are used due to their larger size than mice to study various aspects of the infection (Schmitt *et al.*, 1988; Kirkpatrick *et al.*, 2002; Marr *et al.*, 2004).

Several human and animal cell lines have also been used for research on aspergillosis and interactions of *Aspergillus* and human and animal cells including human lung carcinoma alveolar epithelial cells (A549), and cells of innate (e.g. macrophages) and adaptive immunity (e.g. T-cells), and endothelial cell lines such as (human pulmonary artery endothelial cells (HPAECs), Human microvascular endothelial cell line (HMEC-1)) (Kauffman *et al.*, 2000; Wasylnka and Moore, 2003; Bezerra and Filler, 2004; Zhang *et al.*, 2005; Bellanger *et al.*, 2009). A549 cell line has particularly been used in many studies as alveolar epithelial cells are among the first cells that *Aspergillus fumigatus* conidia/hyphae encounters while creating an infection, hence are the first site of infection. The human alveolar adenocarcinoma cell line (A549) was originally established, together with twelve other cell lines, by Giard *et al.*, 1973 through removal and culturing of tumour specimens from a malignant lung tissue of a 58 year old Caucasian man (Giard *et al.*, 1973). A549 cells are type II human alveolar basal epithelial cells that are squamous in nature and responsible for diffusion of different material such as water and electrolytes across the alveoli of lungs. These cells are able to synthesize lecithin and contain high levels of unsaturated fatty acids which are used in cytidine-diphospho-choline pathway and play an important role in maintaining the membrane phospholipids in the cells (Lieber *et al.*, 1976). The immune system cells are also among the cell lies used for the study of aspergillosis. Immediately after entrance of the *Aspergillus* into the body it will be recognized and dealt with by several cells and molecules of the innate and acquired immunity. Hence, utilization of these cell lines has provided a great media to unravel the mechanism of interaction of the fungus and immune response *in vitro*.

1.8 Cell-death

In mammalian cells, processes involving and/or leading to an irreversible loss of cell membrane are generally referred to as cell-death (Golstein and Kroemer, 2007). In cellular biology, programmed death is a naturally occurring process in many cells, required for the maintenance of multicellular organisms. It can however also occur due to internal or external changes and stimuli. There are two main classes of cell-death in mammalian cells including apoptosis, and necrosis (Figure 1.7). However, recently autophagy is also considered as the third major form of cell-death (Kroemer *et al.*, 2008; Hotchkiss *et al.*, 2009). Since different cellular activities are involved in cell-death processes, a recent classification by the nomenclature committee on cell-death, has recommended classification of these processes according to different morphological, enzymological (involvement of certain classes of enzymes), functional, and immunological criteria (Kroemer *et al.*, 2008). For example, various cell-death processes result in different morphologies in the cell (e.g. cell shrinkage or swelling), hence morphologically cell-death has been classified into apoptosis, necrosis, autophagic or associated with mitosis. Similarly in terms of the enzymes involved these processes are arranged according to the presence of nucleases or certain classes of proteases such as caspases, calpains, cathepsins, and transglutaminase enzymes. Functionally cell-death can be programmed or accidental, and physiological or pathological. Finally from the immunological point of view cell-death can be immunogenic or non-immunogenic (Kroemer *et al.*, 2008).

Since in this research project, the effect of gliotoxin on causing cell-death (apoptosis and necrosis) in pneumocytes has been investigated, these two main types of mammalian cell-death as well as autophagy type are briefly explained in this section.

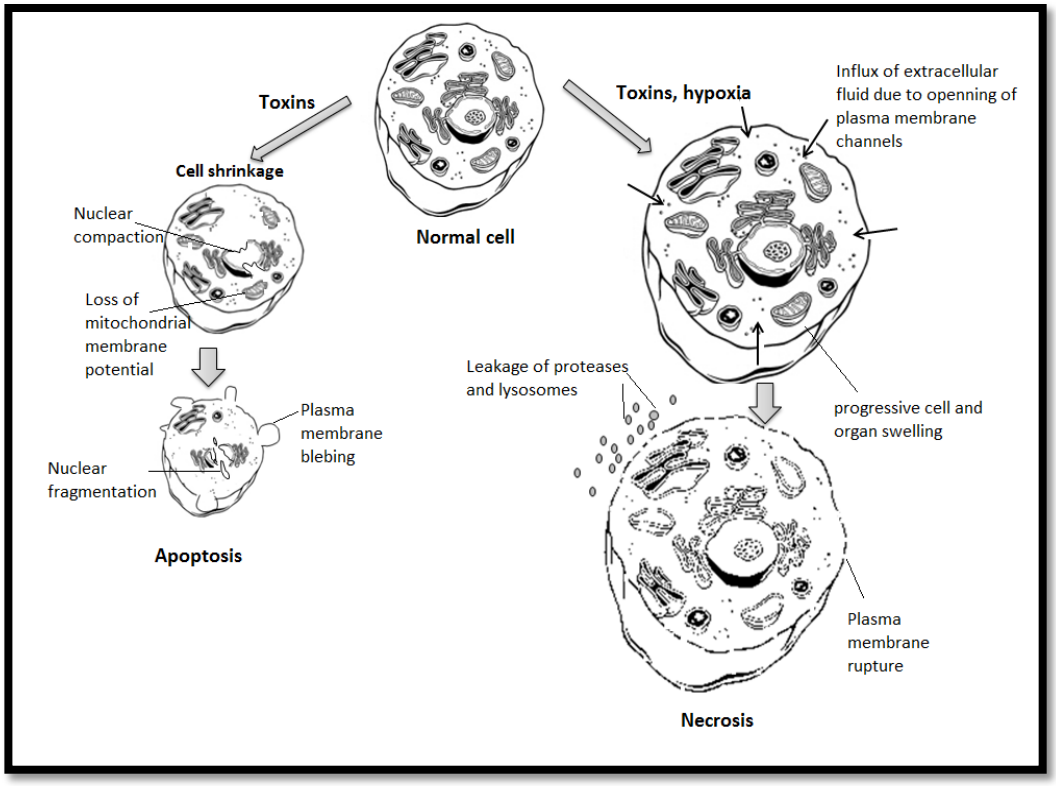


Figure 1.7-

Schematic diagram showing the two main types of cells death including apoptosis and necrosis. Figure adopted from (Hotchkiss *et al.*, 2009) with modifications.

1.8.1 Apoptosis

Apoptosis, also referred to as programmed cell-death, involves a series of events leading to loss of cell functionality. Cell-death is considered programmed when it is a genetically controlled process (Hotchkiss *et al.*, 2009). A main morphological characteristic of apoptosis is shrinkage of the cell and its nucleus. During apoptosis the cytoskeletal proteins are also cleaved by aspartate proteases which lead to cell collapse/failure. Also plasma membrane blabbing (budding) and formation of apoptotic bodies occurs, as well as externalization of phosphatidylserine phospholipids from inner to outer plasma membrane,

chromatin condensation, changes in DNA morphology and DNA fragmentation (Figure 1.7). Loss of membrane integrity is the last thing that happens in an apoptotic cell (Golstein and Kroemer, 2007; Hotchkiss *et al.*, 2009). Apoptosis can be triggered through different cellular pathways including death-receptor pathway, BCL-2 (B cell leukaemia/lymphoma 2 protein family) regulated mitochondrial pathway (Kroemer *et al.*, 2008; Hotchkiss *et al.*, 2009). One of the features of cell-death is activation of the caspases family of proteins and as a result of this, the cell will embark in one of the two main pathways of death-receptor or mitochondrial.

Death receptors are located in the cell membrane and are activated upon binding of the members of tumour necrosis family (e.g. TNF and TRAIL) to them. The result of this binding is formation of a multi-protein complex and subsequent conformational changes in the complex which leads to recruitment and activation of caspases 8 (catalytic activity) and eventually caspases 3, which in turn initiate apoptosis by activating other caspases like caspases 6 and 7, protein cleavage and DNAase activation (Hotchkiss *et al.*, 2009).

The mitochondrial pathway is activated by external stimuli such as toxins, oxygen radicals, γ-irradiation etc., or internal ones such as DNA damage or lack of growth factors, which all lead to mitochondrial injuries. This process is controlled as a result of interaction between pro-apoptotic and anti-apoptotic proteins of the BCL2 family. During this process the presence of stimuli activates pro-apoptotic BH3 proteins (pro-apoptotic BCL2 family that have BCL-2 Homology domain 3) such as BIM and PUMA in the cell cytoplasm; these proteins in turn inactivate anti-apoptotic proteins such as BCL2 or BCL-XL (which are bound to BAK and BAX proteins in the cytosol). Interaction of BH3 proteins with anti-apoptotic proteins sets BAK and BAX free and activates them to induce permeability of the mitochondrial membrane and cytochrome c (a pro-apoptotic protein) release, activation of caspases 9 and ultimately caspases 3, 6, and 7, protein degradation in the cell and DNA damage by DNAase enzymes (Hotchkiss *et al.*, 2009).

1.8.2 Necrosis

Necrosis is mostly considered as an accidental form of cell-death. It involves early swelling of the cells and cytoplasmic organelles, rupture of plasma membrane which leads to the leakage of the intracellular organelles and cytoplasmic vacuolization (Figure 1.7) (Golstein and Kroemer, 2007; Hotchkiss *et al.*, 2009). During necrosis the swelling and damage to the membrane of cellular organelles results in the release of lysosomes and other proteolytic enzymes into the cytoplasm and extracellular matrix of the cells and eventually cell damage and death (Figure 1.7).

Necrosis is caused by various physiological and non-physiological factors. Physiological causes of necrosis include metabolic failure and lack of ATP, acute hypoxia or ischemic injuries such as stroke. Non physiological initiators of necrosis include temperature shock, mechanical damage, and toxins (Hotchkiss *et al.*, 2009). Presence of factors such as increased intracellular calcium (as a result of calcium influx across the plasma membrane), increased presence of reactive oxygen species (ROS) (as result of mitochondrial dysfunction), poly-ADP-ribose-polymerase (PARP) and proteases such as calpains and cathepsins, which are released as a result of cellular damage, mediate the necrosis process (Golstein and Kroemer, 2007; Hotchkiss *et al.*, 2009). Necrosis results in recognition of the cell by the innate immune system cells and their removal. Finally, recent data suggests that necrosis can occur as both accidental and programmed (as it might be regulated by certain signal transduction pathways and catabolic mechanisms) (Golstein and Kroemer, 2007; Kroemer *et al.*, 2008).

1.8.3 Autophagy

Autophagy is a form of programmed cell-death referring to a process in which cells utilize their non-essential, or damaged organelles and macromolecular components in response to environmental stress such as nutrient deficiency, suppression of tumours, or deletion of misfolded proteins (Hotchkiss *et al.*, 2009). Three forms of autophagy include macro-autophagy, micro-autophagy and chaperon-mediated autophagy. The common feature in all three forms is the presence of lysosomal derived vesicles containing cytosolic components in the cell, also known as autophagic vacuolization of the cytoplasm. They however differ in the way lysosomes obtain the material for degradation; i.e. formation of autophagosomes and their fusion with lysosomes (Golstein and Kroemer, 2007; Hotchkiss *et al.*, 2009). Once inside lysosomes (autolysososme), acidic lysosomal hydrolysis degrades the engulfed material into simple metabolic substrates (Golstein and Kroemer, 2007).

Finally, autophagy is more known as a survival process rather than a cell-death mechanism as it provides the cell with required nutrients for cell survival during nutrient deficiency conditions, or protects the cell through removal of tumours and wrongly folded toxic proteins (Hotchkiss *et al.*, 2009).

1.9 Quorum sensing (QS)

1.9.1 History of Quorum Sensing

For many years, microbial cells were considered to be poor in their ability to communicate with each other and with other species in general. However, it has been discovered that they are highly communicative (Nealson *et al.*, 1970). In the microbial world that cells are struggling to out-compete their opponents, the ability to recognize self from non-self is essential and effects a range of activities including secondary metabolite production, adaptation to the new environment, overcoming the defence mechanism of their host, symbiosis, and swarming motility (Fuqua *et al.*, 1994; Williams *et al.*, 2007).

Excluding the direct cell to cell contact, bacterial cell communication is by releasing diffusible substances into the environment. The substances are believed to be pheromones. Pheromones are defined as chemical signal molecules that initiate natural responses in other members of the same species. Bacterial cells produce many different molecules through their metabolic activities and release them to their environment. These molecules include primary and secondary metabolites such as antibiotics, metabolic end products, and bacterial cell-cell signalling molecules (pheromones). An environmental sensing system enables bacteria to check their own population density by releasing the substances to their surroundings during their growth. When the population of the cells reaches a critical density (called critical mass) and the auto inducer accumulates in the environment, the microorganisms modify their responses for a common goal. In fact, the perception of the environment by the microbe influences their expression of genes responsible for directing many pathways. In 1970 Nealson reported that *V. fischeri* emitted light when the accumulated cells on the host (some species of fish and squid) reached a high density compared to their dispersed state in a suspension (Nealson *et al.*, 1970). Although the process was known since 1970, it was only in 1994 that Fuqua and his colleagues, for the first time, named this phenomenon as quorum sensing (QS) and the cell-cell signal molecules released from bacterial cells as

autoinducers (Fuqua *et al.*, 1994). They described the QS mechanism in Gram-negative (G^-) marine bacterium *Vibrio fischeri.* This bacterium has a symbiotic life with the light emitting organs of particular squids and fishes (e.g. Hawaiian Squid, Euprymna scolopes) (Ruby and Lee, 1998). They discovered that when population of the bacterial cells reaches a critical density, the accumulated signal molecule activates the transcription of luciferase operon and eventually leads to light emission or bioluminescence that has been provided by this bacterium for host organisms (Lerat and Moran, 2004).

Investigations also revealed that adding cell free culture supernatant of *V. fischeri* to its fresh cultures had a stimulating effect on the light emission by this bacterium. Soon after, the component responsible for this phenomenon was identified as acyl-homoserine lactone (AHL). Since the discovery of QS in *V. ficsheri* the phenomenon has been described in many other microorganisms via employment of AHL as well as various other signals such as, γ-butryolactone from *Streptomyces griseus*, *2*-heptyl-3-hydroxy-4-quinolone from *Pseudomonas aeruginosa,* and pedtides (e.g. cyclic thiolactone) from type III *Staphylococcus aureus* to sense their cell density (Mayville *et al.*, 1999; Bassler and Losick, 2006).

1.9.2 Quorum Sensing Systems in G^- and G^+ Bacteria

There are different types of quorum sensing mechanisms in G^- and G^+ bacteria. In G^- bacteria (e.g. *Pseudomonas aeruginosa, Agrobacterium tumefaciens, Erwinia carotovora)* a two component system is present, comprised of a LuxR-type signal receptor protein (cytoplasmic protein) and LuxI-type AHL synthase enzyme. When the population density of the cells is low, bacteria produce a low level of AHL signals. Over time, as the cell density increases so does the AHL signal concentration. When the AHL signal reaches a threshold it binds to the R protein and activates it by making R-AHL complex. This complex, in turn, binds

to the related promoter and activates the expression of the corresponding genes (Figure 1.8, A) (Zhang and Dong, 2004; Dong and Zhang, 2005). In G^+ bacteria (e.g. *Bacillus* species, *Lactococcus lactis, Staphylococcus aureus*) on the other hand, QS mechanism appears as a phosphorylation–dephosphorylation system. Here, a membrane bound histidine kinase enzyme acts as a sensor for QS signal molecules. The information detected by this sensor is transmitted through a phosphorelay signal transduction system that ultimately affects the related regulatory proteins and transcription of the corresponding genes (Figure 1.8, B) (Kleerebezem *et al.*, 1997; Kuipers *et al.*, 1998; Lerat and Moran, 2004; Zhang and Dong, 2004; Dong and Zhang, 2005).

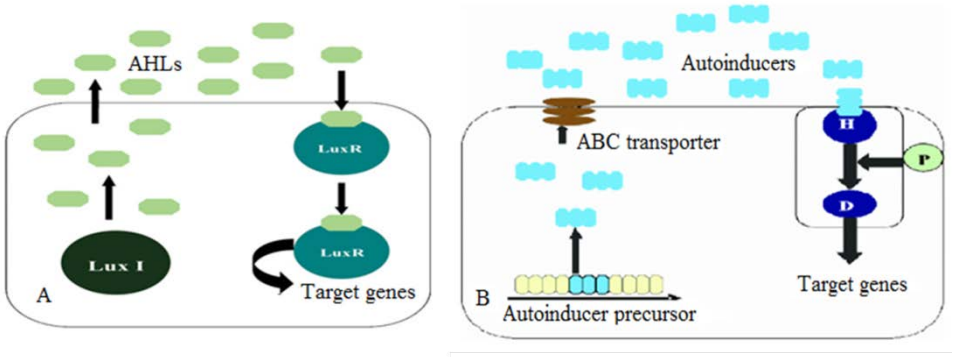


Figure 1.8
Quorum-sensing system in Gram-negative (A) and Gram-positive (B) bacteria. Picture adopted from (Raina *et al.*, 2009).

1.9.3 Quorum sensing in Fungi

There is accumulating evidence that fungi, like bacteria, also use quorum sensing to regulate population density dependent behaviours such as pathogenesis and biofilm formation (Tseng and Fink, 2008). Majority of quorum sending studies on fungi have been carried out on dimorphic fungi (e.g. *Candida albicans*). One characteristic of these types of fungi is that they can switch between the hyphal and yeast morphology in response to the

environmental changes. Among dimorphic fungi, some species are human pathogens. *Candida albicans* is an opportunistic human pathogen that has been extensively studied for quorum sensing. These dimorphic fungi pathogens (e.g. Candida albicans) are able to grow as the yeast like shape in the body fluids for proliferation (they can easily proliferate by budding); however they switch to hyphal morphology when they invade tissues (Deacon, 2006; Tseng and Fink, 2008).

For sensing and adaptation to the environment, Candida uses a two component phosphorelay systems. Fungal histidine kinases are hybrid enzymes. That means they act in several steps of phosphorelay. During these steps the phosphate is transferred from the response regulator domain of the hybrid histidine kinase to a second histidine residue in a histidine phosphotransfer domain, and that in turn is transferred to a second response regulator domain. These systems have been involved in the regulation of virulence in human and plant fungal pathogens (Tekaia and Latgé, 2005). For instance the human fungal pathogen, *Cryptococcus neoformans* utilises the mitogen-activated protein kinase system (MAPK), a two component system, to control several cellular events including virulence, sexual reproduction, and drug sensitivity (Bahn *et al.*, 2006). In *Candida albicans*, these systems are involved in the regulation of cell wall biosynthesis and adherence to the host cell, osmotic and oxidant adaptation, morphogenesis and virulence (Kruppa and Calderone, 2006). In the genome of *A. fumigatus* 13 genes have been identified that encode for histidine kinases with orthologues which are either limited to other *Aspergillus* species or common to all eukaryotes (Tekaia and Latgé, 2005).

Recently, it has been found that filamentous fungi also use quorum sensing to regulate genes encoding for cell density dependent behaviour like secondary metabolite production or virulence. For example, environmental strains of *Aspergillus* species have been shown to elicit quorum-sensing behaviour. (EC FP6 proposal, 2006; Raina, 2008). The existence of butyrolactone I, which is an auto inducing factor in some bacteria, as one of the secondary metabolites of

A. terreus opened an insight to investigate its role as a self-regulating factor in this fungus. As a result, research showed that addition of butyrolactone I resulted in an increase in the lovastatin production in *A. terreus.* Furthermore, the corresponding gene cluster responsible for the biosynthesis of lovastatin in *A. terreus* has been identified (EC FP6 project, 2009). Moreover, research carried out by the Quorum Group at School of Life Sciences, University of Westminster has provided evidence for quorum sensing process in variety of filamentous fungi (Raina, 2008; Sorrentino, 2009; Williams, 2009).

1.10 Molecular tools in studying aspergillosis

Various molecular techniques are used in biological sciences for the study of genomes, transcriptomes, proteomes, lipidomes, and metabolomes. In this research project two methods of proteomics and transcriptomics have been employed and are further explained below.

1.10.1 Transcriptomics

Transcriptomics is defined as a comprehensive analysis of the mRNA species of a cell under defined conditions. Studying mRNA species in a cell will provide an extensive knowledge of the expressed genes under described conditions, as well as providing an understanding of the changes in the level of gene expression after certain treatments or alterations in the environmental conditions (Burdine *et al.*, 2002). This technique allows simultaneous studying of the expression level of a large number of genes under tested conditions (especially if microarray is used) and provides a comprehensive knowledge of the mechanisms of gene regulation.

In the present study, qRT PCR method was used in order to study the expression level of certain *Aspergillus* genes after treatment with hydrocortisone. For this purpose, total RNA was extracted from *Aspergillus* cultures. RNA was reverse transcribed into cDNA in order to generate a cDNA

library, and the generated cDNA library was subsequently subjected to quantitative real time PCR (qRT PCR) using gene specific primers. Further details of the experimental methods are explained in section 2.8 of this thesis.

1.10.2 Proteomics

Proteomics is another Omics tool that provides an understanding of different cellular processes via studying the protein complement of the genome (Burdine *et al.*, 2002). Combining proteomics with mass spectroscopy (MS) analysis allows concurrent identification of multiple gene products (proteins) that are modulated in response to a changing environment (Rhodes and Askew, 2010). In the present study a proteomics approach was employed using two-dimensional gel electrophoresis (2DGE) combined with MS. This was done in order to further understand the changes in cytoplasmic protein of *A. fumigatus* after hydrocortisone treatment. Further explanation of the experimental procedures can be found in section 2.7 of this thesis.

1.11 Aim and objectives

Earlier studies of the host pathogen interactions during aspergillosis have been mostly focused on the understanding of the interactions between fungal metabolites and the cells of human immune system. However, once *Aspergillus* enters the host, the lung alveolar cells are typically the first point of encounter and one of the main sites of infections. Additionally, majority of the patients who develop aspergillosis infection are immunosuppressed (as a result of extensive immunosuppressive therapy).

Considering these factors, the overall aim of this project is to investigate the interaction of *Aspergillus fumigatus* and human alveolar epithelial cells through the produced metabolites and the environmental factors.

In order to achieve this aim the following objectives are explored:

- To investigate the ability of different clinical strains of *A. terreus* and *A. fumigatus* for production of secondary metabolites, gliotoxin and butyrolactone I. Also to investigate the time and level of production of these secondary metabolites.
- To investigate the effect of commercially available gliotoxin on the human alveolar epithelial cell line (A549) (Cell viability assay), and to determine the half maximal inhibitory concentration (IC_{50}), (if any), of this compounds on A549 cells.
- To understand, through *in vitro* stimulation studies, the effect of gliotoxin and other metabolites produced by *Aspergillus fumigatus*, on the lung alveolar cells. Also to determine to what extent the cell damage/death caused by gliotoxin in *Aspergillus* culture broth is due to the production of gliotoxin.
- To investigate the mechanism of cell-death in A549 cells by *Aspergillus* produced metabolites.

- To investigate the effect of hydrocortisone treatment on growth, morphology and metabolite production of *Aspergillus fumigatus* clinical isolates.
- To implement proteomic studies in order to gain an insight into the effect of hydrocortisone on cellular mechanisms and protein expression levels in this fungus.
- To investigate binding of *Aspergillus fumigatus* spores to the hydrocortisone treated lung alveolar cells (A549).
- To implement transcriptomic studies to further examine the effect of hydrocortisone treatment on the expression of *Aspergillus* genes.

Chapter II: Materials and Methods

2 Materials and Methods

2.1 Chemical and reagents

Most of the chemicals used in this study were obtained from Sigma Aldrich (Poole, Dorset, UK), Fisher Scientific (Loughborough, UK), and Qiagen Ltd. (Crawley, UK). High Performance Liquid Chromatography (HPLC) assays were performed using HPLC-grade solvents from VWR, UK.

2.2 Strains

Various strains of *Aspergillus terreus* and *Aspergillus fumigatus* were kindly donated by different medical centres. All the strains and their sources used in this work are shown in Table 2.1.

Strain name	Origin
***Aspergillus terre* MUCL38669; Environmental isolate**	UOW*, Lab C7.01 culture collection
***Aspergillus terreus* 1027F; Clinical isolate**	UCL**- Dr. Patrick Kimmitt
Aspergillus fumigatus 1025F; Clinical isolate	UCL**- Dr. Patrick Kimmitt
***Aspergillus fumigatus* 1026F; Clinical isolate**	NUI MAYNOOTH- Dr. Kevin Kavanagh
***Aspergillus fumigatus* 1024F; Clinical isolate**	UCL**- Dr. John Holton
***Aspergillus fumigatus* 1030F; Clinical isolate**	UCL**- Dr. John Holton
***Aspergillus fumigatus* 1031F; Clinical isolate**	UCL**- Dr. John Holton
***Aspergillus fumigatus* 1032F; Clinical isolate**	UCL**- Dr. John Holton
***Aspergillus fumigatus* AF-10; Clinical isolate**	UHSM***- Prof. David Denning
***Aspergillus fumigatus* AF-293; Clinical isolate**	MSU****- Dr. Robert Cramer
***Aspergillus fumigatus* ARC2; gliotoxin Knockout strain (ΔgliP)**	MSU****- Dr. Robert Cramer

Table 2.1
Different clinical strains used in this study were collected from different centres: NUI MAYNOOTH- National University of Ireland Maynooth;*UOW-University of Westminster, UK; **UCl-University Collage of London, UK; ***UHSM- University Hospital of South Manchester; UK, **** MSU-Montana State University, USA.

2.3 Media and growth conditions

2.3.1 Aspergillus terreus

Stock culture of the environmental strain of *A. terreus* MUCL 38669 was maintained on potato dextrose agar (PDA) (Sigma Aldrich, UK). Spores of *Aspergillus* were grown on agar slants at 28° C for seven days and subsequently stored at 4° C. The clinical isolate of *A. terreus* was grown on Sabouraud Dextrose Agar (SDA), on both slants and petri dishes at 37° C for five days. *Aspergillus* cultures were subsequently stored at 4° C. For all experiments spores were harvested using a sterile solution of 0.01% (vol./vol.) Tween 80 (BDH Laboaratory supplies) in 5 ml of distilled water supplemented with 2mm glass beads (VWR). Spores were then counted using a haemocytometer and adjusted to the desired concentration.

2.3.2 Butyrolactone I production medium

Production of butyrolactone I from *A. terreus* MUCL 38669 was carried out using a two-step process consisting of growth and production. For the growth stage *A. terreus was* grown in 500 ml Erlenmeyer flasks, containing 100 ml of inoculum medium of the following composition (for 1 L): 5 g of corn steep liquor, 40 g of tomato paste (Sainsbury's stores), 10 g of oat flour (Tesco stores), 10 g of glucose and 10 ml of a trace element solution. The pH of the solution was adjusted to 6.8 using NaOH, 1.0 M, before autoclaving (121° C, 15 psi, 10 min). Trace element solution contained (for 1 L of solution): 1 g of $FeSO_4 \bullet 7H_2O$, 1 g of $MnSO_4 \bullet 4H_2O$ (BDH Laboratory Suppliers), 200 mg of $ZnSO_4 \bullet 7H_2O$ (BDH Laboratory Suppliers), 100 mg of $CaCl_2 \bullet 2H_2O$ (Merck), 56 mg of H_3BO_3, 25 mg of $CuCl_2 \bullet 2H_2O$ and 19 mg of $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$. Cultures were inoculated with 1 ml of a spore suspension (10^7 spores per ml concentration of the spore suspension) prepared using 0.01% Tween 80 (vol/vol) as mentioned earlier. The inoculated flasks were shaken at 220 rpm (5.1 cm throw) and incubated at 27° C for 1 day for the environmental strain of *A. terreus* and at 37° C and 1 day for the clinical strains of *A. terreus.*

For the production stage a complex production medium was used containing (per L): 50 g of lactose, 25 g of glucose, 24 g of peptonised milk (Oxoid) and 2.5 g of yeast extract (Oxoid). The pH of the solution was adjusted to 7.4 using NaOH, 1.0 M, before autoclaving (121° C, 15 psi, 10 min). For the production step, 10 ml of mycelia grown in the growth medium were used to inoculate 500 ml Erlenmeyer

flasks containing 90 ml of the sterilized production medium. Flasks were then shaken at 220rpm for 72 h.

2.3.3 Butyrolactone I production in cell culture medium

It was necessary to investigate the possibility of butyrolactone I (a putative quorum sensing signal molecule) production in a medium similar to the human cells medium. *Aspergillus* clinical isolates were grown in cell culture medium (Dulbecco's Modified Eagle's Medium) (DMEM) (Lonza, Wokingham Ltd., UK). For this purpose DMEM medium containing 10% and/or 5% Foetal Calf Serum (FCS), 1% antibiotics, and 1% L-glutamine was used as the production medium. This medium is used to grow human lung carcinoma epithelial cells (A549). DMEM medium was inoculated with 10% of the growth medium (as mentioned in section 2.3.2). Flasks were then shaken at 220rpm for 72 h.

2.3.4 Aspergillus fumigatus

All *A. fumigatus* strains used in this study were clinical isolates from patients suffering from various types of aspergillosis. All clinical isolates were maintained on both Sabouraud Dextrose Agar SDA and Malt Extract Agar (MEA) media. Stock cultures of *A. fumigatus* were grown at 37°C for five days and stored at 4°C afterwards. For all experiments using any of the clinical strains of *A. fumigatus,* Approximately 2 mm x 2 mm square pieces of agar covered with the culture were cut using a sterile scalpel and added to a sterile solution of 0.01% Tween 80 containing 2 mm glass beads. The spore suspension was subsequently shaken and spores counted using a haemocytometer and adjusted to the desired spore concentrations for each experiment.

2.3.5 Gliotoxin production medium

Gliotoxin production was carried out based on the method described by (Reeves *et al.*, 2004; Kupfahl *et al.*, 2008). For this purpose flasks containing 25 ml of Roswell Park Memorial Institute medium (RPMI 1640) (Lonza, Wokingham Ltd., UK) (without sodium bicarbonate, with 25mM HEPES) supplemented with 5% FCS , 1% antibiotics, and 1% L-glutamine were inoculated with $1x10^6$ A*spergillus* conidia and incubated at 37° C and 250 rpm for 72h. For gliotoxin extraction, a flask was removed every 24 hours, pH of the culture was checked and the content was filtered through a 0.22µm filter (Millipore, UK).

The same method was used to test the gliotoxin production in *A. terreus* strains.

2.3.6 Medium used to study the effect of hydrocortisone on *A. fumigatus* and all molecular experiments

Vogel's medium, a defined minimal medium, was used for all experiments regarding the effect of hydrocortisone on *A. fumigatus* (Vogel, 1956). For all experiments a stock solution of 50x concentrated Vogel's salts was prepared and subsequently diluted using sterile distilled water. This was supplemented with the appropriate amounts of a sterile solution of 1% glucose as the carbon source to achieve 5mM glucose concentration. A 50x solution of Vogel's salt medium contained per litre: 130g ($C_6H_5Na_3O_7 \bullet 2H_2O$), 126g KNO_3, 144g $(NH_4)H_2PO_4$, 80g KH_2PO_4, 10g $MgSO_4.7H_2O$, $CaCl_2.2H_2$ 5g in 20ml distilled water, 5ml of trace element solution, 2.5ml of a 0.1mg/ml biotin solution (filter sterilized). The trace elements solution contained per 100ml: 5g ($C_6H_8O_7.H_20$), 5g $ZnSO_47H_2O$, 1g $Fe(NH_4)_2(SO_4)_2.6H_2O$, 250mg $CuSO_4.5H_2O$, 50mg $MnSO_4.H_2O$, 50mg H_3BO_3, and 50mg $Na_2Mo_4.2H_2O$. Vogel's salt solution was then filter-sterilized using a 1000 ml, 2µm pore size Stericup vacuum filtration unit (Merc Millipore, UK), and stored at 4°C.

For experiments using agar-solidified Vogel's medium 15 g of agar was also added to the salt solution. Fungal cultures were inoculated on agar plates using spore suspension, overlaid by sterile cellophane to ensure that the mycelia grew on single plane. Plates were incubated at 37° C for 15 to 48 h.

For all experiment aimed to study the effect of hydrocortisone on *A. fumigatus* growth, solutions of hydrocortisone sodium succinate SoloCortef (Pharmachia, UK) and hydrocortisone sodium phosphate (Efcottesol™) (Sovereign Medical) were added to either solidified or liquid Vogel's medium before inoculation to prepare the medium with a final concentration of 10^{-5} M and 10^{-6} M hydrocortisone. Vogel's medium without hydrocortisone was used as control.

2.3.7 Medium used for *in vitro* stimulation studies

For all *in vitro* stimulation experiments *A. fumigatus* AF-10, *A. fumigatus* AF-293, and gliotoxin knockout strain of *A. fumigatus* (ARC-2), were grown in RPMI 1640 medium (Lonza, Wokingham Ltd., UK) (without sodium bicarbonate, with 25mM HEPES) supplemented with 5% FCS , 1% antibiotics, and 1% L-glutamine. Strains were inoculated with conidia at concentration of $1x10^6$ per ml and incubated at 37°C for minimum 48h.

2.4 Assay procedures

2.4.1 pH and biomass measurement

Fungal growth-curves were drawn by plotting the dry biomass weight against time. At particular time intervals cultures were harvested using grade No. 54 filter paper (Whatman international Ltd., UK) that had been pre-weighted and stored under desiccating conditions. Filter papers containing fungal biomass were dried at 80°C in the oven for 24 h and placed under desiccating conditions for 10 minutes before weighing. The difference between the tare weight of the filter paper and the combined weight of the filter paper together with fungal biomass was considered as biomass dry weight.

pH of the samples taken from shaken flask cultures were monitored using a pH probe (VWR, UK).

2.4.2 Butyrolactone I extraction

For this assay 2 ml samples from *Aspergillus* cultures were taken every 24h and equal volume of methanol was added to them. They were then thoroughly mixed in a homogeniser (MPbio, UK) for 40 seconds at a speed of 4.5 M/S. The extracted samples were then injected into an HLPC system for both detection and quantification. Butyrolactone I from the methanolic layer was quantified by High-Performance liquid chromatography.

2.4.3 Butyrolactone I quantification by High Performance Liquid Chromatography (HPLC)

Butyrolactone I was quantified by High-Performance liquid chromatography (HPLC) using a C18 column (Dionex, Acclaim 120; C18, 5µM x 4.6mm x 150mm). The mobile phase was 0.1% aqueous phosphoric acid and acetonitrile in the ratios of

45/55 (v/v). Butyrolactone I was eluted isocratically at a flow-rate of 1ml/min and detected at 308 nm and quantified using butyrolactone I standard obtained from BioMOL International, UK.

2.4.4 Gliotoxin extraction

Gliotoxin extraction was carried out according to the method of Reeves, 2004 and Kupfhal, 2008. Fungal cultures were harvested post-inoculation by filtration every 24 hours, using a grade No. 54 filter paper. Culture-filtrate was then mixed with an equal volume of chloroform for 30 minutes in room temperature. The chloroform layer was then separated using a separating funnel and was evaporated to dryness in a vacuum rotary evaporator, reconstituted in 500 µl of acetonitrile. Samples were then transferred to HPLC vials and stored at -20 until use.

2.4.5 Gliotoxin detection by Thin Layer Chromatography (TLC)

After extraction, Gliotoxin was detected by loading approximately 15 µl of each sample onto the silica TLC plate containing a fluorescent indicator (Sigma, UK) along with gliotoxin standard (sigma, UK). TLC plates were then developed in toluene-ethyl acetate-formic acid (5:4:1, v/v/v) (Xiao *et al.*, 2010). Photographs of TLC plates were taken following exposure to UV radiation using a digital camera.

2.4.6 Gliotoxin quantification by High Performance Liquid Chromatography (HPLC)

For detection and quantification of gliotoxin a C18 column (Dionex, Acclaim 120; C18, 5µM x 4.6mm x 150mm) was used and 20 µl of each sample were injected into the HPLC column. The mobile phase consisted of 0.1% Trifluoroacetic acid (TFA) and acetonitrile in the ratios of 65.1/34.9 V/V. Gliotoxin was eluted at a flow rate of 1ml/min and detected at 270nm and quantified using gliotoxin standard obtained from Sigma, UK.

2.4.7 Cell viability assay (MTT assay)

Cellular metabolism (cell viability) was assessed using 3(4,5-dimethylthiazol-2yl) 2,5-diphenyltetrazolium bromide (MTT) assay for which 5mg/ml reagent in Dulbecco's Phosphate-Buffered Saline (DPBS) (Lonza, Wokingham, UK) prepared. Monolayers of lung epithelial cells (A549 cell line) were cultured in DMEM medium containing 10 % FCS, 1% L-glutamine, and 1% penicillin-streptomycin (as

described in section 2.5.3). Cells were cultured at 37° C in an incubator with 5 % CO_2 in the air. Cells were then harvested at around 70-80% confluency using 1x trypsin-EDTA solution centrifuged at 400 relative centrifugal force (rcf) for 10 min at room temperature. Following the removal of supernatant, the obtained pellet was re-suspended in fresh medium and cells were counted using a haemocytometer.

A549 cells were then seeded in a 96 well tissue culture plate (triple Red Ltd., UK) at the concentration of 10^5 cells per well and grown over night at 37° C in the presence of 5% CO_2 to become confluent in the wells. Plates were washed with DPBS solution and Cells were exposed to gliotoxin (Sigma, UK) and cortisol (Sigma, UK) for different time-spans ranging from 4 to 48 h before response was quantified. For each treatment, 10 µl of MTT dye solution was then added to the 100µl of medium in each well at the end of exposure time. The plates were incubated at 37°C for 2 hours. After the 2 h exposure to MTT dye, all wells in each plate were drained completely and then 120 µl of Dimethyl Sulfoxide (DMSO) was added to each well to dissolve the crystalline formazan. The plates were then hand- swirled for 3-5 min until the crystals were dissolved. Absorbance was measured at 540 nm on a THERMOmax plate-reader ROM version 1.72 (Molecular Devices, Hampshire, UK) (Peterson *et al.*, 2010). The results of the MTT bioassay were used as a parameter for viability. The results are presented in percentage viability (%viability= 100x OD mean sample/OD mean control).

For each experiment two independent assays were carried out with triplicates for each concentration as well as the controls. The relative toxicity of each tested substance was quantified after each time-point by determining the average effective concentration (EC50). The average effective concentration was calculated as the concentration required to reduce the cell concentration to 50% of that measured in the control (Gräbsch *et al.*, 2006).

2.4.8 Protein assay

Protein concentration was determined using the Bradford method. 1ml of Single strength Bradford reagent (Sigma Aldrich, UK) was added to 20 µl of each sample.

The contents were mixed by vortexing and after incubation for 5-10 min the absorbance was measured at 595nm. A standard calibration curve was prepared by plotting the protein concentration versus absorbance at 595nm. Bovine Serum Albumin (BSA) was used to prepare all standard concentrations ranging from 125 to 1000 µg/ml. Concentrations of proteins in the samples were determined using the standard calibration curve.

2.5 Animal cell culture conditions

2.5.1 The human alveolar adenocarcinoma cell line (A549) and reagents

The human alveolar adenocarcinoma cell line (A549) was originally established, together with twelve other cell lines, by Giard DJ *et al.*, 1973 through removal and culturing of tumor specimens from a malignant lung tissue of a 58 year old Caucasian man. After establishing the cell line, the cells were capable of maintaining their morphology over continuous propagation for more than 80 passages when cultured in monolayer in a medium containing 10 % serum.

A549 cells are adherent i.e. they attach to the bottom of the cell culture flask, and grow as monolayers *in vitro*.

The A549 cell line used in this study was kindly provided by Dr. John Holton from UCL, Windeyer Building.

The following animal cell culture reagents were purchased from Lonza, Wokingham, UK: Dulbecco's Modified Eagle's Medium (DMEM), containing 4.5 g/L glucose and without L-glutamine, penicillin-streptomycin mixture (10,000 units/ml potassium penicillin G/10,000 units/ml streptomycin sulphate), 1x Dulbecco's Phosphate Buffered Saline (DPBS) without calcium (Ca^{2+}) and magnesium (Mg^{2+}), 10x trypsin-ethylenedaminetetraacetic acid (EDTA) [5 g/L trypsin, 2 g/L EDTA] solution, and L-glutamine solution (200 mM).

Heat inactivated foetal calf serum (FCS) (South American origin) was supplied by Biosera Ltd., Ringmer, UK, and dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich, Poole, UK. Cell culture flasks (25 and 75 cm^2), 6 well and 96 well tissue culture plates, Millipore Millex GP PES 0.2 µm sterile syringe filters, 15 ml and 50 ml centrifuge tubes, 10 ml and 25 ml disposable pipettes were all obtained

from Fisher Scientific, Loughborough, UK; while 2 ml CRYO.S cell freezing vials were bought from Greiner Bio-one Stonehouse, UK.

2.5.2 Continuous culture conditions for A549 cells

The A549 cells were cultured in DMEM medium supplemented with 10x FCS, 1x L-glutamine, and 1x penicillin/streptomycin. Cell viability, morphology and confluence were observed by light microscopy. When the cells in tissue culture flasks were fully confluent (normally confluence was achieved within 3-4 days) they were split into more flasks at a 1:2 split ratio. For this purpose, the cell culture medium in the original flask was emptied and cells rinsed with 10 ml of Ca^{2+} and Mg^{2+} free DPBS followed by addition of 5 ml of 1x trypsin-EDTA solution. This was done to detach the cells from tissue culture flasks. Trypsin-EDTA was inactivated by adding an equal volume of serum supplemented DMEM medium and the resulting cell suspension was centrifuged at 400 rcf for 10 min at room temperature. Following the removal of the supernatant, the obtained pellet was suspended in fresh medium and stored in a Galaxy carbon dioxide humidifier incubator (Wolf Laboratories, York, UK), in the presence of 5 % CO_2 at 37° C.

Culturing was routinely performed in 75 cm^2 tissue culture flasks in a total volume of 15 ml per flask and subcultured twice weekly. All tissue culture maintenance and experiments were carried out in a BIOAIR Aura 2000 Class II cabinet (Wolf Laboratories, York, UK). Cells were checked daily for morphology and bacterial and fungal infection by observing the status of the medium as well as microscopic examination (clarity of the medium and lack of any unusual substances indicates that there is no contamination). Cells used for the experiment were between 5-30 passages old after which cultures were discarded.

2.5.3 Preparation of cells for storage

In order to ensure a continuous supply of cells for future use A549 cells were stored in Liquid Nitrogen to maintain the maximum possible viability of the cells in long term storage. For this purpose cells were first harvested by addition of trypsin and centrifugation (section 2.5.2). The pellet was then re-suspended in 6 ml of freezing medium containing 50 % FCS, 20 % DMSO, and 30 % DMEM without supplement. Aliquots (2 ml) of cell suspension were added to CYRO.S cryo vials. Vials were then transferred to a Mr. Frosty freezing chamber (Merk, Leicester, UK)

containing isopropanol (to ensure a controlled rate of freezing) for a minimum of 24 h and subsequently transferred to Liquid Nitrogen container.

2.6 *In vitro* stimulation of A549 cells and Cell death

The number of apoptotic and necrotic cell populations was determined using flow cytometry to investigate the cytotoxicity of aspergillus culture broth as a result of gliotoxin production and to check the possibility of changes in cytotoxity of the fungal culture broth before and after hydrocortisone treatment.

Hydrocortisone sodium phosphate (Efcottesol™) (Sovereign Medical) was kindly provided by Dr. John Holton. Glitoxin was produced by *A. fumigatus* AF 293 and released into the culture broth. Gliotoxin was detected and quantified as mentioned in sections 2.4.4 to 2.4.6.

Southern Biotech Aposcreen Annexinä V Apoptosis kit containing Annexin V-Fluorescein isothiocyanate (FITC) conjugate (200 µg/ml stock solution supplied in 1x binding buffer), Aposcreen™ 10x Annexin V binding buffer, propidium iodide, (100 µg/ml stock solution, supplied in PBS/sodium azide-NaN_3) was purchased from Cambridge Bioscience Ltd. Cambridge, UK, and used for flow-cytometry studies.

2.6.1 *In vitro* stimulation of A549 cells

In vitro stimulation of lung epithelial cells for apoptosis and necrosis investigation was carried in a two stage process. The first part of the experiment involved inoculation and growth of *A. fumigatus* wild type strains AF-293, AF-10 and gliotoxin knockout strain ARC-2 (Δglip) into 250 ml shaken flasks containing 50 ml of the RPMI 1640 medium without sodium bicarbonate with 25 mM HEPES, supplemented with 5% FCS (25ml in a bottle of RPMI containing 500ml) and 1% penicillin-streptomycin (5ml in 500ml), with L-glutamine. Spore suspension of each strain was used for inoculation to make final concentration of 1 x 10^6 conidia/ml in each flask. Fungal cells were incubated for more than 48 h in 37° C in order to ensure the gliotoxin production.

The second part, which involved the stimulation of lung cells, was carried out by seeding A549 cells on 6 well tissue culture plates at a density of 4.5 x 10^5 cells per well to reach approximately 70-80 % confluency (~ 5 x10^5 cells per well).

A549 cells were then treated for 4 h, 8 h, 12 h, 24 h and 48 h with 40% sterile filtered culture broth of *A. fumigatus* AF 293 and *A .fumigatus* ARC-2 (ΔgliP). The control cells were only treated with DMEM medium.

A. fumigatus strains AF-293 and ΔgliP were also used for the investigation of the effect of hydrocortisone treatment on gliotoxin production by *A. fumigatus* strains. The strains were grown in RPMI 1640 medium containing 10^{-6} M and 10^{-5} M hydrocortisone. The control cultures were without any hydrocortisone treatment.

In order to treat the A549 cells with fungal culture broth, fungal cultures were harvested by filtration through a 0.2µm pore size Stericup vacuum filtration unit (Merc Millipore, UK) and the filter-sterilized culture broth was then added to A549 cells as mentioned above.

Following the treatment, A549 cells were harvested and prepared for flow cytometry analysis as mentioned in section 2.6.2.

2.6.2 Flow cytometry experiments

The assessment of apoptosis and necrosis following treatment with above mentioned stimuli was determined by Annexin-V-FITC/propidium iodide (PI) labelling followed by flow cytometry.

The Annexin-V-PI labelling is based on the observation that during the early stages of apoptosis in the cells the phosphatidylserine (PS) phospholipids that are normally located in the inner cell membrane translocate to the outer membrane followed by loss of the plasma membrane lipid symmetry. This universal process, which occurs during the early stages of apoptosis, is independent of the cell type, species and the apoptosis induction factor. This externalization of the PS to the extracellular environment can be detected by binding of the Annexin V, a calcium dependent binding protein, that has a high affinity to PS. The binding of the Annexin V to PS can be visualised by conjugating Annexin V to a fluorochrome such as Fluorescein isothiocyanate (FITC)(Fig. 2.1)(Van Engeland *et al.*, 1998).

During the latter stages of the programmed cell-death, apoptotic cells may lose the cell membrane integrity and become permeable to PI. Therefore, as a result of Annexin-V-FITC/PI labelling, cells represent the following: viable cells (Annexin-V-

FITC negative, PI negative), early apoptotic cells (Annexin-V-FITC positive, PI negative), late apoptotic and necrotic cells (Annexin-V-FITC positive, PI positive) can be detected and visualised by flow cytometry.

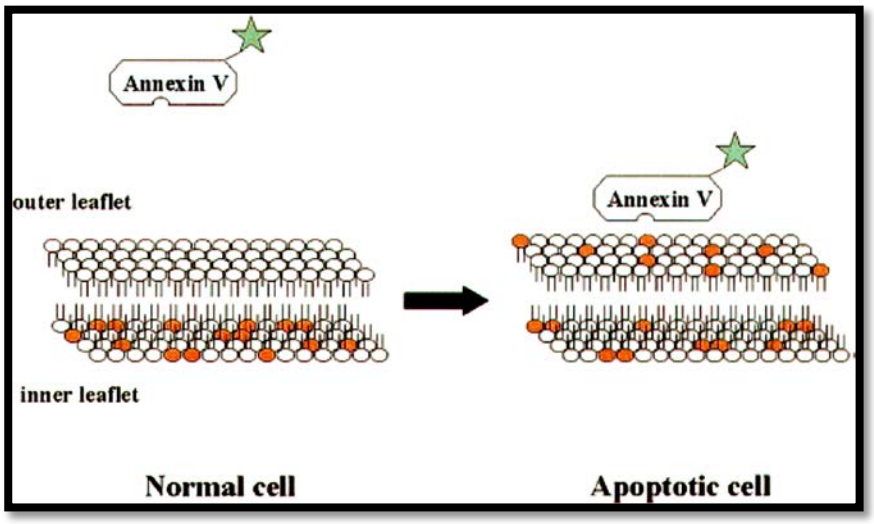


Figure 2.1
A Schematic representation of the Annexin-V-FITC conjugate calcium dependent binding to phosphatidylserine (red circles) following its translocation to the outer membrane and loss of cell membrane lipid symmetry. Picture adapted from (Van Engeland *et al*., 1998).

2.6.3 Detection of apoptosis and necrosis in A549 cells following Annexin-V-FITC/PI labelling using Flow cytometry

The flow cytometry experiment was initially optimised in a 4 tube protocol: tube 1: unstained cells (cells in 500 µl of Aposcreen™ 1x Annexin V binding buffer without staining); tube 2: cells stained with Annexin-V-FITC only; tube 3: cells stained with PI only and tube 4: Annexin-V FITC and PI labelled cells. The assay required optimisation in order to determine the correct voltage for focusing the light emission from the fluorochromes onto the photon detector as well as establishing the fluorescent compensation on the flow cytometer required for post data analysis.

Following protocol optimisation, the number of apoptotic and necrotic A549 cells was determined using a Southern Biotech Aposcreen Annexinä V Apoptosis kit.

Following the treatment of the cells for any of the time points (4 h, 8 h, 12 h, 24 h, and 48 h) with filter sterilised culture broth of aspergillus strains in 6 well tissue culture plates, A549 cells were harvested using the following steps: first the supernatant (containing the spent medium and the detached cells) was collected

and the remaining cells were rinsed with 1 ml of DPBS which was also collected. Adherent cells were detached from the bottom of the plate by trypsin treatment and added to a centrifuge tube containing the collected supernatant. The tube was subsequently centrifuged at 400 rcf for 10 min. After centrifugation the supernatant was drained and the pellet was suspended in 1 ml of cold DPBS to avoid any remnants of the culture medium used to deactivate the trypsin. Cells were then centrifuged again at 4° C, 400 rcf for 5 min. After centrifugation the supernatant was discarded and pellet was disaggregated by gently flicking the tubes. The cells were then re-suspended in 100 µl of the Aposcreen™ 1x Annexin-V binding buffer. Tubes were placed in ice and to each tube 5µl of Aposcreen™ Annexin-V-FITC conjugate (0.5 µg/ml) was added and incubated in ice and in the dark for 15 min. A further 390 µl of the Aposcreen™ 1x Annexin-V binding buffer was added to each tube followed by addition of 5 µl of Propidium iodide (1 µg/ml) and gentle mixing. Samples (500 µl total volume) were analysed by flow cytometry using Cyan™ ADP flow cytometer (Dako cytomation, Ely, UK). Unstained samples were prepared following protocol optimization for each experiment which consisted of cells in 500 µl Aposcreen™ 1x Annexin-V binding buffer without Annexin-V-FITC and PI staining. The unstained cell population was used in the post analysis of data to determine the quadrant regions used for establishing viable, early apoptotic, late apoptotic and necrotic cell populations generated from the FITC and PI fluorescent channels. A total of approximately 5x 10^5 cells were prepared for each sample of which 10,000 cells/events were counted and analysed per experimental condition. Cells were gated on forward scatter (FSC) and side scatter (SSC) dot plot histogram to exclude cellular debris. Percentage apoptosis was determined from the 10,000 cell/even gated into four quadrants (indicating different subpopulations of cells: viable cells, early apoptotic, late apoptotic and necrotic) and the number of events/cells in each quadrant indicates the number of apoptotic, necrotic or viable cells.

Subsequent analysis of the flow cytometry data was carried out using Summit v4.3 software (Dako cytomation, Ely, UK).

2.7 Proteomic studies

A. fumigatus clinical isolate, AF-10, isolated from a patient with invasive pulmonary aspergillosis treated with adrenocorticotrophic hormone that results in increases levels of cortisol, was used throughout all proteome experiments (Ng *et al.*, 1994). Sabouraud Dextrose Agar (SDA) medium was used for the maintenance of the cultures. All cultures on slants and plates were grown at 37° C for 3-5 days and subsequently stored at 4° C. Vogel's minimal medium containing 5mM glucose was used for all liquid culture experiments (i.e. shaken flask) as mentioned in section 2.3.6. For inoculation of the medium, spores of *A. fumigatus* were harvested with a sterile solution of 0.01% Tween 80 (v/v) (BDH Laboratory Supplies) in phosphate buffered saline (PBS) supplemented with glass beads (VWR) and then counted using a haemocytometer. Spore concentration in the medium was adjusted to $1x10^5$ spores/ml in all experiments. All shaken flask cultures were grown at 37°C at 220 rpm.

2.7.1 Protein extraction and cell fractionation

For protein extraction, *Aspergillus* mycelium was harvested by filtration through Whattman filter paper no.54 using a vacuum pump connected to a side arm conical flask. Mycelium was then rinsed with cold distilled water and 0.6 M $MgSO_4$. The excess liquid was removed by sandwiching the mycelia between filter paper layers. The mycelia were then stored at -80° C and subsequently freeze dried in a vacuum freeze dryer (Savant modulyo D, Thermo Fisher Scientific, Loughborough, UK). To lyse the cells and extract the total protein, 50 mg freeze dried mycelium was added to Lysing matrix C tubes (Q-BIO Gene, MP Biomedicals) containing glass beads together with lysing buffer (lysing buffer was consisted of 20mM Tris buffer pH 7.5, 1mM EDTA, 1mM DTT, and 40µl/ml fungal protease inhibitor cocktail (Sigma, Poole, UK). The contents of all tubes were homogenized in homogenizer (FastPrep® 24, MP Biomedicals, UK) at a speed of 6.5M/S for 8-10min in 30 second bursts of homogenization followed by cooling on ice. Cell fractionation was carried out according to the method described by (Cagas *et al.*, 2011). Based on this method, after protein extraction, samples were centrifuged at 5000 x g for 10 min to remove the cell wall and plasma membrane fractions (the pellet). To isolate the cytoplasmic proteins the supernatant of the centrifuged samples were centrifuged again in an ultracentrifuge (Sorvall® Discovery™ 100SE, Kendro®) at a speed of

100, 000 x g for the period of 1 h at 4° C. The supernatants from these samples were considered as cytoplasmic proteins and used for all subsequent experiments including affinity chromatography and 2-dimentional gel electrophoresis proteomic studies (2D).

2.7.2 Binding of hydrocortisone to the column and Affinity chromatography

The initial aim of the experiments involved affinity chromatography and proteomics. In order to investigate the presence of a cortisol binding protein in *A. fumigatus* cytoplasmic protein content, affinity chromatography experiments were carried out by binding hydrocortisone sodium succinate to an activated Sepharose gel (GE Healthcare) using a Bio-Rad 10ml column. For this purpose Hydrocortisone sodium succinate SoloCortef (Pfizer Pharmachia, UK) was bound to the column according to the manufacturer's instructions and an earlier method by (Rosner and Bradlow, 1971; Mahajan *et al.*, 1980). The following protocol was followed: 2 mls of the activated medium (EAH Sepharose 4B), pre-swollen in 20% ethanol solution (GE Healthcare), was centrifuged at 3090 x g for 5 min to discard the ethanol. [EAH Sepharose™ 4B contains an activated amino group and is used to immobilize carboxyl-containing ligand]. It was subsequently washed with 2 ml of distilled water. In the next step distilled water was added, to make a final volume of 2 ml, followed by centrifugation. Hydrocortisone sodium succinate solution (0.5 ml of 0.1 M) was added to 0.5 ml of Dioxane (Sigma-Aldrich) and mixed thoroughly. Then 1 ml of 0.2 M carbodiimide [to encourage the condensation between a free amino and a free carboxyl group to form a peptide link] (sigma, UK) in distilled water (pH 4.5) was added to the above solution, mixed and added to the gel. The gel was left shaking for 24 h at the room temperature. In the first hour pH was adjusted using 0.1 M HCl if necessary. After 24 h the medium was centrifuged at 3090 x g for 5 min and the supernatant was discarded. These steps were then followed by addition of 2 ml of acetic acid and centrifugation after 2 min. The supernatant was discarded. The pellet was then washed thoroughly with 4 cycles of alternating pH using 2 ml of 0.1 M acetate buffer pH 4 containing 0.5 M NaCl followed by 0.1 M Tris buffer pH 8.3 containing 0.5M NaCl, for each wash cycle. Finally, pH was checked and the gel was poured into the column.

After packing the gel, partial purification of the cytoplasmic proteome of the *A. fumigatus* was carried out as the steps described below:

1. Prepared gel (as above) containing 20% ethanol was added to the column (Bio-Rad, 10 ml) and the column was left open for the ethanol to drain off.
2. Equilibration buffer: 10 ml of the equilibrium buffer (0.05M phosphate buffer pH 7.0 containing 0.2M Nacl) was added and left until it all flow through the column.
3. Then 5ml of the sample (cytoplasmic fraction of the total extracted protein from *A. fumigatus* AF-10) was added and the sample that passed through the column was collected (containing non-bonded proteins)
4. Wash buffer (0.001M phosphate buffer pH 7.0) (10ml) was added and passed through the column.
5. The first elution buffer (wash buffer containing 200µg/ml cortisol) (10ml) was added and ten samples of 1ml elution fractions were collected. The protein content was checked using Bradford protein assay (as mentioned in section 2.4.8). No protein was detected in any fractions at this stage.
6. The second elution buffer (0.05M phosphate buffer pH 7.0 containing 0.2 M Nacl and 200 µg/ml cortisol) (10ml) was added and again ten samples of 1ml elution fractions were collected and the protein content was checked. The first eluted fraction contained no protein, the second had the highest protein content and from the second fraction until the tenth fraction the protein content decreased gradually. The protein content in the second fraction was used for 2D gel electrophoresis analysis.

2.7.3 Protein precipitation and one-dimensional (1D) gel electrophoresis

Separation of proteins in the elution fractions from the sepharose-cortisol sodium succinate column was carried out using two dimensional gel electrophoresis after a protein precipitation step.

2.7.3.1 Protein precipitation

To a 150 µl of sample (containing approximately 150-300 µg of protein), 600µl of methanol was added and mixed by vortexing. Then 150 µl of chloroform and 450 µl of ultrapure water was added and mixed thoroughly. The mixture was then

centrifuged at 12000 rpm for 5 min. After centrifugation the upper layer was discarded. The white disc that formed between the upper and the lower phases was kept by carefully pipetting out as much as possible of the upper and lower layers without disturbing the disc. Finally 450 µl of methanol was added to the tube containing the disc and mixed. The mixture was then centrifuged at 12000 rpm for 5 min. The supernatant was discarded and the pellet was air dried. Precipitated proteins were then re-suspended in 200µl of buffer containing (per 10ml buffer) 6M urea, 2M thiourea, 0.5% (v/v) CHAPS, and 0.5% (v/v) pharmalyte pH 3-10 (Amersham, UK). After precipitation the protein content was quantified using Bradford assay.

2.7.4 1-D Gel electrophoresis; isoelectric focusing

For the first (one-dimensional) dimensional gel electrophoresis, Isoelectric focusing (IEF) of the purified cytoplasmic fraction from *A. fumigatus* mycelium was carried out with IPG strips (7cm length-NL (nonlinear) covering a pH range from 3 to 10 (GE Healthcare). A total of 80 µg of proteins in a total of 150 µl of rehydration buffer (6 M Urea, 2 M thiourea, 0.5% (v/v) CHAPS, 0.5% (v/v) pharmalyte (pH 3-10) (Amersham, UK), 0.4% (w/v) DTT, and Bromophenol Blue) was applied to the 7cm-NL Immobiline IPG strips (pH 3-10), overlaid with mineral oil (Sigma-Aldrich, UK) and left for overnight rehydration in a swelling tray (Amersham, UK). Proteins were allowed to absorb onto the 7 cm immobilised gel strip (IPG). All experiments were carried out in replicates of 6, using protein samples from different cultures.

First dimension was carried out on a Multiphore II (Pharmacia Biotech) electrophoresis system for a total focusing time of 4 h and 5 min. Temperature during isoelectric focusing was maintained at 17° C using Amersham Multiptemp III thermostatic circulator cooling unit. Strips were placed gel-side-up onto the dry strip aligner tray and electrode strips were placed on both anode and cathode ends of the IPG strips after soaking them in distilled water. A small amount of mineral oil was poured onto the cooling ceramic block before placing the aligner tray over the block. This was done to minimize evaporation and urea crystallization. Electrodes where then connected to the IEF electrophoresis unit and the IPG strips were focused for 4 h and 5 min using an Amersham EPS 3501 power pack with the following voltage profile: A linear increase from 0 to 300 V for 30 min, A linear

increase from 300 to 600 V for 30 min followed by a linear increase from 600 to 3500 V for 3h and 5 min.

At the end of the first dimension run strips were equilibrated for unfolding, avoiding point streaking, and easier transfer of proteins from the IPG strips to the SDS-PAGE using two buffers. The first equilibration buffer contained 5mM Tris-HCl, pH 8.4, 6M Urea, 30% glycerol, 2% sodium dodecyl sulphate (SDS), 0.02% Bromophennol Blue, 50mM DTT). Strips were equilibrated in this buffer for 15 min while rocking in a speed of 40. The strips were then further equilibrated in the second equilibration buffer (pH 8.4) containing (5 mM Tris-HCl, 6 M Urea, 30% glycerol, 2% SDS, 0.02% Bromophennol Blue, and 100 mM iodoacetamide) while rocking at 40 speed for 15 min the strips were then loaded on a 12.0 % SDS-PAGE gel for the second dimension.

2.7.5 Two-dimensional (2D) gel electrophoresis and protein identification

The second dimension was carried out by loading the equilibrated strips onto a 1mm thick 8 x 7 cm, 12.0% (w/v) SDS-PAGE resolving gel containing 3.3 ml distilled water, 2.5 ml of 1.5 MTris-Hcl (pH 8.8), 4.0 ml of 30% Bio-Rad Acrylamide Mix, 100 µl of 10% SDS stock solution, 100 µl of 10% ammonium persulfate (APS), and 10 µl of N,N,N',N'-tetramethylethylenediamine (TEMED). Molecular weight marker (Pierce Prestained Protein Molecular Weight Marker, Thermo Scientific, UK) was loaded onto a small piece of filter paper and placed on the low pH end of the strip on the gel. IPG strips were sealed onto the SDS-PAGE resolving gel by adding the sealing buffer containing (0.25 g agarose, 50 ml Laemmli electrolyte buffer (containing 1% SDS (w/v), 0.25 M Tris base, 1.92 M Glycine, and 200 µl of 0.002% Bromophennol Blue) over the strips. SDS-PAGE electrophoresis was carried out using a Bio-Rad protein electrophoresis unit at 150 V for about 90 min until the dye-front reached the bottom of the gel. After second dimensional electrophoresis, the gels were stained with Coomassie brilliant blue G-250 (Sigma-Aldrich, UK) dye in 10% (v/v) acetic acid by microwave-heating. This was followed by distaining with 10% (v/v) acetic acid to remove the excess dye until the protein spots became visible. Gels were then stored in 2% acetic acid at 4° C.

2.7.6 Image analysis

Stained gels were scanned using GS800 densitometer (Biorad, Uk) and the resulting images were obtained with Quantity II software. All images were converted to a TIFF file and analysed using Progenesis PG240 Samespot software (Nonlinear Dynamics, UK). Six gel pictures from each of the test and control experiments were uploaded onto the progenesis software for analysis. The amount of protein in each spot was determined according to spot intensity. The images from the test cultures were compared to the control counterparts for each time-point of the experiment. Fold changes in protein spots between the test and control images were calculated based on the *p*-value from one way ANOVA and only spots with *p*-value ≤0.05 and maximum fold change ≥1.5 were considered in the final analysis.

2.7.7 Protein identification

Spots that differed significantly in the intensity between test and control cultures at 48 h were cut out and sent to the University of York Mass Spectrometry unit (UK). The spots were identified by MALDI-TOF/TOF (Matrix-Assisted Laser Desorption Ionization tandem Time of Flight) which allows identifications by including tandem spectral data in Mascot search. The resulting data was further identified by the input of the resulting sequences into protein Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins)

2.8 Transcriptomic studies

2.8.1 Molecular biology reagents

2.8.1.1 RNA extraction, agarose gel electrophoresis (AGE) and sequence analysis

RNeasy® plant Mini kit and Qiaquick® Gel Extraction kit were obtained from Qiagen Ltd., Crawley, UK.

Ethidium bromide (10mg/ml) and 5x tris base boric acid EDTA (TBE) buffer (pH 8.0) composed of (in 500 ml) Tris base, 27 g; boric acid, 13.75 g and disodium EDTA 1.9 g were purchased from Sigma-Aldrich, Poole, UK.

Molecular biology grade agarose, Hyperladder™ IV (100-1000 bp) DNA ladder and 5x sample loading buffer were obtained from Bioline Ltd., London, UK.

2.8.1.2 Reverse transcriptase-Polymerase Chain Reaction (RT-PCR) and quantitative PCR (qPCR)

ImProm-II Reverse Transcription system/kit was purchased from Promega, UK and used for reverse transcription of total RNA.

PCR Master Mix was also obtained from Promega and was used to carry out Polymerase Chain Reaction (PCR).

In order to remove any genomic DNA contamination from the extracted RNA, TURBO DNA-*free™*kit was obtained from Ambion® Applied Biosystems®, UK.

All quantitative PCR experiments were carried out using a Rotor Gene™ SYBR® Green real-time PCR kit from Qiagen Ltd., Crawley, UK.

Forward and reverse primers were obtained from Invitrogen™ Fisher Scientific, Loughborough, UK for the following genes: TEF-1, HSP70, MMSAD, ALP-2, gliZ and gliP, FBA.

The primer sets used in PCR and real-time qPCR experiments are detailed in Table 2.2. PrimerBlast software (URL http://www.ncbi.nlm.nih.gov/tools/primer-blast) was used to design all primers except for ALP-2 and gliP. The latter two primers were predesigned from the published data (Reichard *et al.*, 2000; Gardiner and Howlett, 2005). Where appropriate primers were designed to span exon splicing junctions in order to ensure that amplicon generation are from mRNA, avoiding amplification of genomic DNA.

Specificity of all primers for *A. fumigatus* genome was verified by input of the sequences into the Basic Local Alignment Search Tool (BLAST) (URL http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene	Accession number	Forward Primer	Reverse Primer	Expected product size (bp)	Annealing temperature°C
TEF-1	XM_745295	5'TCCCCGGTGACAACGTCGGT3'	5'GGTGACCTTACCGGCACCGC3'	449	56
HSP70	XM_745397	5'CGAGGGCGAGCGTAACGTCC3'	5'GCGTTGGTGGTGAGATCCTGTGA3'	261	56
MMSAD	XM_746452	5'TGCCGATCTCGGCCCTGTCA3'	5'GCCACAGGCTGGTCACGGTC3'	500	56
Alp-2	XM_748625	5'GCCTACACCATTGACACTGG3'	5'GTTGACTGCGTGCTTAGAGC3'	591	55
Glip	XM_745762	5'AAACCCCTGTGAATGCAGAC3'	5'CCCCTTGAGATGAAAGGTGA3'	118	53
Gliz	XM_745759	5'AAGCTGCTGCGCTCCTGCAA3'	5'GGCGGTGGCGGTCTTTTCCA3'	137	54
FBA	XM_749359	5'AGCTTTCCCGCAAGTCCGGC3'	5'CTTGGCGCAGTGGTCGGTGT3'	326	55

Table 2.2
Sequences of the primer sets used in PCR and two-step RT-PCR experiments

2.8.2 Total RNA isolation from *A. fumigatus*

Aspergillus fumigatus was grown on Vogel's minimal medium with or without hydrocortisone (section 2.3.6.). The cultures were harvested after 48h by filtration through a grade 54 Whatman filter paper. The fungal biomass was then frozen at -80° C and subsequently freeze-dried in a vacuum freeze dryer (Savant modulyo D, Thermo Fisher Scientific, Loughborough, UK) and stored at -20° C freezer to be used later.

To extract total RNA, 1ml of RLT buffer (Qiagen Ltd. Crawley, UK) was added to 0.05-0.08 g of the freeze-dried culture in Lysing Matrix C tubes (Q-BIO Gene) and disrupted in a bench top homogeniser (FastPrep® 24, MP Biomedicals, UK). Following the cell disruption step, total RNA was extracted from cells using RNeasy Mini Plant kit (Qiagen) according to manufacturer's instructions. To avoid RNase contamination, sterile disposable plastic-ware and RNase free water (Sigma-Aldrich, UK) were used. Subsequent addition of appropriate extraction reagents and centrifugation steps resulted in the elution of total RNA from the Spin column membrane into 50 µl of nuclease free water.

RNA purity and concentration (ng/µl) was determined by measuring the Optical Density (OD) at A_{260}/A_{280} using Thermo Scientific NanoDrop™ 1000 v3.7.1 (Fisher Scientific, Loughborough, UK). Typically when the A_{260}/A_{280} absorption ratio is between 1.8 to 2, it indicates that isolated RNA is pure, is not degraded and is of good quality without protein interference (Fleige *et al.*, 2006).

RNA integrity was verified by gel electrophoresis on a 1% (w/v) agarose gel in 1x TBE buffer and visualised after Ethidium bromide (10mg/ml) staining using a UV trans-illuminator (UViTec, Cambridge, UK).

RNA samples were then stored in the -80° C freezer.

2.8.3 Reverse transcriptase, Polymerase chain reaction

Complementary DNA (cDNA) synthesis from *A. fumigatus* mRNA was carried out using ImProm-II Reverse Transcription system (Promega, UK) according to manufacturer's instructions. Total RNA (250ng, 3µl) was reverse transcribed into cDNA in an overall reaction volume of 20 µl containing 2 µl oligo $(dT)_{15}$ primer, 1µl dNTP mix 0.5 mM, 0.5 µl recombinant RNasin® ribonuclease inhibitor (20-40 units/µl), 1µl Reverse Transcriptase (RT) 5% (vol/vol) enzyme, 1.8µl Magnesium chloride $MgCl_2$ 2 mM, 4 µl 5x reaction buffer and 6.7µl RNase-free water. RNA was initially incubated with the oligo $(dT)_{15}$ primers at 70° C for 5 min in order to denature the secondary structure of RNA and allow annealing of primers to the template RNA. The mixture was then chilled on ice until the reverse transcription reaction was added. The reverse transcription was performed for a total of 1 h 20min at annealing temperature of 25°C for 5 min, extension at 42° C for 1h and inactivation of the reverse transcriptase at 70° C for 15 min. The resulting cDNA was subsequently stored at -20° C to be used later.

In order to ensure that the designed primers gave individual specific products of the desired size, the cDNA obtained from the reverse transcription reaction was directly used as template and amplified using PCR in a thermal cycler (MJ Mini, Biorad, Thermo Fisher Scientific, Loughborough, UK) using a PCR Master Mix (Promega, Ltd., UK). The reaction mixture had a final volume of 25 µl and consisted of 12.5 µl Master Mix, 1 µl Forward primer (10 µM), 1 µl Reverse primer (10 µM), 1 µl template cDNA, and 9.5 µl Nuclease free water. A negative control was also

prepared for each PCR reaction containing all **the** above reagents but replacing cDNA template with nuclease-free water in order to detect any contamination from reagents or water used.

The PCR programme consisted of the following steps: initial denaturation at 95° C for 2 min, 35 cycles of denaturation at 95° C for 1 min, primer annealing at 55° C ±10 (i.e. 45-65° C) for 1 min, elongation at 72° C for 1 min and a final elongation step at 72° C for 5 min. Products from PCR amplification were electrophoresed through a 1.5% (w/v) agarose gel in 1x TBE buffer containing ethidium bromide and visualised under a UV trans-illuminator (UViTec, Cambridge, UK). The size of PCR products were determined by comparison with a molecular weight marker Hyper ladder IV (Bioline, UK).

2.8.4 Preparation of PCR products for sequencing

PCR products generated from two-step PCR amplification were excised from a 1.5% agarose gel. The products were isolated using a Qiaquick® Gel Extraction kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions. The resulting PCR products were eluted in DNase free water and verified by sequence analysis carried out by UCL DNA sequencing unit (Wolfson Institute for Biomedical Research, University College London, UK) in which PCR fragments were sequenced from both ends (using both forward and reverse primers). Homology of the resulted sequences to *A. fumigatus* target genes were certified by the input of the resulting sequences into BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast).

2.8.5 Two step real time quantitative PCR

In order to determine any changes in the expression level of mRNA of genes of interest (*TEF-1, HSP70, MMSAD, Alp-2, gliZ,*) following treatment of *A. fumigatus* cultures with different concentrations of hydrocortisone, two-step qPCR experiments were conducted consisting of cDNA synthesis from mRNA template (section 2.7.3) and real time qPCR experiments. For qPCR experiments relative expression analysis was performed using comparative C_q (or $2^{-\Delta\Delta cq}$) method. This method requires PCR efficiencies of both reference and target genes to be similar or within 10% of each other as this ensures the reproducible results and more accurate estimation of gene expression (Schmittgen and Livak, 2008).

qPCR experiments were conducted by generating standard calibration curves for all target genes as well as the reference gene by plotting the log of the amount of RNA template (on X axis) against the C_q values of the reference and target genes (on Y axis). PCR efficiency was then calculated from the following formula: $E=10^{(1/m)}-1$, where E is PCR efficiency and m is the slope of the line. If the slop of the line is less than 0.1 or PCR efficiencies of different genes are within 10% from each other than PCR efficiencies are comparable and the relative quantification method can be used to examine the relative quantification of changes in gene expression (Schmittgen and Livak, 2008). Alternatively if PCR efficiencies are not comparable the Relative Expression Software Tool (REST) can be used for analysis of the results. The REST mathematical model contains an efficiency corrected calculation program in order to provide a more accurate quantification of the relative gene expression (Pfaffl, 2001).

In this study standard curves for the target genes (*HSP70, MMSAD, Alp-2, and gliZ*) and reference gene (TEF-1) was constructed using a Rotor-Gene SYBER Green PCR kit (Qiagen Ltd., Crawley, UK) according to manufacturer's instructions. The kit allows amplification of cDNA using a combined primer annealing and extension time.

In order to generate the standard curve the following concentrations of cDNA was made: 25, 2.5, 0.25, 0.025, and 0.0025 ng/reaction to confirm the efficiency of the primers. Real time PCR was carried out using a total reaction volume of 25 µl containing 12.5 µl 2x Rotor-Gene SYBER Green PCR master mix, 0.5 µl of each forward and reverse gene specific primers (10µM), RNase free water, and template cDNA corresponding to the required final concentration of cDNA as mentioned above. For standard curve experiments each cDNA concentration was performed in triplicates.

The following cycling conditions used to perform real time quantitative PCR experiments: Initial activation step to activate HotStar Taq Plus DNA polymerase 95° C for 5 min; this was followed by a two-step cycling consisting of denaturation of cDNA template at 95° C for 5 min and a combined primer annealing and extension step at 60° C for 25 sec. All real-time PCR cycling experiments were

carried out using a Rotor-Gene Q 2plex HRM system PCR machine (Qiagen Ltd., Crawley, UK). A melt curve analysis of PCR amplicons was also performed to determine the specificity of primers. Details of all primers used in real time qPCR experiments and their conditions are mentioned in Table 2.2.

2.8.6 qPCR for relative quantification of gene expression

Real time qPCR experiments were conducted to determine the changes in the expression of HSP70, MMSAD, Alp-2 and gliZ following treatment of *A. fumigatus* strain AF-10 with 10^{-6}M and 10^{-5}M hydrocortisone for 24h and 48h in Vogel's modified medium. The real time experiment was carried out on 25ng/reaction of cDNA template using a Rotor-Gene SYBER Green PCR kit (Qiagen Ltd., Crawley, UK) according to manufacturer's protocol using reagents and cycling conditions mentioned earlier (section 2.7.5). A negative control containing all reagents except for cDNA template was also included in each experiment for each gene. All experiments for all conditions were carried out in triplicates of tree experiments for each gene.

2.9 Additional assays to investigate the effect of hydrocortisone on both *A. fumigatus* and A549 cells

2.9.1 Spore-binding assay

In order to investigate the effect of hydrocortisone treatment on the ability of *A. fumigatus* spores to bind to pulmonary cells, spore binding assay was carried out (Bromley and Donaldson, 1996a). Fresh cultures of *A. fumigatus* AF-10 were prepared on Sabouraud Dextrose Agar (SDA) at 37° C for 4 days. Spores were collected and added to phosphate buffered Saline solution (PBS) (pH 7.4). The spores were counted using a haemocytometer (counting chamber) and adjusted to 10^6 spores per ml.

2.9.1.1 Binding of spores to A549 cells

To prepare the monolayers of lung epithelial cells (A549 cell line), the cells were cultured in DMEM medium containing 10 % FCS, 1% L-glutamine, and 1% penicillin-streptomycin (section 2.5.3). The cells were cultured at 37° C in an incubator with 5 % CO_2 in air. Cells were then harvested at around 70-80% confluency using 1x trypsin-EDTA solution centrifuged at 400 rcf for 10 min at

room temperature. Following the removal of the supernatant, the obtained pellet was suspended in fresh medium and cells were counted using a haemocytometer.

A549 cells were then seeded in a 96 well tissue culture plate (triple Red Ltd., UK) at the concentration of 10^5 cells per well and grown over night at 37° C in the presence of 5% CO_2 to confluency in the wells. Prior to infecting the A549 cells with Aspergillus spores, A549 cells in the test wells were pre-treated with hydrocortisone (10^{-6} M and 10^{-5} M final concentration in the medium in each well) for 4 h at 37° C with 5 % CO_2. Plates were then washed with PBS solution (PH 7.4) and 200 µl of the spore suspension was added to each well and the plates were incubated at 37° C in an atmosphere of 5% CO_2 in the air for 30 min. Following the incubation, the plates were washed three times for five min with PBS containing 0.01% tween 80 (v/v) (Sigma-Aldrich, UK). The remaining spores were fixed in 2.5% (v/v) glutaraldehyde in PBS for 15min. These spores were then counted using an inverted microscope (Leica Microsystems, Germany) by phase contrast microscopy at x 400 magnification. Five fields of view were counted for each well, in triplicate wells, and the results were expressed as the average number of spores per five high power fields (x 400).

2.10 Statistical analysis

All statistical analyses for differences between the treatment groups were determined by one-way analysis of variance (ANOVA). Data analysis was performed using Microsoft® Excel 2010 software (Microsoft, USA) for testing the normal distribution of the data. Data sets were compared using unpaired T-test and significant differences were given as $P \leq 0.05$ (unless stated otherwise as $P \leq 0.01$). A *P* value equal or below 0.05 was regarded as statistically significant. All results are presented as mean ± standard error of two or three independent experiments (SEM).

The comparative C_q or ($2^{-\Delta\Delta c}q$) method was used for analysis of qPCR data and relative expression analysis. Calculations for ($2^{-\Delta\Delta c}q$) were carried out using Microsoft® Excel (Microsoft, USA) and the unpaired T-test of the delta Cq (ΔCq) values determined the statistical significance of the relative expression changes of the target genes. A *P* value equal or below 0.05 was regarded as statistically significant.

Chapter III: Results

3 Results

The focus of this thesis is on the interactions of *A. fumigatus* and human lung cells through cell communications and involvement of environmental factors such as hydrocortisone (as a result of hydrocortisone treatment of patients suffering from aspergillosis) effecting the host pathogen relation during aspergillosis. In this context, this chapter presents the results of all experiments including both cellular and morphological studies as well as molecular experiments related to this investigation.

The Results chapter is divided into five main sections. The selected secondary metabolites, butyrolactone I and gliotoxin, produced by *A. terreus* and *A. fumigatus* are studied in section 3.1. Investigation of the effect of hydrocortisone stimulation of *A. fumigatus* at cellular and morphological level is presented in section 3.2. Section 3.3 is devoted to the results of a brief experiment on the effect of hydrocortisone treatment of lung cells on the binding of *A. fumigatus* spores to the pneumocytes. Proteomic studies of the effect of hydrocortisone treatment on protein expression in the cytosolic fraction of *A. fumigatus* are presented in section 3.4. Finally the transcriptome studies of the effect of hydrocortisone on the relative mRNA expression of some of *A. fumigatus* genes are given in section 3.5.

3.1 Investigation of the effect of selected secondary metabolites produced by *Aspergillus* sp.

3.1.1 Butyrolactone I; investigating quorum sensing in *A. terreus* and *A. fumigatus*

Earlier studies have demonstrated that butyrolactone I, a lactone-containing molecule and a secondary metabolite of *Aspergillus* sp. especially *A. terreus,* can act as a putative quorum sensing signal (Raina, 2008). This observation was further investigated in clinical isolates of *A. terreus* (1027F) and *A. fumigatus* (1025F, 1026F). Butyrolactone I production and the effect of its exogenous addition on its own production (100 nM) was studied. For this purpose butyrolactone I was added to the early growth stage cultures of these strains grown in a complex medium and in human cell culture medium (DMEM). Butyrolactone I was detected in the supernatants of both the control (with no exogenous addition of butyrolactone I) and the test where the commercially available butyrolactone I was added (Figure 3.1) in both complex medium and DMEM medium. The butyrolactone I production level was much higher in the complex medium (Figure 3.2) compared to the cell culture medium (Figure 3.3) which indicates the effect of different types of media (media ingredients) on both the time and the level of secondary metabolite production. As it is evident from the graphs, in the culture supernatants of the complex production medium in both the control and the test cultures production of butyrolactone I started at around 24 h post-inoculation and increased up to 72 h post-inoculation. After this, there was a decline in the level of production of this compound (Figure 3.2). This pattern of production is similar to the butyrolactone I production pattern observed in the previous studies (Schimmel *et al.*, 1998; Raina, 2008). In the cell culture medium, butyrolactone I was detected in the culture supernatants of both control and test cultures at 0 h post-inoculation and reached the highest level at 24 h after which a decline was observed. Detection of butyrolactone I in DMEM at 0 h post-inoculation could be due to carry over from the inoculum culture. In the cell culture medium the level of butyrolactone I production was significantly lower than its level in the complex medium ($p<0.05$). (Further details regarding the media used for this experiment are given in sections 2.3.2 and 2.3.3).

However as it can be seen from the graphs 3.2 and 3.3, in the experiments presented here addition of butyrolactone I to the cultures of *A. terreus* did not significantly (p>0.05) affect the production level of this compound compared to control cultures where no exogenous butyrolactone I addition was carried out, nor did it effect the biomass production and pH pattern in this fungus (Figures 8.1 and 8.2 in appendix). Moreover, butyrolactone I was not detected in the culture supernatants of *A. fumigatus* strains used in this experiment (Figure 8. 3 in Appendix).

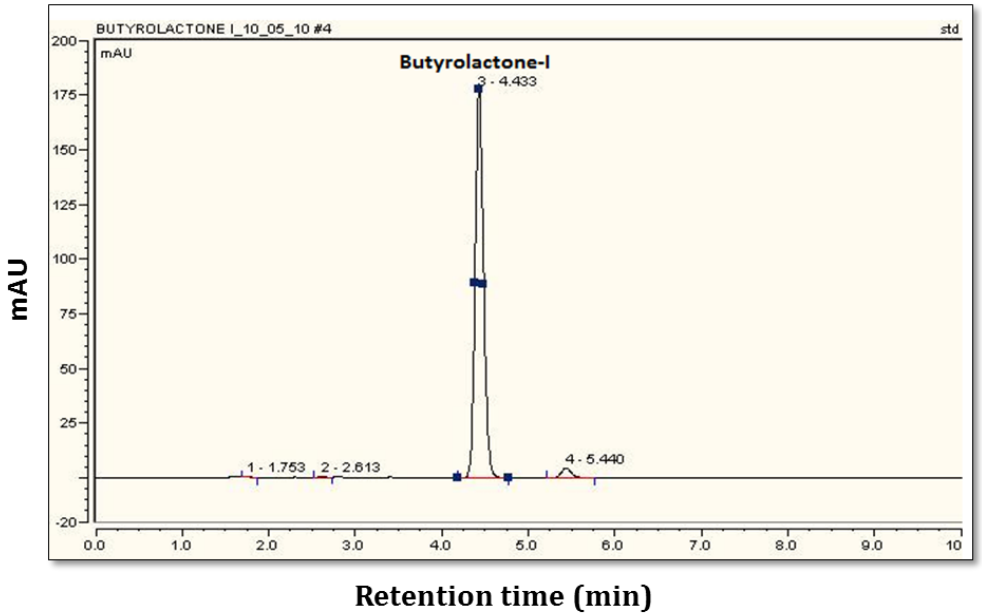


Figure 3.1
HPLC detection of butyrolactone I. Analysis was carried out using a C18 silica HPLC column and butyrolactone was eluted after 4.433 min (retention time). The labelled peak shows butyrolactone I in the chromatogram.

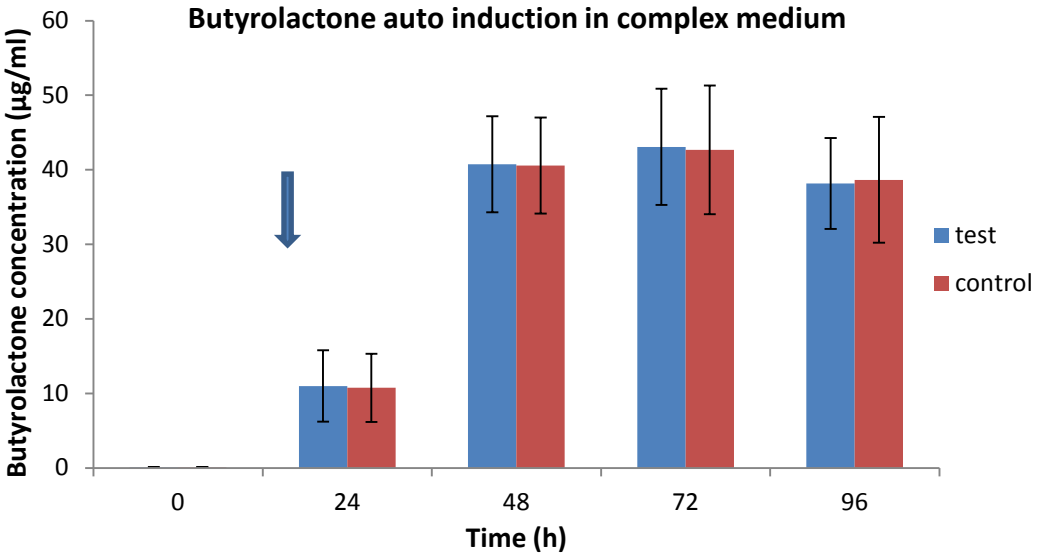


Figure 3.2
Butyrolactone I levels in clinical isolates of *Aspergillus terreus* culture supernatant in the absence and presence of exogenous addition of 100 nM butyrolactone I in complex medium. Standard error of the mean of triplicate is represented by error bars. Arrow indicates the time of butyrolactone I addition. No significant difference (P>0.05) was observed in the level of butyrolactone I production between the test and the control.

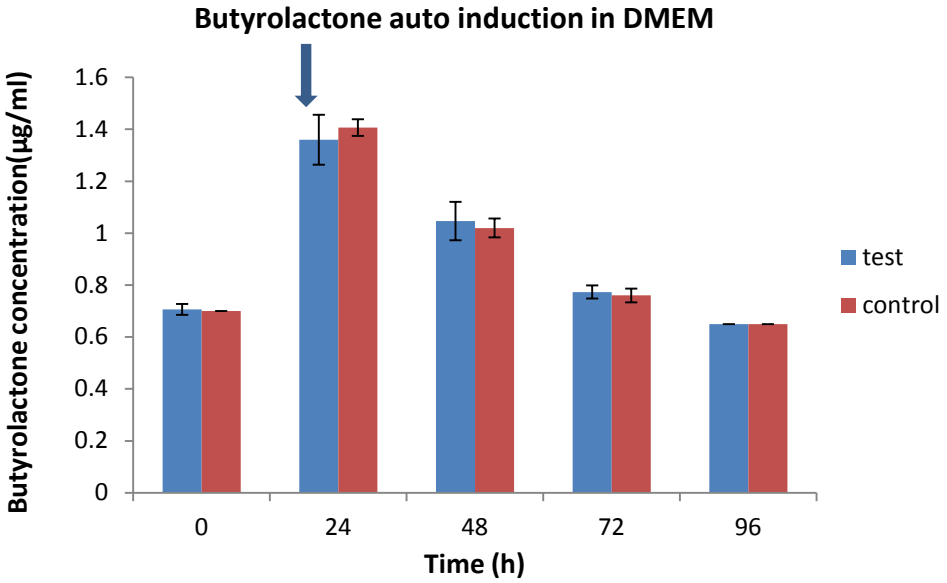


Figure 3.3
Butyrolactone I levels in clinical isolates of *A. terreus* culture supernatant in the absence and presence of exogenous addition of 100 nM butyrolactone I in DMEM medium with 25mM HEPES. The arrow indicates the time of butyrolactone I addition. No significant difference (P >0.05) was observed in the level of butyrolactone I production between the test and the control.

3.1.2 Gliotoxin production by *A. fumigatus* clinical strains

Gliotoxin, as described earlier in section 1.3.2.1, is a non-ribosomal dipeptide, and a secondary metabolite of *A. fumigatus*. This compound is a toxic metabolite that plays an important role in the virulence of this fungus. To determine the gliotoxin production, Shaken flask studies were carried out to screen different isolates of *A. fumigatus* for their ability to produce gliotoxin; Figure 3.8 (Sections 2.3.5, 2.4.4, 2.4.5 and 2.4.6 of the Materials and Methods chapter). Gliotoxin was detected initially using TLC to determine the presence of the toxin (Figure 3.4). After the presence of gliotoxin was established, HPLC (Figure 3.5) was used in order to determine the level of its production. The highest gliotoxin concentration was detected after 72 h post-inoculation in all strains. Figure 3.6 shows gliotoxin production pattern in the culture supernatant of a clinical strain of A. *fumigatus* (1028F). Production was observed after 24 h post-inoculation and reached the highest level at 72 h after which a decline was observed. The growth curve of A. *fumigatus* also corresponds with the gliotoxin production pattern (Figure 3.7). A standard curve was obtained using gliotoxin standards ranging from 100 to 25,000 ng/ml. Samples at 24 h, 48 h and 72 h were diluted x100 and x1000 with acetonitrile in order to fit within the linear range of the standard curve.

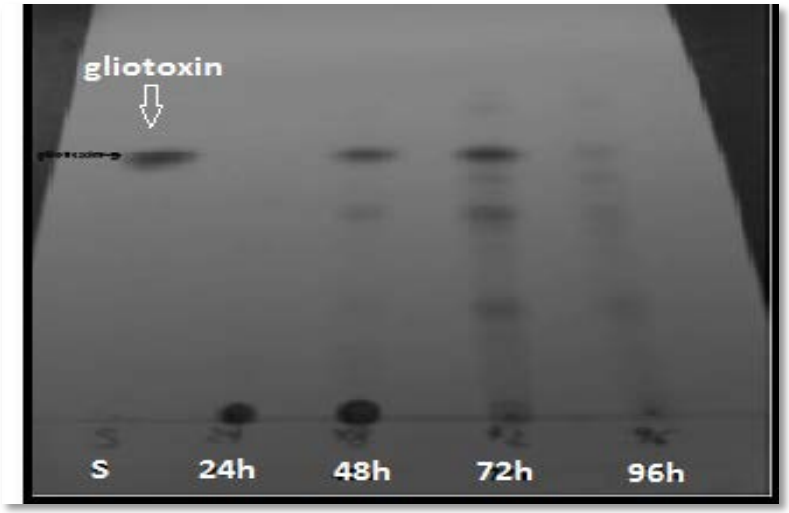


Figure 3.4
TLC of gliotoxin produced after 4 days of liquid submerged culture of the *A. fumigatus* clinical isolate (1028F). Toluene-ethylacetate-formic acid (5:4:1, v/v/v) was used as developing solvent. S; gliotoxin standard. 24, 48, 72, and 96h indicate the time that samples were taken for gliotoxin detection post-inoculation. TLC pattern also follows the gliotoxin production pattern obtained from HPLC analysis

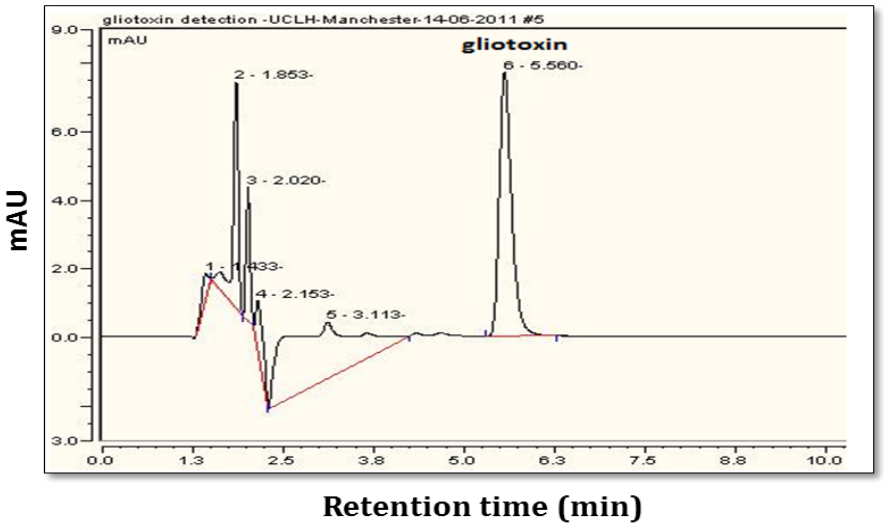


Figure 3.5
HPLC detection of gliotoxin. Analysis was carried out using a C18 silica HPLC column. Gliotoxin was eluted after approximately 5.5 min (retention time). The labelled peak shows the gliotoxin in the chromatogram.

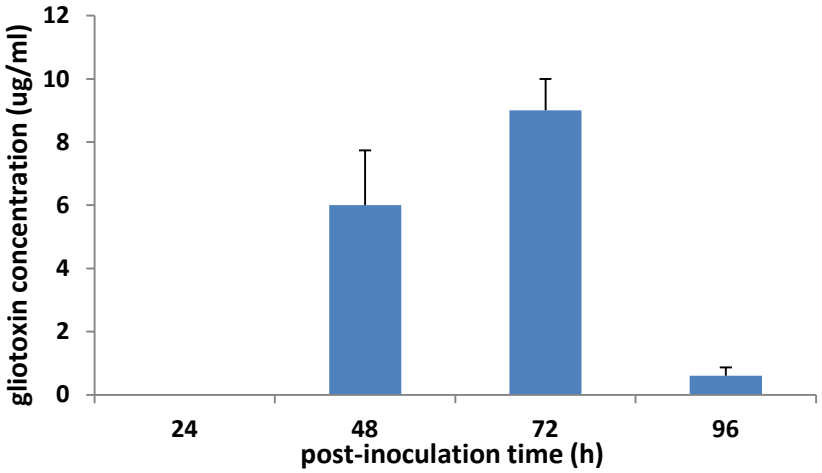


Figure 3.6
Mean concentrations of gliotoxin in culture filtrates of *A. fumigatus* (strain 1028F) as determined with HPLC. Maximum gliotoxin concentration was detected 72h post-inoculation. Error bars represent the standard error of the mean of two independent experiments.

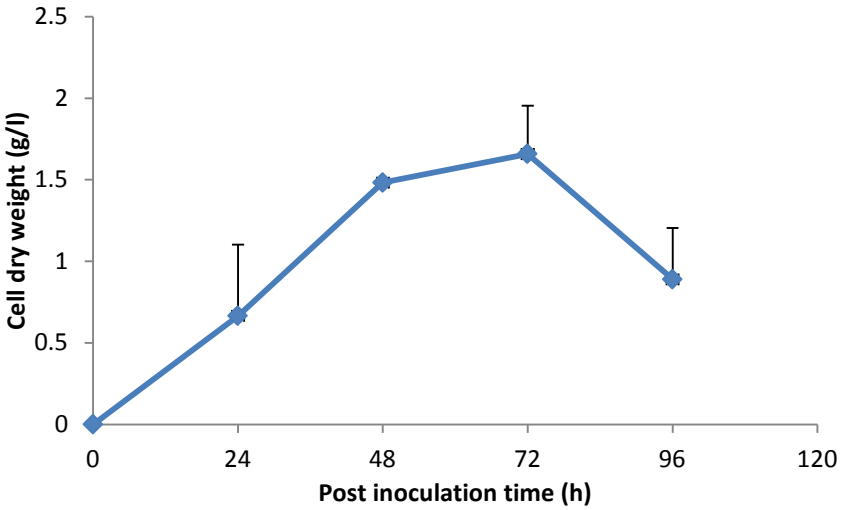


Figure 3.7
Growth curve of *A. fumigatus* (1028F) in cell culture medium. Shaken flask runs were done in triplicates and error bars represent the standard error of the mean of two independent experiments.

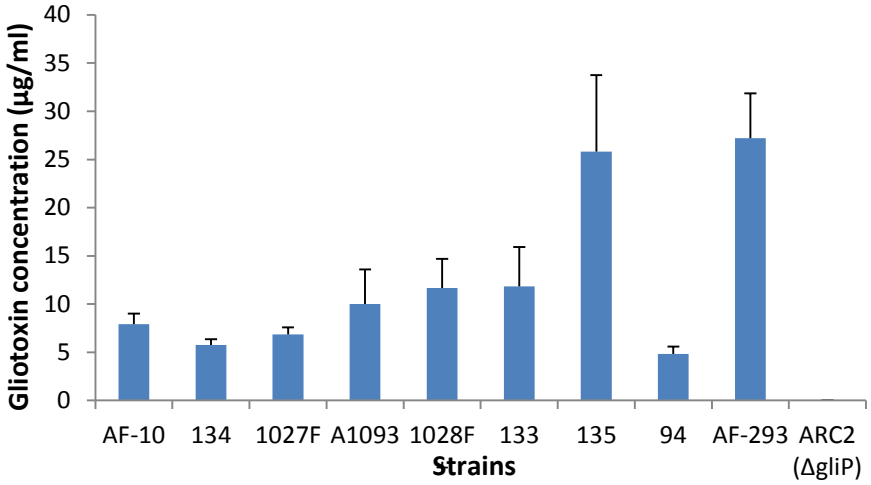


Figure 3.8
Gliotoxin production by different clinical isolates of *A. fumigatus*. Apart from ARC2, a gliotoxin knockout strain without the ability to produce gliotoxin, all other strains were able to produce gliotoxin at different levels.

3.1.2.1 *Effect of gliotoxin on the viability of the A549 cells using MTT assay*

In order to determine the minimum effective gliotoxin concentration on lung cells, A549 cells (lung alveolar epithelial cells) were exposed to different concentrations of the commercially available toxin and the toxicity was measured using MTT assay. Ethanol (98%) in the final concentration of 0.01 (v/v) did not affect the development of the A549 cells (data not shown) and therefore, it was used for making the solution and for the serial dilution of gliotoxin. During the first 4 to 24 h generation times, under the described conditions, gliotoxin caused a decrease in cell viability in a dose dependent manner. The treated cultures were analysed after 24 h and 48 h in order to show any long term effect. The cell viability, as determined by MTT assay, was strongly affected after 4 h, 24 h, and 48 h exposure to gliotoxin in both experiments with sub-confluent and confluent cells. However, confluent cells showed more resistance to lower concentrations of gliotoxin Figures 3.9 [and 8.5 in appendix]. The EC_{50} value for the proliferation following the 4h, 24h, and 48 incubation was found to be a concentration of 1µM gliotoxin for the non-confluent cells (Figure 3.9) and 5µM for the confluent cells (Figures 8.4 and 8.5 in Appendix).

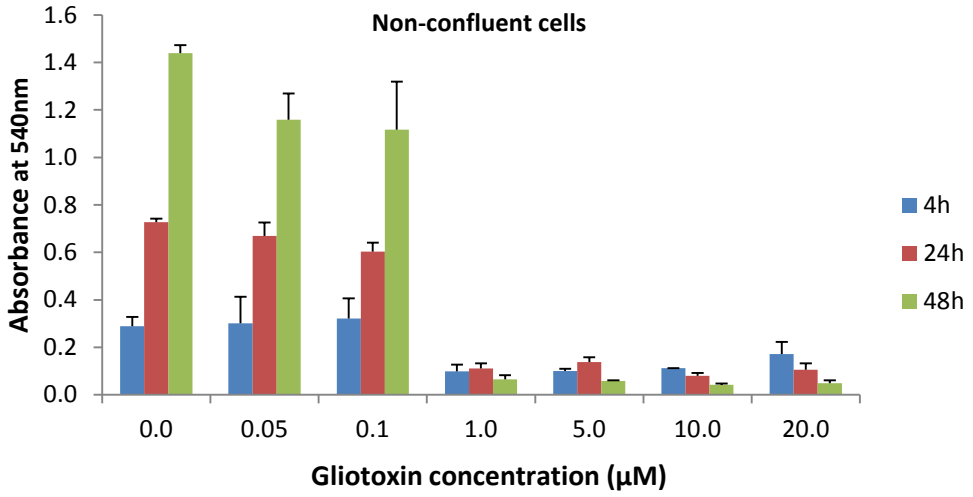


Figure 3.9
Effect of gliotoxin on the concentration of the A549 cells after 4h, 24h and 48h exposure. Cell concentration was measured in absorbance at 540nm. Up to 1µM gliotoxin concentration there is no significant difference between the control (0 concentration of toxin) and the tests (0.05 and 0.1 µM) (P>0.05) whereas from 1µM gliotoxin concentration a significant decrease in the cell concentration was observed in all three exposure time points (P<0.05) this also showed an inhibition in the growth of the sub-confluent cells. Error bars indicate the standard error of the mean between the two independent experiments.

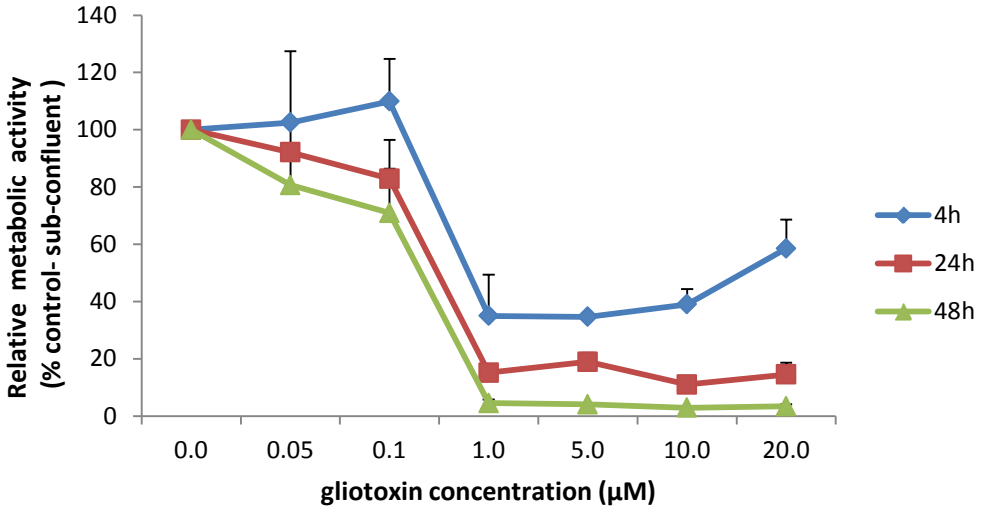


Figure 3.10
Response of A549 cells to gliotoxin quantified as metabolic activity after 4, 24 and 48 h exposure to the toxin. Error bars indicate the standard error of the mean between the two independent experiments.

3.1.2.2 *Effect of gliotoxin in causing apoptosis and necrosis in A549 cells measured by flow-cytometry*

Lung epithelial cells were exposed to 40% filter-sterilized culture broth of *A. fumigatus* wild type as well as the gliotoxin knockout strain. Flow-cytometry assay was carried out for further understanding of the mechanism of cell death by gliotoxin in lung epithelial cells. Studies were carried out to investigate the extent of the damage caused by the metabolites produced and released by *A. fumigatus*. Figure 3.11 shows a flow-cytometric histogram (A) and its corresponding Annexin-v-FITC and PI double labelled histogram (B) for an unstained population of cells which was carried out at the beginning of each experiment to ensure quadrants were correctly placed.

Within histogram A there is a rectangular "gate" which represents the population for analysis after exclusion of debris which appears below and to the left of the gate. The Forward Scatter (FS) versus Side Scatter (SS) histogram analysis (plotted in a linear scale) gives information regarding the size of the cells/events on FS axis and their granularity, refractiveness and internal complexity on SS axis allowing cells to be distinguished from debris and to avoid including debris in the data analysis.

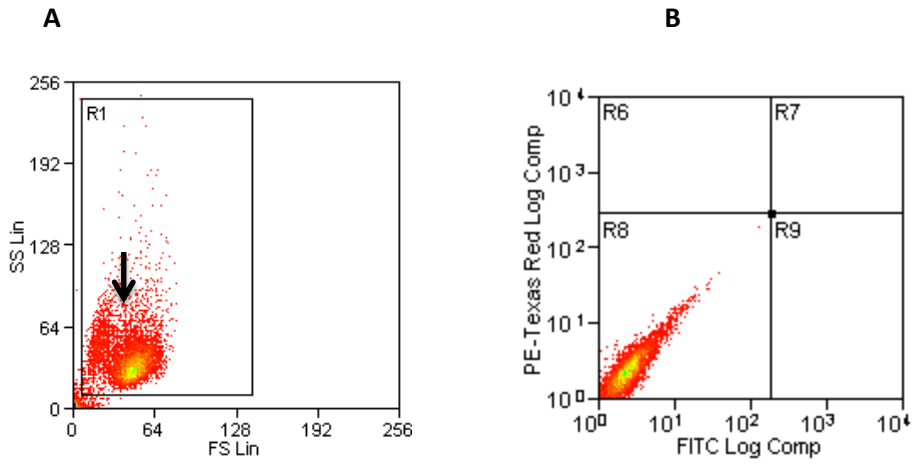


Figure 3.11
The figure represents an un-stained population of cells. Histogram A is for forward scatter (FS) and side Scatter (SS) and histogram B is for Annexin-V-FITC vs PI fluorescence staining. A total of 10,000 cells/events were counted per samples. (Section R1 in histogram A indicates the gating of the cells so that a total of 10,000 cells within the gate would be counted by the machine and all cell debris (below and to the left of the R1) would be excluded from data analysis. Arrow indicates the viable cell population. In histogram B the number of cells in each quadrant is equivalent to percentage of viable (R8), apoptotic (R7 and R9) and necrotic cells

(R6). Results presented here are from one experiment representative of the three performed runs.

3.1.2.2.1 Flow-cytometric analysis of the effect of sterile filtered culture broth of *A. fumigatus* strains on A549 cells

3.1.2.2.1.1 Apoptosis in A549 cells

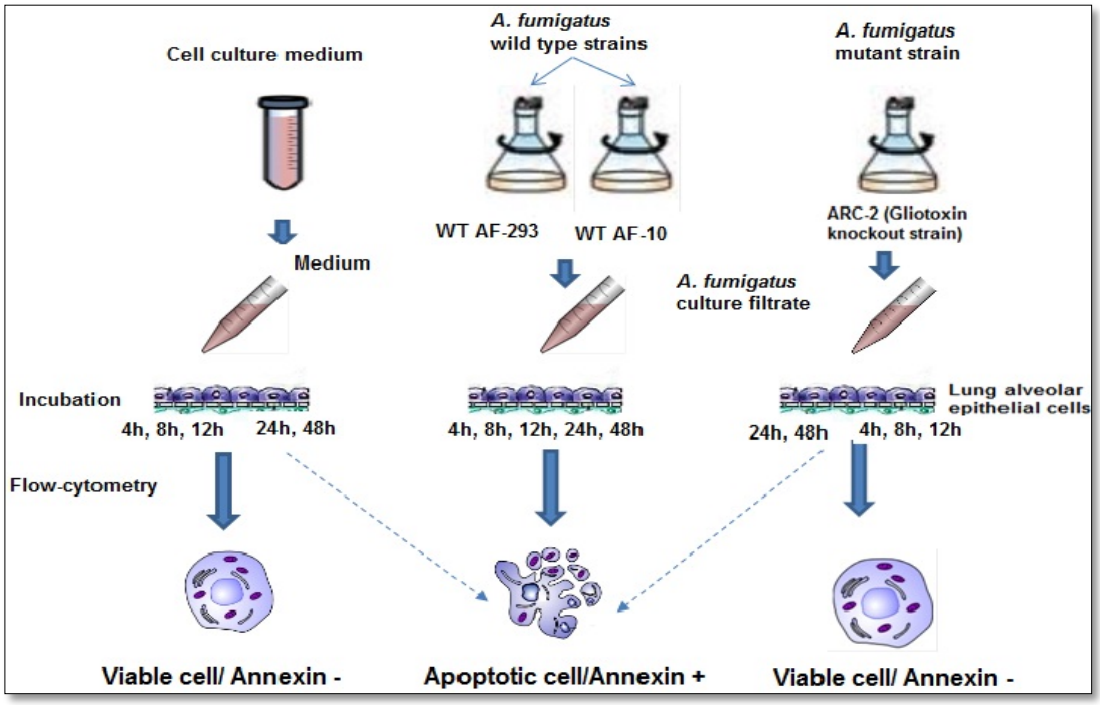


Figure 3.12
Schematic diagram of the flow cytometry results. Lung alveolar epithelial cells (A549) were exposed to 40% filter-sterilized culture broth of *A. fumigatus* wild type as well as the gliotoxin knockout strain. A549 cells treated with wild type fungal culture filtrates (WT AF-293/AF-10) underwent measurable apoptosis from early hours of incubation. Cells treated with culture filtrate of gliotoxin mutant and Control cells (where no fungal culture broth added) did not undergo any significant measurable apoptosis.

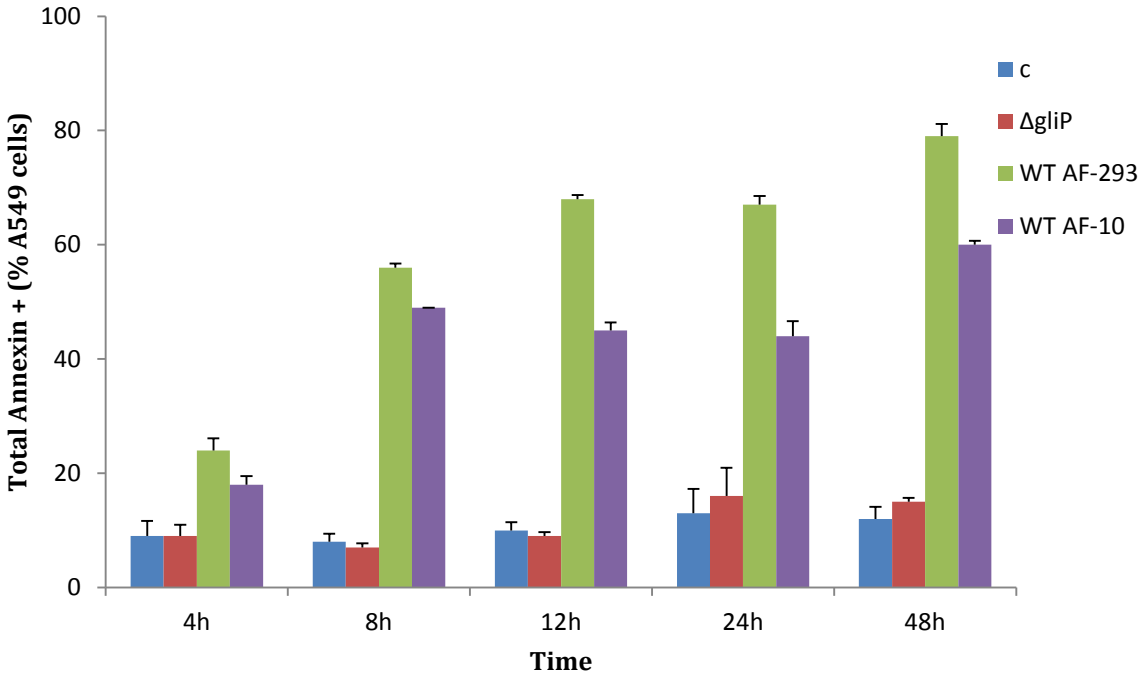


Figure 3.13
A549 apoptosis after exposure to 40% culture filtrate from *A. fumigatus* strains: Wild type (WT)-AF-10, wild type-AF-293, gliotoxin knockout (*ΔgliP*) for 4h, 8h, 12h, 24h, and 48h. Percentage of total cells that were Annexin V positive (apoptotic) is shown. The control was untreated A549 cells. The data represent the means (± standard deviations) calculated from triplicate measurements in three different experiments.

Figure 3.13 shows A549 total apoptosis after exposure to 40% culture filtrate of different strains of *A. fumigatus* (wild type AF-10, wild type AF-293, and gliotoxin knockout strain (*ΔgliP*)) for 4h, 8h, 12h, 24h, and 48h. After only 4h treatment of the A549 cells with different types of culture broth there is a significant increase ($p<0.05$) in the number of apoptotic cells. The increase is from around 9% (total apoptotic cells) in the control to 24% when cells are treated with the culture filtrate of *A. fumigatus* AF-293 strain. When cells are treated with the culture filtrate of the *A. fumigatus* AF-10 strain the number of annexin positive cells rises to 18%.

A. fumigatus AF-293, as shown earlier in Figure 3.8, produces higher amounts of gliotoxin. However the total apoptotic population in cells treated with the gliotoxin mutant strain of *A. fumigatus* (*ΔgliP*) remains similar to the control cells which are around 9%. After 8h incubating A549 cells with 40% culture broth of different fungal strains, around 56% of the cells treated with WT AF-293 and 49% of the cell treated with WT AF-10 undergo apoptosis (both early and late stages). Again, the

total number of apoptotic cells in cells treated with culture filtrate of mutant strain (*ΔgliP)* is very similar to the control, around 8% Annexin positive A549 cells.

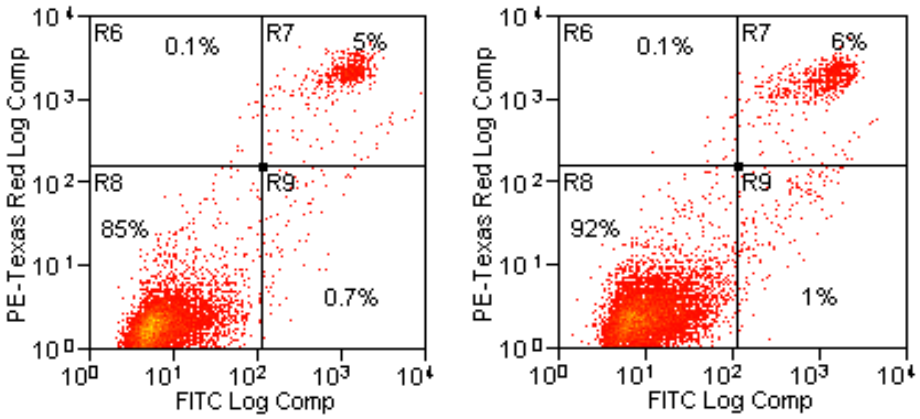
After 12h of treatment, the population of apoptotic A549 cells exposed to both wild type strains show an increase compared to 8h data and still significantly higher than the control A549 cells. The number of apoptotic A549 cells are higher in the cells treated with WT AF-293 (68%) in comparison to the cells treated with WT-AF-10 (45%). This is in agreement with the graph of toxin distribution (Figure 3.6) showing a higher production of toxin for this strain. The number of apoptotic cells (7% total apoptotic cells) is again very similar to the control (8%), (Figure 3.13).

To investigate the longer term effects, lung epithelial cells were exposed to the culture broths of *A. fumigatus* strains for 24h and 48h (Figure 3.13). The percentage apoptotic A549 cells in the cells treated with culture filtrate of both wild-type strains of *A. fumigatus* (AF-293 and AF-10) remained almost similar to the results from 12h incubation (67% and 44% apoptotic A549 cells respectively). However, an increase in the total apoptotic cells treated with gliotoxin knockout strain, and the control was observed when compared to earlier data sets (16% and 13% respectively). A comparison between the three tests (cells treated with *A. fumigatus* AF-293, AF-10, and ARC2 (*ΔgliP)*) with the control at this time set (24h), demonstrates a similar cell death pattern as observed earlier for 4h, 8h, and 12h data. Again the total number of apoptotic A549 cells in cells treated with culture filtrate of wild type strains is significantly higher compared to the control cells. The profile of cell death in cells treated with culture filtrate of the gliotoxin mutant strain (*ΔgliP*) is very close to the control population though. Compared to the control (with 12% total cell death), After 48h treatment, the majority of the A549 cells treated with 40% culture filtrate of wild type *A. fumigatus* strains (AF-293 and AF-10) are Annexin positive with 79% and 60% total apoptotic cell populations respectively (Figure 3.13). For A549 cells treated with the mutant strain of *A. fumigatus* (*ΔgliP*) a similar pattern to the control cells is observed. There are only 15% total apoptotic cells treated with *ΔgliP* strain after 48h, compared to the control with 12% total Annexin positive (apoptotic) A549 cells. A diagrammatic representation of flow cytometry experiments can be found in section 8.3.11 of Appendix (Figures 8.6 to 8.10).

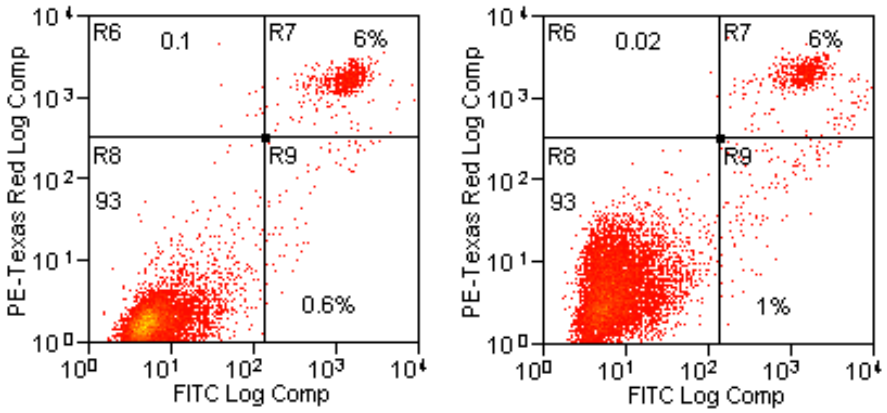
3.1.2.2.1.2 Changes in the morphology of the viable A549 cells treated with culture filtrate from ΔgliP strain of A. fumigatus

After 4 h treatment

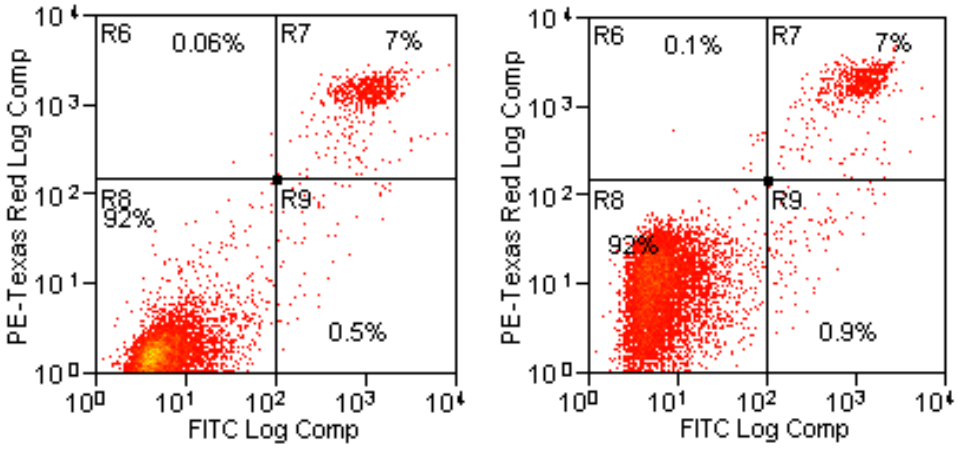
A-Control population **B-ARC2 (*gliP* knockout strain)**



After 8 h treatment



After 12 h treatment



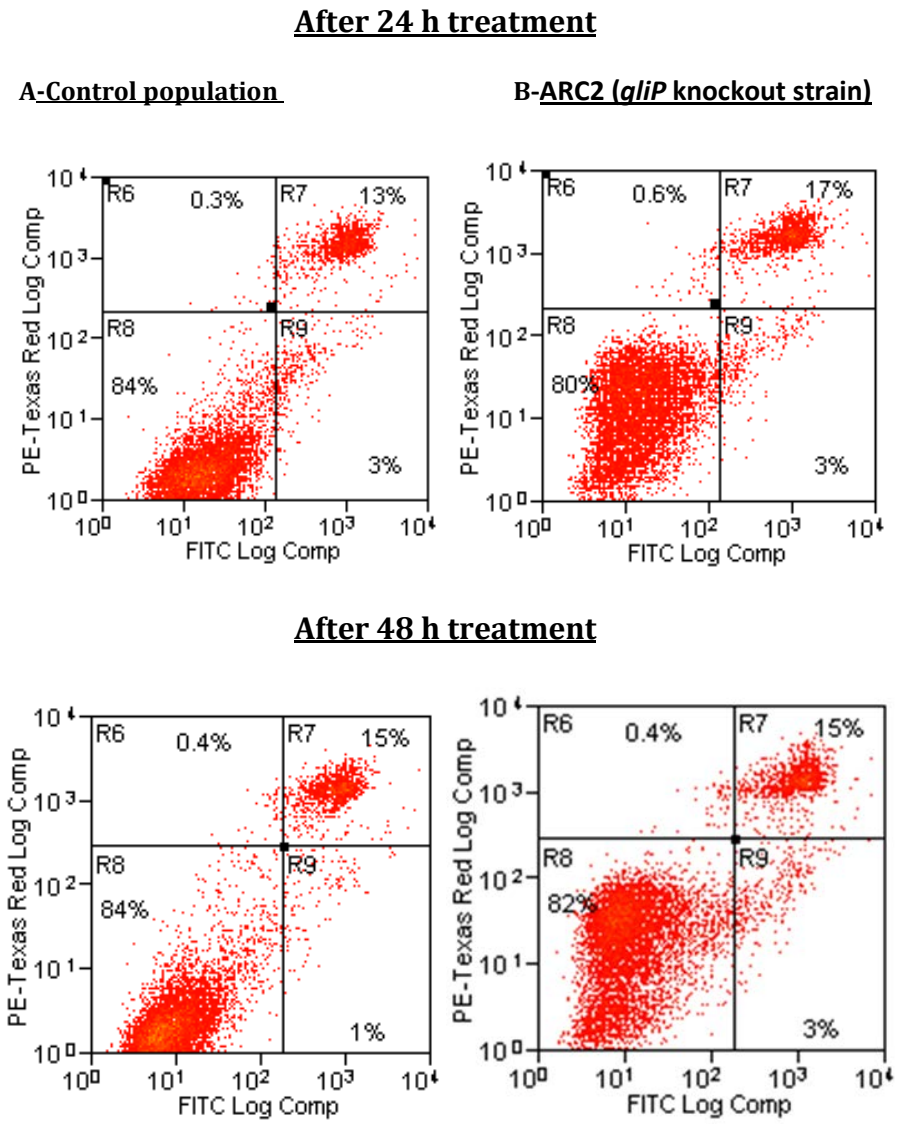


Figure 3.14
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture filtrate of 48h old culture of *A. fumigatus* ARC2 strain in RPMI medium after 4h, 8h, 12h, 24, and 48h treatment. Section (A) represents the control A549 cells without any treatment; (B) shows A549 cells treated with 40% culture broth from *A. fumigatus gliP* knockout strain ARC2 (*ΔgliP*). Following treatment of A549 cells with culture filtrate of mutant strains, morphology changes in the viable cell in the R8 quadrant was observed. Cells have gradually moved towards the PI+ section along the PI axis. Results presented here are from one experiment representative of the three performed.

Figure 3.14 demonstrates double labelled histograms that are plotted on a logarithmic scale and show the Annexin-V-FITC and PI staining patterns obtained from the fluorescent channels (FL1: FITC fluorescence and FL3: PI/PE-Texas Red fluorescence). For analysis, the data is divided into four quadrants in order to distinguish different cell populations. The lower left quadrant (R8) of the Figure 3.14 represents cells that are both Annexin-V-FITC and PI negative i.e. viable cells not undergoing any measureable apoptosis. The lower right quadrant (R9) shows cells that are positive for Annexin-V-FITC but negative for PI representing early apoptotic cells. The cells in the upper right quadrant (R7) are positive for both Annexin-V-FITC and PI. They represent cells in the latter stages of apoptosis. The upper left hand quadrant (R6) contains cells positive for PI but negative for Annexin-V-FITC indicating necrotic cells.

Following treatment of A549 cells with the culture filtrate of mutant strain *ΔgliP* (group B), morphology changes in the viable cell in the R8 quadrant was observed when compared to the same quadrant in the control population (group A). Most of the morphological changes in viable A549 cells occurred after 8h incubation of the cells with the culture filtrate of the *ΔgliP* strain.

Figure 3.14 (after 8h), shows that viable cells in quadrant R8 of the histogram B have undergone some changes in their morphology and internal complexity where there is an upward move towards the direction of PI axis. However, no changes in the morphology are observed for the control cells (histogram A) in the R8 quadrant.

After 12h, there is an increase in the changes observed in the morphology of the viable cells in quadrant R8 of the histogram B (Figure 3.14, after 12h) compared to the control group (A).

The number of viable cells with morphology change in the R8 quadrant of histogram B (*ΔgliP* treated) is further increased after 24h as compared with the same section in the control cells (histogram A) (Figure 3.14, after 24h).

Finally, after 48h the cells in the R8 quadrant show further change in the shape/size in the histogram B (*ΔgliP* treated) as compared with the control (Figure 3.14, after 48h).

3.1.2.2.1.3 Necrosis in A549 cells

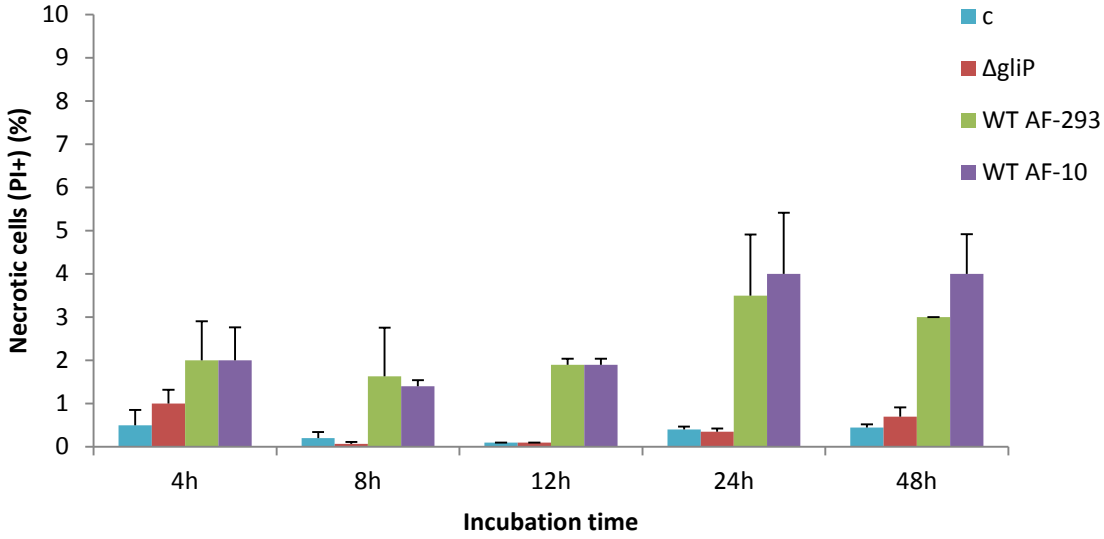


Figure 3.15
A549 PI positivity after exposure to 40% culture filtrate from *A. fumigatus* strains: Wild type (WT)-AF-293, wild type-AF-10, gliotoxin knockout (*ΔgliP*) for 4h, 8h, 12h, 24h, and 48h. Percentage of the cells that were PI positive (necrotic) is shown. Control was untreated A549 cells. Data represent means (± standard deviations) calculated from triplicate measurements in three different experiments.

The percentage of A549 cells that were PI positive (necrotic) were considerably lower than Annexin V positive (apoptosis) cells (Figure 3.13) in all three exposure groups (wild type AF-293, wild type-AF-10, gliotoxin knockout (*ΔgliP*)) tested for 4h, 8h, 12h, 24h, and 48h incubation time.

Figure 3.15 shows percentage A549 cells undergone necrosis. The percentage necrotic A549 cells treated with the *ΔgliP,* is similar to the control throughout all time sets tested. Maximum PI positive cells after 48h incubation of A549 cells with culture filtrate of *ΔgliP* strain was 0.7%, compared to control with maximum 0.5% necrotic cells after 48h. Comparing this data to that of A549 cells treated with wild type strain of *Aspergillus* the percentage PI positive cells are considerably higher throughout all time sets tested.

With regards to the A549 cells treated with culture filtrate of both wild type strains of *A. fumigatus* (WT AF-293 and WT AF-10) the percentage necrotic cells remain almost similar with around 2% after 4h to 12h incubation time sets.

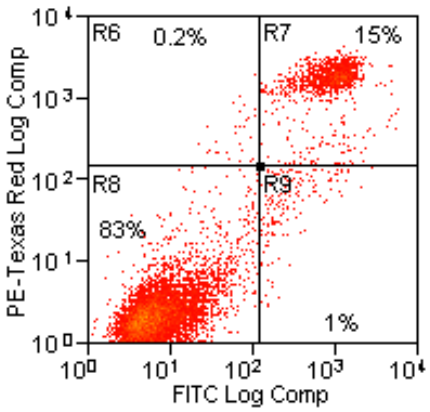
After 24h and 48h incubation of A549 cells with culture filtrate of wild type strains (WT AF-293 and WT AF-10), the number of PI positive cells increases to 4% in cells treated with culture filtrate of WT AF-10 and around 3.5% in the cells treated with WT AF-293. This shows that longer term treatment of the cells with culture filtrate of wild type strains almost doubles the number of necrotic cells as compared to the earlier time sets of the same group. However, comparing this data to the percentage Annexin V positive cells after the same incubation period (Figure 3.13), the number of PI positive cells (Figure 3.15) are significantly lower ($P<0.05$).

3.1.2.2.2 Flow-cytometric analysis of the effect of culture broth younger than 48h old (36-38h post inoculation harvest) on A549 cells

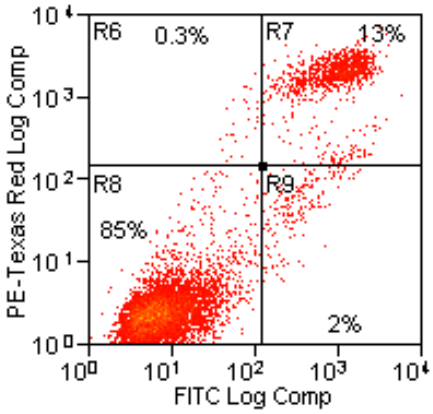
The human lung epithelium cells were also treated with fungal culture filtrates harvested before 48h post inoculation (at 36-38h). This was done to establish the time required for the accumulation of minimum effective concentrations of the toxin (gliotoxin). As before, cultures of the three strains of *A. fumigatus* (the wild type strains AF-10 and AF-293 and the mutant ARC2 (*ΔgliP*)) were used.

After 8 h treatment

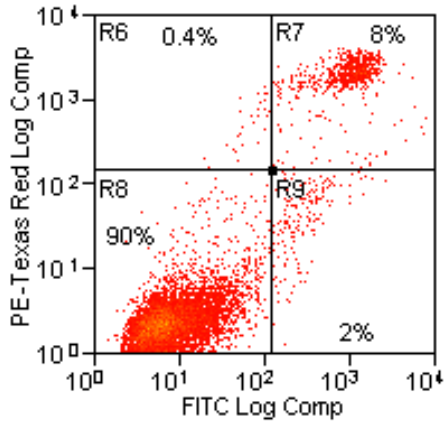
A-Control population



B- AF -10 strain (low gliotoxin producer)



C-ARC2 (*gliP* knockout strain)



D- AF-293 (high gliotoxin producer)

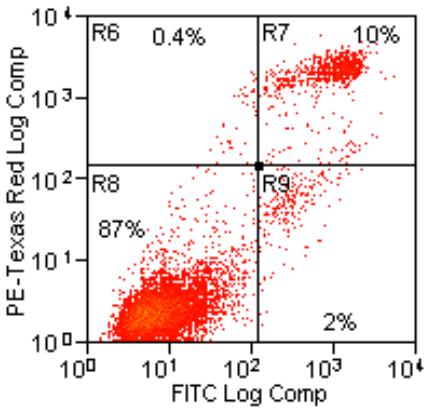


Figure 3.16
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of less than 48h old (38h) culture of *A. fumigatus* AF-10, AF-293 and ARC2 strains in RPMI medium for 8h. Section (A) represents the control A549 cells without any treatment; (B) represents A549 cells treated with 40% culture broth from *A. fumigatus* AF-10 strain (wild type); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus gliP* knockout strain ARC2 (not able to produce gliotoxin); (D) representing A549 cells treated with 40% culture broth of *A. fumigatus* AF-293 strain (wild type). The results presented here are from one experiment representative of the two performed runs.

Figure 3.16 demonstrates double labelled histograms that are plotted on a logarithmic scale and show the Annexin-V-FITC and PI staining patterns obtained from the fluorescent channels (FL1: FITC fluorescence and FL3: PI/PE-Texas Red

fluorescence). For analysis, the data is divided into four quadrants in order to distinguish different cell populations. In all figures above, the lower left quadrant (R8) represents cells that are both Annexin-V-FITC and PI negative i.e. viable cells not undergoing any measureable apoptosis. The lower right quadrant (R9) shows cells that are positive for Annexin-V-FITC but negative for PI representing early apoptotic cells. Cells in the upper right quadrant (R7) are positive for both Annexin-V-FITC and PI representing cells in the latter stages of apoptosis whilst the upper left hand quadrant (R6) contains cells positive for PI but negative for Annexin-V-FITC indicating necrotic cells.

Figure 3.16 shows that after 8h incubation of A549 cells with culture filtrate of wild type and gliotoxin mutant strains, there is little change in the number of apoptotic/necrotic cells in A549 cells treated with culture broth of any of the strains (wild type AF-10 and AF-293 and mutant ARC2) as compared to the control (untreated) cells. Comparing these results with the cells treated with 48h old fungal culture filtrates (Figure 3.13) a significant difference is observed ($p<0.05$) in the number of Annexin positive A549 cells. Therefore, supplementation with the younger culture broth is less effective, suggesting lesser amount/no toxin is produced at around 36 to 38h post inoculation.

3.2 Investigation of the effect of hydrocortisone treatment on enhancing growth and toxin production in *A. fumigatus*

Hydrocortisone has been shown in literature to alter the growth of *A. fumigatus*. Corticosteroid therapy is associated with an increase in invasive aspergillosis. Corticosteroids such as hydrocortisone impair immune function in mammals and specifically the conidicidal activity of human macrophages, which was thought of as sufficient explanation for this increased risk. However research has shown a 30-40% increase in the growth rate of *A. fumigatus* and *A. flavus* when exposed to pharmaceutical doses of hydrocortisone (a human glucocorticoid), suggesting an alternative or additional mechanism for the association. The presence of a ligand receptor has therefore been suggested (Ng *et al.*, 1994).

This section of the results chapter will therefore be focused on reporting the result of experiments carried out in order to understand the effect of hydrocortisone, as a compound present in the environment where the *A. fumigatus* grows inside the lungs, on morphology, growth and gliotoxin production in this fungus. Further investigations of the effect of this hormone are reported in future sections.

3.2.1 Morphological changes in *A. fumigatus* in response to hydrocortisone treatment

Following the addition of hydrocortisone sodium succinate in 10^{-5}M and 10^{-6}M concentrations to the culture medium of *A. fumigatus* AF-10 strain (wild-type), the cells were examined for morphological changes using light microscopy. At each time-set at least 10 slides were prepared from fungal cell pellets and changes in their morphology were examined under a light microscope. In control cultures no hydrocortisone was added to the medium prior to inoculation. Figure 3.17 demonstrates morphological observations of *Aspergillus* cells while growing in the presence and absence of hydrocortisone in the medium. At early growth stage, the 12h old cells in the test flasks containing hydrocortisone (10^{-5}M and 10^{-6}M) demonstrated similar morphology compared to the control flask. Samples were taken at the same time; mainly consisting of young conidiophore stalks prior to vesicle formation stage. The vesicle (spore producing structure) was not formed at

this stage of growth under any of the conditions (with or without hydrocortisone) in this experiment. Examination of the 15h old culture however displays a significant difference in the morphology of the cells in the test flasks where vesicles have been developed and cells mostly have developed phialid as well, compared to the control (untreated) fungal cells where vesicles are still developing at the tip of the conidiophore. Aspergillus cells treated with hydrocortisone both at 10^{-5} M and 10^{-6}M concentrations thus seem to have undergone an early onset of sporulation compared to the control cells at 15h time set, as they are in a further stage of conidiation. From 15h onwards cells in the control and all the test groups seem to have completed the phialide formation and have started sporulation. From this stage onwards no significant difference in the morphology of the test and the control cells is observed. At 24h all spore forming structures are formed and the tips of the mature conidiophores bear numerous conidia. Again at this stage no significant phonotypical difference was observed between the test and the control cultures.

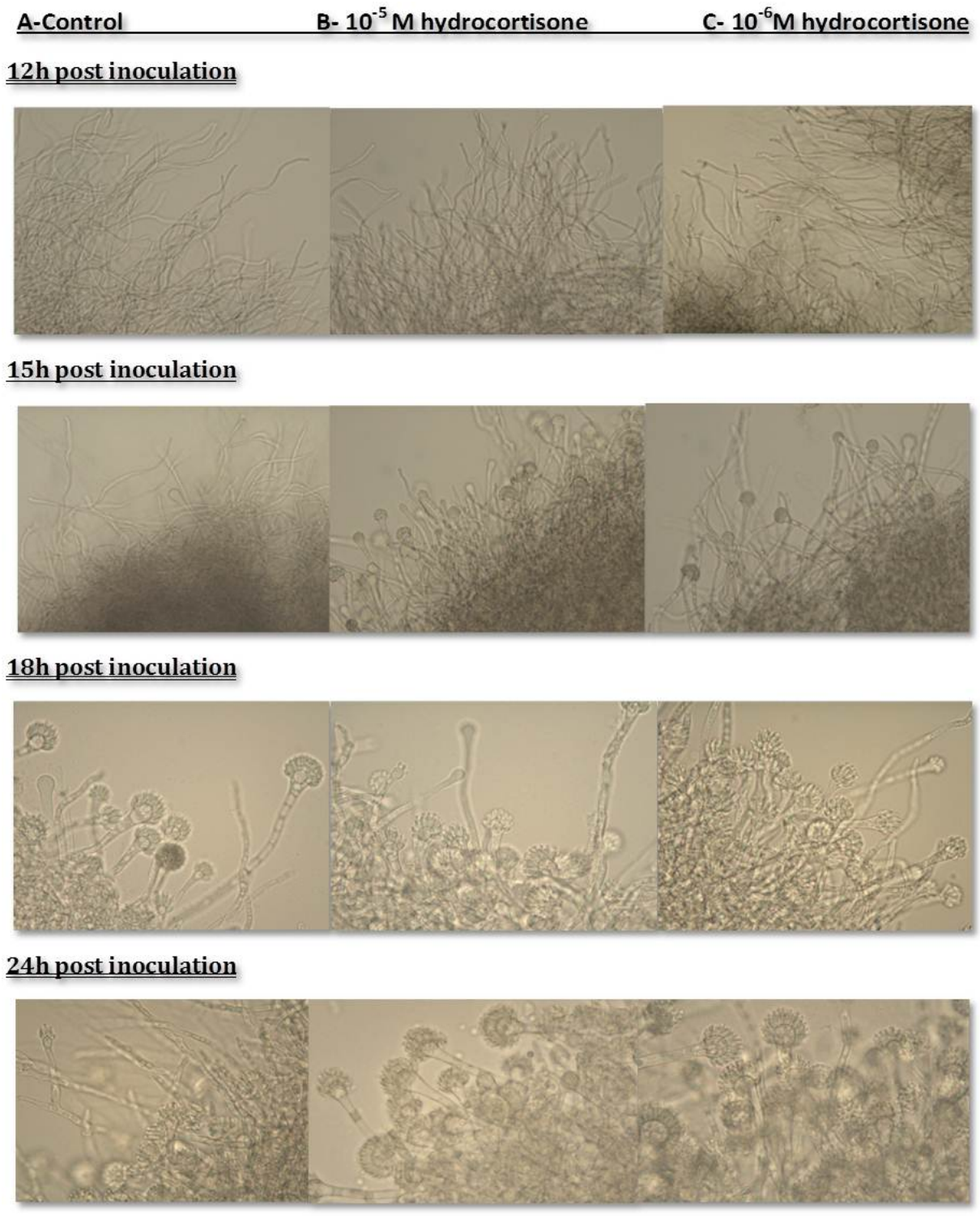


Figure 3.17
Morphological changes in the cultures of *A. fumigatus* AF-10 from 12h to 24h post inoculation. Fungal cells were grown in Vogel's modified medium. At 15h test cultures B and C (treated with 10^{-5}M and 10^{-6}M hydrocortisone respectively) undergo an early unset of sporulation compared to the control cells. At each time set at least 10 slides were prepared from fungal cell pellets and changes in the morphology were examined under microscope. All pictures were taken at x200 magnification.

3.2.2 Effect of hydrocortisone treatment on growth profile and biomass formation in A. *fumigatus*

Following the morphological observations of the *A. fumigatus* cultures during growth under the influence of hydrocortisone, the effect of hydrocortisone supplementation of the *A. fumigatus* on the biomass concentration was investigated. The liquid cultures were monitored during their growth in shaken flasks containing Vogel's modified medium and the total biomass produced in the flasks at specific time intervals was measured. Figure 3.18 demonstrates a typical (the profile is one of the three fermentations) growth profile of the *A. fumigatus* cultures grown in the presence or absence of hydrocortisone in the medium. As the Figure shows, there is little difference in the biomass and pH between the control and the test cultures.

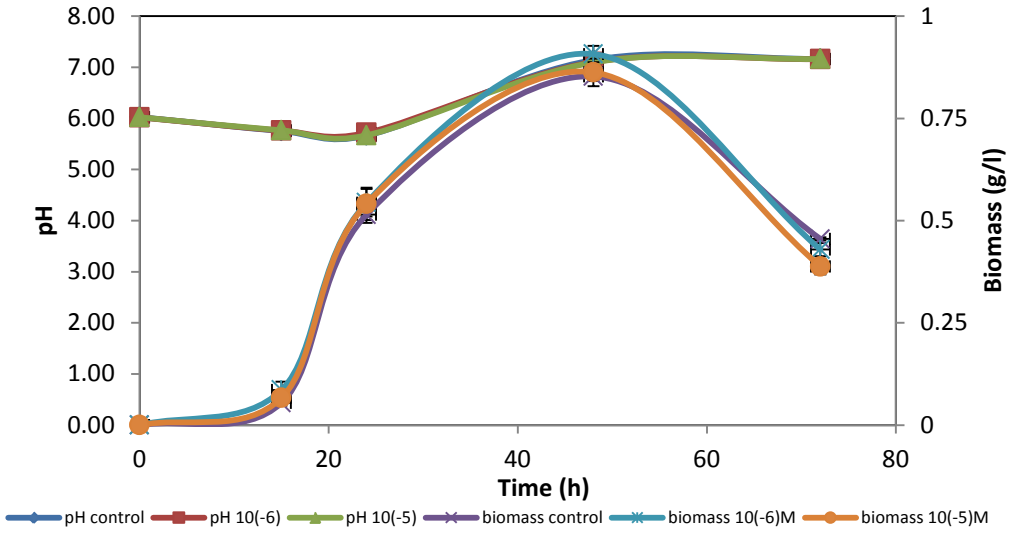


Figure 3.18
Biomass production and pH profile in the cultures of *A. fumigatus* AF-10 treated with and without hydrocortisone during the course of fermentation. Shaken flask runs were done in triplicates.

3.2.3 Effect of hydrocortisone treatment on the level of gliotoxin production in *A. fumigatus*

This study was carried out in two parts. Firstly the level of the gliotoxin produced with and without the presence of hydrocortisone (at 10^{-5}M and 10^{-6}M concentrations) in the medium was measured by HPLC analysis. However there was reliability difficulties associated with the results due to the filamentous nature of the fungus (non-homogeneity of the samples) as well as the number of steps involved in the extraction process. Consequently, there were large variations between the repeats of the samples using this technique. So a clear comparison could not be drawn between the amount of gliotoxin produced in the test and the control samples.

However, an alternative technique, flow-cytometry, was used to study the effect of culture broth of the wild type *A. fumigatus* (AF-293). The culture filtrate contained all the metabolites produced by this fungus including gliotoxin. An isogenic gliotoxin knockout strain (unable to produce gliotoxin) on A549 cells was also used in parallel to AF-293.

3.2.3.1 *Effect of hydrocortisone treated A. fumigatus culture broth on A549 cells*

In order to investigate whether stimulating A549 cells with the culture filtrate of the hydrocortisone treated cultures of *A. fumigatus* (*A. fumigatus* AF-293, wild-type and gliotoxin knockout strain ARC2 (ΔgliP)) would induce a further response in pneumocytes compared to non-treated cells, a series of cell-death investigations were carried out using flow-cytometry (Figures 3.19, 3.20, and 3.21).

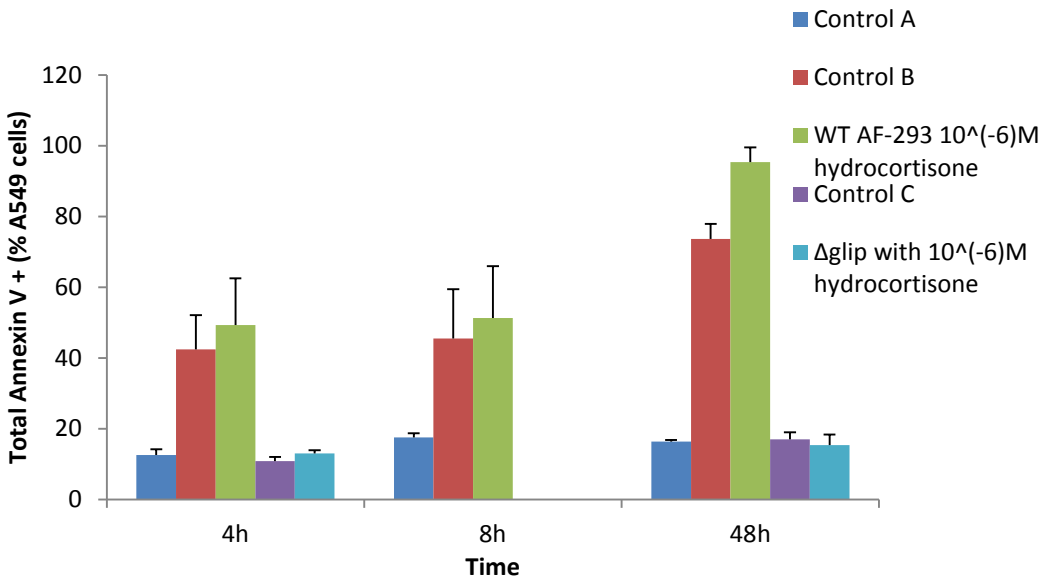


Figure 3.19

A549 apoptosis after exposure to 40% culture filtrate from A. *fumigatus* strains: wild type AF-293 for 4h, 8h, and 48h and gliotoxin knockout (*ΔgliP*) for 4h and 48h with and without hydrocortisone treatment. Percentage of total cells that were Annexin V positive (apoptotic) is shown. **Control A:** untreated A549 cells (treated with medium only). **Control B**: A549 cells exposed to WT AF-293 with no hydrocortisone supplementation. **Control C**: A549 cells exposed to *ΔgliP* strain with no hydrocortisone supplementation. Data represent means (± standard deviations) calculated from triplicate measurements in two different experiments.

Figure 3.19 shows the effect of hydrocortisone treatment of *A. fumigatus* on A549 total apoptosis after exposure to 40% culture filtrate of wild type AF-293 for 4h, 8h, and 48h, and gliotoxin knockout (*ΔgliP*) for 4h, and 48h. The wild type AF-293 and *ΔgliP* with no hydrocortisone supplementation were used as controls. The test experiments contained hydrocortisone supplemented strains. This was done in order to signify the effect of hydrocortisone, on gliotoxin production.

After 4h and 48h treatment of A549 cells with 40% culture broth of *A. fumigatus* gliotoxin knockout strain (*ΔgliP*), no significant changes in the annexin positive (apoptoic) A549 cells were observed compared to the control A cells (solely treated with cell culture medium) and control C (A549 cells exposed to *ΔgliP* strain with no hydrocortisone supplementation). At the R8 quadrant of the histogram C, viable cells have undergone a significant morphology change (up-ward move along the PI axis) compared to the R8 quadrant of the histogram B (*ΔgliP* with no

hydrocortisone treatment) (Figure 3.20).This suggests that production of other metabolites produced by the *ΔgliP* strain might also be affected by the presence of hydrocortisone in the culture medium.

Treatment of A549 cells with the wild type *Aspergillus* strain AF-293 (Figure 3.19) shows that after 4h and 8h treatment of the cells with hydrocortisone treated cultures of this fungus a slight increase in the percentage of Annexin V positive cells observed compared to the A549 cells exposed to the untreated fungus. However, the increase was insignificant for these time sets.

After 48h, a significant ($P<0.05$) increase in the number of Annexin V positive A549 cells exposed to the culture filtrate of hydrocortisone treated fungal cultures was observed when compared to A549 cells exposed to the control B. The increase was from around 74% in average to about 95% leaving almost no viable cells in the A549 cells treated with hydrocortisone treated WT AF-293 culture filtrate of *A. fumigatus*.

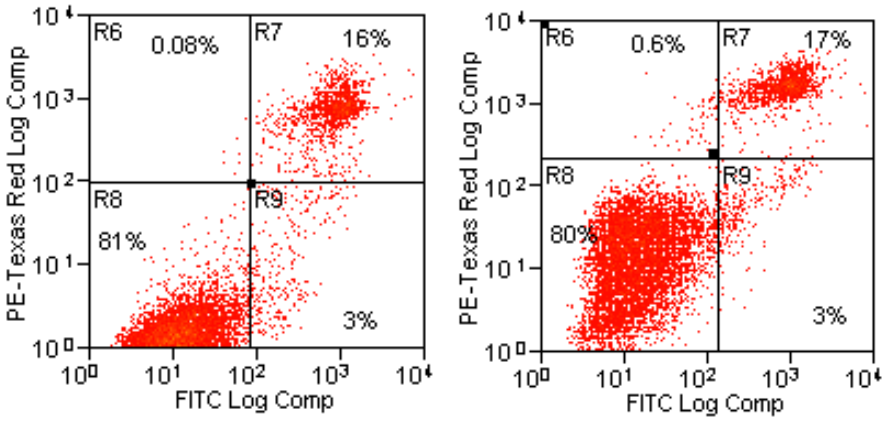
The observed results indicate that hydrocortisone enhances the degree of damage caused by the culture filtrate of *A. fumigatus* AF-293 on A549 cells.

Aspergillus fumigatus ARC2 (*ΔgliP*)

After 48 h treatment

A-Control population

B- *ΔgliP* without hydrocortisone treatment



C-*ΔgliP* with 10^{-6}M hydrocortisone treatment

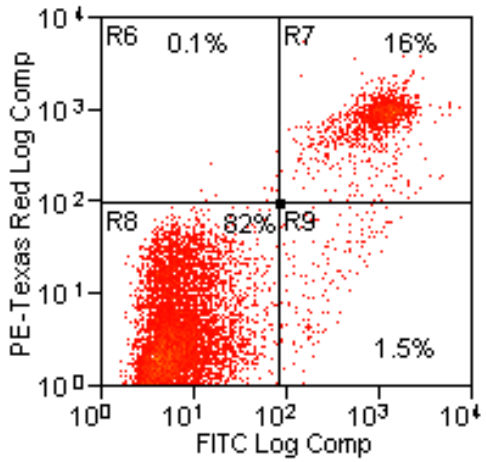


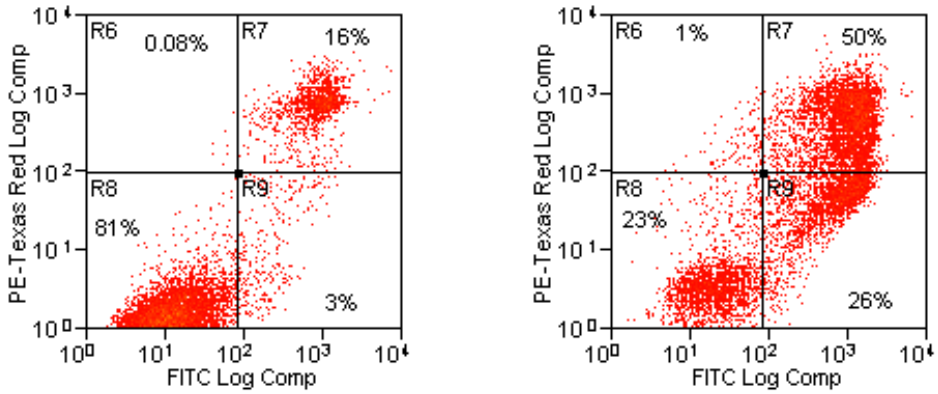
Figure 3.20
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of 48h old culture of *A. fumigatus* ARC2 strain treated with or without hydrocortisone in RPMI medium after 48h treatment. Section (A) represents the control A549 cells without any treatment with Aspergillus culture broth; (B) represents A549 cells treated with 40% culture broth from *A fumigatus* ARC2 (without any hydrocortisone treatment); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus gliP* knockout strain (ARC2) treated with 10^{-6}M hydrocortisone. Results presented here are from one experiment that is representative of the two performed.

Aspergillus fumigatus AF-293

After 48 h treatment

A-Control population treatment

B- AF-293 without hydrocortisone



C-AF-293 with 10^{-6}M hydrocortisone treatment

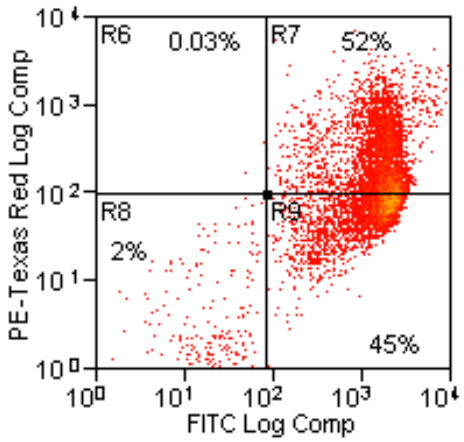


Figure 3.21
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of a 48h old culture of *A. fumigatus* AF-293 strain treated with or without hydrocortisone in RPMI medium after 48h treatment. Section (A) represents the control A549 cells without any treatment with Aspergillus culture broth; (B) represents A549 cells treated with 40% culture broth from *A fumigatus* AF-293 (without any hydrocortisone treatment); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus* AF-293 treated with 10^{-6}M hydrocortisone. Results presented here are from one experiment that is representative of the two performed.

Figure 3.20 and 3.21 demonstrate the flow-cytometric analysis of the A549 cells treated for 48h with the culture broth of *A. fumigatus* ARC2 (*ΔgliP*) and wild type AF-293 pre-treated with (group C) and without (groups B) hydrocortisone.

Figure 3.20 indicates that the total number of Annexin positive cells of group B is still very similar to the control population (group A). However, change in the morphology of the viable cells, displayed in the R8 quadrant, is observed compared to the cells in the same region of the control group. Comparison of the group B with the group C indicates a further increase in the morphological changes observed in the live cell population in R8 quadrant of the histogram C (hydrocortisone treated) suggesting other metabolites produced by the fungus might also be affected by the presence of hydrocortisone in the culture medium.

Figure 3.21 demonstrates the effect of a longer term exposure of the A549 cells to the culture broth from a hydrocortisone treated (groups C) and untreated (group B) wild type strain of *A. fumigatus.* Majority of the cells show apoptosis compared to the control untreated cells. Comparing the histogram in group B (cells exposed to the culture broth of the untreated fungus) with that of groups C (cells exposed to the hydrocortisone treated culture of *A. fumigatus*) a considerable increase in the total percentage of apoptotic cells was observed.

3.3 Binding of *A. fumigatus* spores to lung epithelial cells after hydrocortisone pre-treatment of pneumocytes

This experiment was conducted in order to investigate the effect of hydrocortisone in physiological and pharmacological levels, in the lung environment, on the binding of the spores of *A. fumigatus* to pneumocytes.

The A549 cells were pre-treated with 10^{-5}M and 10^{-6}M concentrations of hydrocortisone sodium phosphate for a period of 4 hours prior to the addition of *A. fumigatus* AF-10 spores. Pre-treatment of A549 cells with different concentrations of hydrocortisone caused a significant ($p<0.01$) increase in the number of adhered spores per 10 cells counted in each field (Figure 3.22). The untreated A549 cells (only containing cell culture medium) were considered as control. Mean (SE) number of spores adhered per five fields counted, in triplicate wells of two separate experiments are presented in the graph.

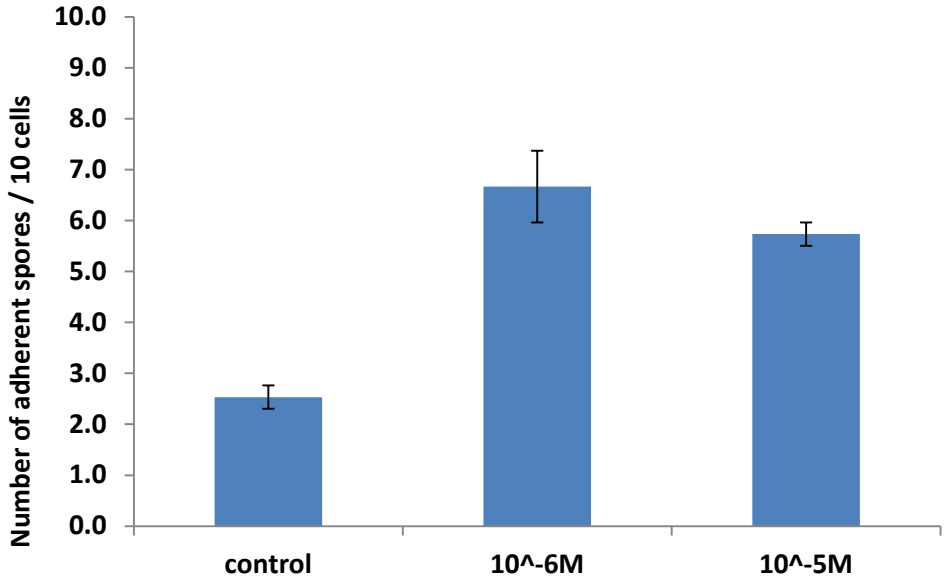


Figure 3.22
Adherence of *A. fumigatus* spores (at a concentration of $1x10^6$ spores/ml) to A549 cell monolayers after pre-treatment of the cells with 10^{-5}M and 10^{-6}M hydrocortisone sodium phosphate. Results represents standard error of the mean (SEM) spores adhered per five fields for triplicate wells in 2 separate experiments and is expressed as the number of adhered spores per 10 cells. Spore binding was significantly ($P<0.01$) increased after pre-treatment of the cells with hydrocortisone.

3.4 Proteomics: Changes in *A. fumigatus* cytosolic protein profile under the influence of hydrocortisone

Following initial observations of the effect of hydrocortisone on *A. fumigatus* morphology and gliotoxin production, to investigate the signalling mechanism of this hormone in *A. fumigatus* as well as exploring the presence of a glucocorticoid receptor in this fungus, a classic proteomic study was carried out. The proteomic response of *A. fumigatus* AF-10 to hydrocortisone was evaluated by 2D gel electrophoresis (PAGE) which allows separation of proteins based on their size and charge on a gel matrix and provides a comprehensive overview about the *A. fumigatus* proteins that are present or are induced as a result of changes in the environment and stress conditions. Prior to the 2D-PAGE step a protein purification step was carried out using an affinity chromatography system in order to enrich the hydrocortisone binding proteins. Hydrocortisone was bound to an activated sepharose gel (according to the process mentioned in section 2.7.2 of the Materials and Methods) and the purified protein fraction was subsequently used in the 2D gel electrophoresis process. The whole proteome of *A. fumigatus* was not used for analysis. In the experiment where the whole proteome was obtained, the protein spots were too numerous and too clustered resulting in an insufficient resolution and identification of individual spots. Therefore, to overcome this problem a cell fractionation approach was carried out in order to separate various cell compartments such as the cell wall and plasma membrane fraction and cytosolic fraction. The cytosolic fraction was subsequently used for further analysis. Utilization of the cytoplasmic fraction among other subcellular fractions was based on the hypothesis that the possibility of detecting any cortisol binding protein or any other proteins affected as a result of this treatment was higher in this fraction. This in turn was based on the earlier observations in the works of similar nature in other higher organisms (human/animals) or other eukaryotic microorganisms such as in *Candida albicans* where the hydrocortisone binding protein was identified in the cytoplasm (Loose *et al.*, 1981). Following cell fractionation and protein purification the protein spots were resolved at a greater level where the identification of the proteins of interest was more accurate and efficient. Each of the gels contained about 400-500 resolvable protein spots (Figure 3.23). The cytosolic fraction mainly contained soluble cytoplasmic proteins although an

overlap between subcellular fractions was also observed such as the detection of some cell wall proteins among the cytosolic fraction proteins.

A total of 13 protein spots within the cytosolic fraction were identified by MALDI TOF TOF mass spectrometry following gel excision. A number of proteins were identified in several tandem spots of the same molecular weight on the gel representing a single protein with different post-translational modifications. As mentioned earlier the initial principle used in this study was the identification of a cortisol binding proteins or identification of proteins that change in abundance when the fungus is grown in a medium containing 10^{-6}M hydrocortisone sodium succinate, when compared to the same proteins spots in the control gel with no hydrocortisone treatment . Among identified proteins within the cytosolic fraction, in the presence of hydrocortisone in the medium, a change in abundance of 1.5-fold or greater was considered significant. The ratios were obtained from the statistical tables of the Progenesis Same Spot software after analysis. Total of 38 proteins were significantly changed in abundance (both increased and decreased) in the cytosolic fraction after 48h. Figure 3.23 demonstrates the test (when fungus treated with 10^{-6}M hydrocortisone) and control (without any treatment) gels containing purified cytosolic proteins. Figure 3.24 displays the 2-D reference gel which was created after all the repeated gels from 6 different experiments in the test and control were aligned for comparison using the Progenesis Same Spot software.

The results of the proteome analysis are shown in Figure 3.23 and Table 3.1. Protein sequences obtained was identified through searching the spectral data against the 20101130 NCBI database restricted to fungi (1205507 sequences) and yielded matches presented in Table 3.1. Some proteins appeared in gels in more than one spot with the same apparent molecular weight, but different PI values and abundance possibly due to post-translational modifications. Moreover some identified spots represented more than one type of protein after identification. Therefore, the 13 identified protein spots represented 11 different proteins (Table 3.1 and Figure 3.23).

Stress response-related proteins such as heat-shock protein (hsp70), and spermidine synthase showed an increase in their abundance in the cultures grown in the presence of hydrocortisone (Table 3.2). It has been shown that particularly in pathogens, Hsp70 is involved in direct interaction with host phagocytes and plays a role in their survival in the host environment (Young and Garbe, 1991; Zügel and Kaufmann, 1999).

Spermidine synthases are involved in the biosynthesis of polyamines, which play a vital role in defence against environmental stress. Different types of proteases were also among the up-regulated proteins after hydrocortisone stimulation including peptidases, aminopertidase, prolidase pepP, serin protease Alp2 (Table 3.2). Several other proteins related to the amino acid metabolism and gluconeogenesis including methylmalonate semialdehyde dehydrogenase, fructose bis-phosphate aldolase class II and aldehyde dehydrogenase were also among the identified protein spots (Tables 3.1 and 3.2) and displayed an increase in intensity due to hydrocortisone stimulation. Lipases, transketolases together with conserved hypothetical proteins were also among the up-regulated protein spots identified in the hydrocortisone treated cultures. Lipases are reported to be involved in the virulence of *A. fumigatus* contributing to the tissue invasion and disease progress in the lungs (Rementeria *et al.*, 2005). Table 3.2 shows a summary of all identified proteins involved in various cellular activities in the cytosolic fraction of *A. fumigatus*.

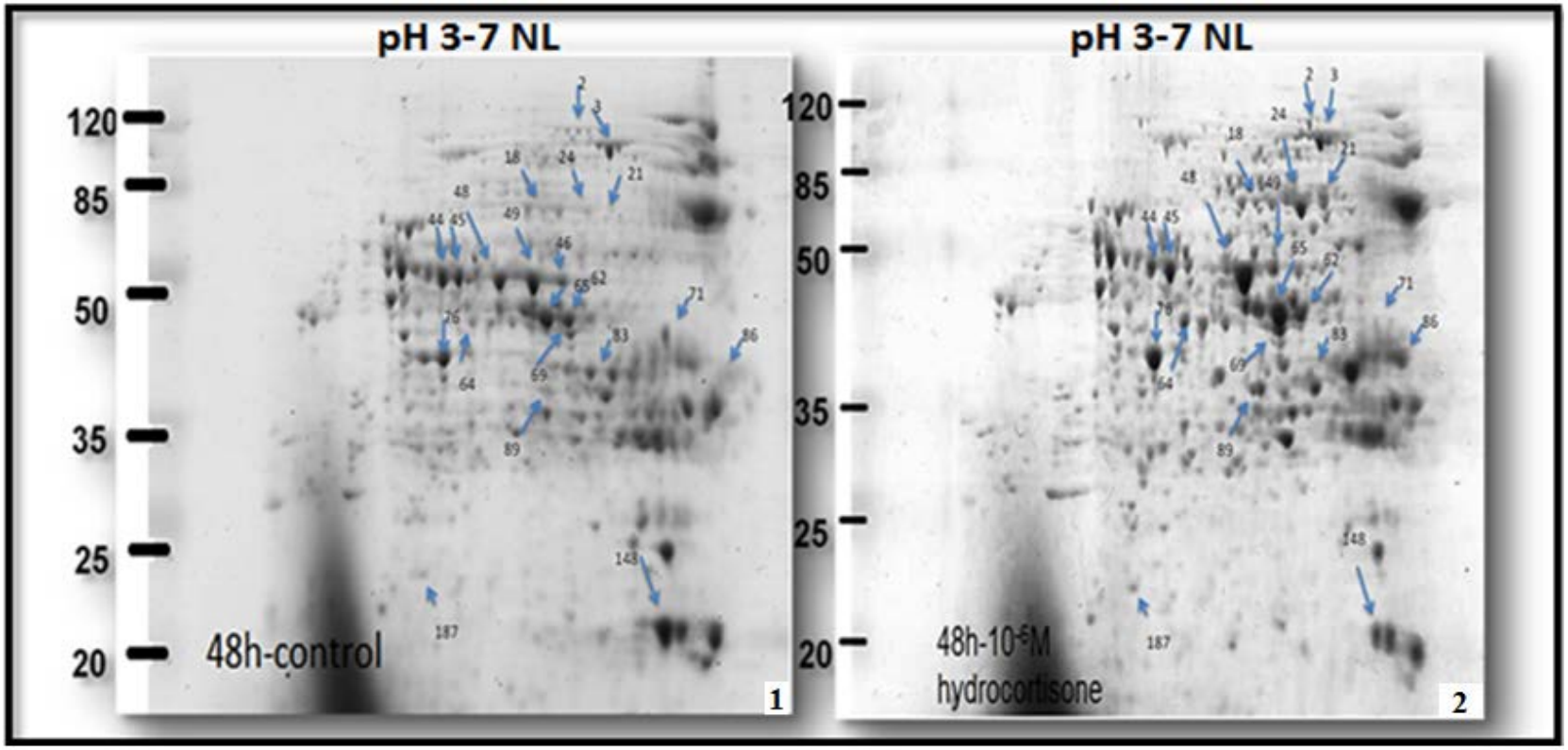


Figure 3.23
Comparative 2-D gel electrophoresis of cytosolic protein extracts of the wild type strain of *A. fumigatus* AF-10 grown for 48h with (panel 2) and without (panel 1) 10^{-6}M hydrocortisone in the medium. Proteins were focused on a pH 3-7 non-linear IPG strip and further separated by 2-D gel electrophoresis. Proteins spots were identified with MALDI-TOF-TOF.

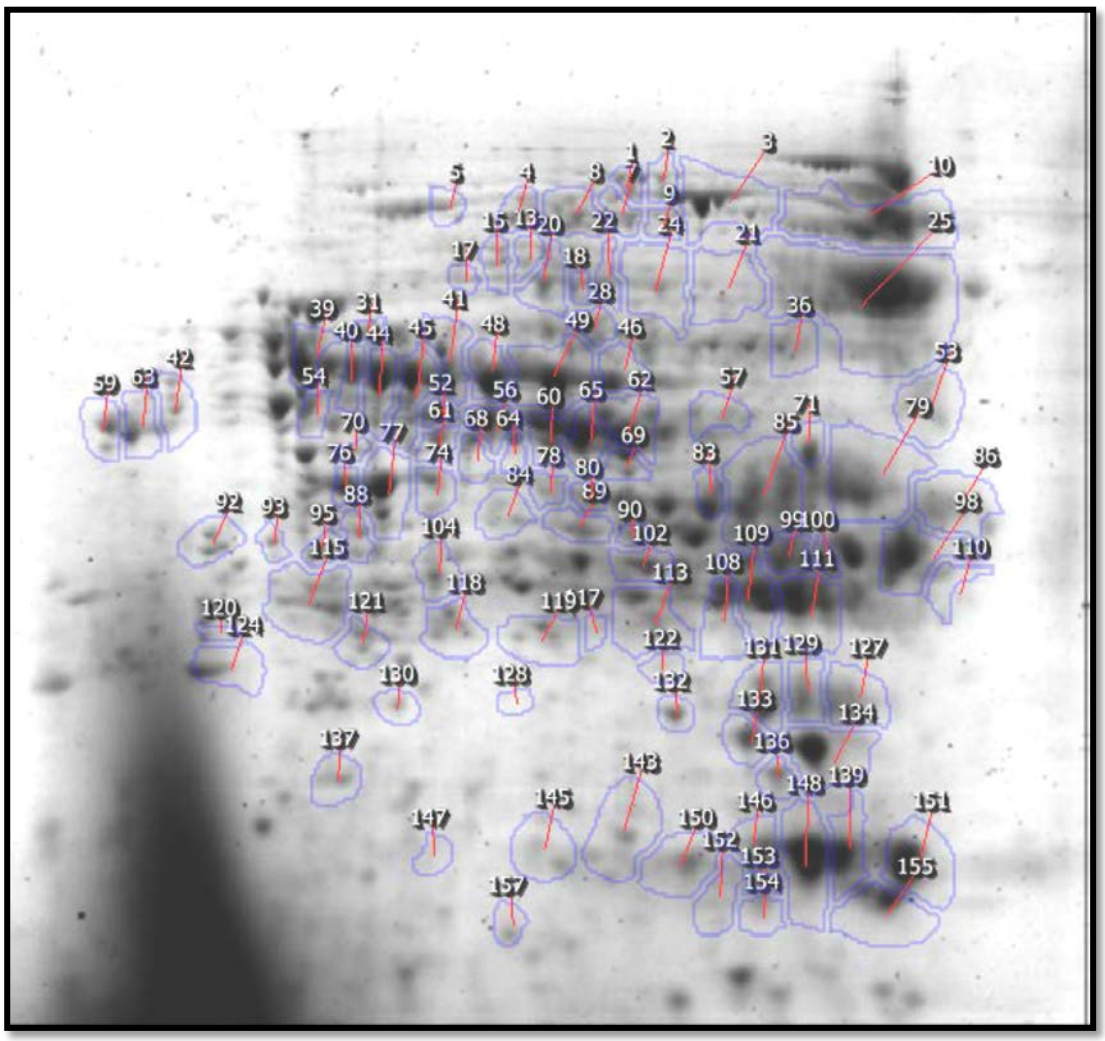


Figure 3.24
Reference 2-D gel electrophoresis of cytosolic protein extracts of the wild type strain of *A. fumigatus* AF-10 grown for 48h with and without 10^{-6}M hydrocortisone in the medium. The reference gene was generated by Progenesis same Spot software after aligning the test and the control gels. Proteins were stained with Coomassie blue. The numbers refer to the proteins whose level changed during growth with hydrocortisone.

Table 3.1
Identified protein spots excised from the 2-D gels. proteins were identified by MALDI-TOF-TOF. Various proteins of *A. fumigatus* AF-10 were differently synthesized after incubation with 10^{-6}M hydrocortisone. Fold changes were extracted from statistical tables of the Progenesis Same Spot after analysis.

Spot no.	Name	MW	pI	Pathways involved in	Fold increase	score	Match to	Peptide sequence
48	***Hsp70***	**67300**	**5.28**	***Chaperone, protein export, Splisosome, Endocytosis, subunit in human GR receptor***	***2.1***	**931/ 499**	**gi|45356863**	**K.DAGLIAGLNVLR.I, R.IEIIANDQGNR.T, R.FEELCQDLFR.S, K.SSVHEIVLVGGSTR.I, R.TTPSFVAFTDTER.L, K.ATAGDTHLGGEDFDNR.L/ K.DAGLIAGLNVLR.I, R.IEIIANDQGNR.T, R.FEELCQDLFR.S, K.SSVHEIVLVGGSTR.I, R.TTPSFVAFTDTER.L, K.ATAGDTHLGGEDFDNR.L, M.APAVGIDLGTTYSCVGVFR.D, R.ETAEAYLGGTVNNAVITVPAYF NDSQR.Q, R.TLSSAAQTSIEIDSLFEGIDFYTS ITR.A**
49	**Hsp70**	**67300**	**5.28**	***Chaperone, Splisosome, protein export , Endocytosis, subunit in human GR receptor***	**1.4**	**887**	**gi|45356863**	**K.DAGLIAGLNVLR.I, R.LVNHFVNEFK.R, R.FEELCQDLFR.S, K.SSVHEIVLVGGSTR.I, R.TTPSFVAFTDTER.L, K.ATAGDTHLGGEDFDNR.L, M.APAVGIDLGTTYSCVGVFR.D, R.ETAEAYLGGTVNNAVITVPAYF NDSQR.Q, R.TLSSAAQTSIEIDSLFEGIDFYTS ITR.A**

69-i	**Fru-BP-aldolase,Class II+**	**39937**	**5.55**	**Glycolysis / Gluconeogenesis, Pentose phosphate pathway, Fructose and mannose metabolism, Methane metabolism, Biosynthesis of secondary metabolites**	**2.0**	**541/451**	**gi|70999466**	**R.LHPELLSK.H, R.LFEYAQEK.N, K.SGVIVGDDVLR.L, R.KSGVIVGDDVLR.L, K.ASIAGSIAAAHYIR.S, K.RVQVALEDFNTAGQL.-, K.DKPVYFVFHGGSGSTK.E**
69-ii	**peptidase**	**46350**	**5.24**	**proteolysis/ protein catabolism**		**75**	**gi|70991449**	**K.AFSQSTHLNPSPSR.F**
46-i	**Hsp70 +**	**67300**	**5.28**	***Chaperone, Splisosome, protein export , Endocytosis, subunit in human GR receptor***	**1.7**	**630**	**gi|45356863**	**K.DAGLIAGLNVLR.I, R.IEIIANDQGNR.T, R.LVNHFVNEFK.R, K.SSVHEIVLVGGSTR.I, R.TTPSFVAFTDTER.L, K.ATAGDTHLGGEDFDNR.L, M.APAVGIDLGTTYSCVGVFR.D**
46-ii	**peptidyl-prolyl cis-trans isomerase - CPr-7**	**41935**	**5.99**	**Homologue of cyp-40 in human which is a co-chaperone in steroid receptor complex, it regulates ATP-ase activity during receptor-hsp90 assembly**	**1.7**	**59**	**gi|159128753**	**R.VYFDIQIGR.Q**
62	**Fru-BP-aldolase,Class II**	**39937**	**5.55**	**Glycolysis / Gluconeogenesis, Pentose phosphate pathway, Fructose and mannose metabolism, Methane metabolism, Biosynthesis of secondary metabolites**	**1.3**	**300**	**gi|70999466**	**K.ASIAGSIAAAHYIR.S, K.RVQVALEDFNTAGQL.-, K.LLPWLDGMLDEDER.Y, K.NFAIPAVNVTSSSTVVACLEAAR.D**
83-	**Methyltransferase SirN-like**	**29535**	**5.21**	**Histidine metabolism, Tyrosine metabolism**	**1.4**	**563**	**gi|70983245**	**K.LGHQPELLR.L, K.ILDSGTADGTWLR.N, K.SSFTGEQLDALPAR.V, K.DPWPQQYLGFFDLVHIR.G, K.AKPELHDQSIHGVTGPIVPLTSVAR.T**
71-i	**Methyltransferase SirN-like +**	**29535**	**5.21**	**Histidine metabolism, Tyrosine metabolism**	***1.3***	**481**	**gi|70983245**	**K.ILDSGTADGTWLR.N, K.SSFTGEQLDALPAR.V, K.DPWPQQYLGFFDLVHIR.G, K.AKPELHDQSIHGVTGPIVPLTSVAR.T**

71-ii	**extracellular *Lipase***	**31823**	**5.59**	***Glycerolipid metabolism***	***1.3***	**57**	**gi|70985264**	**K.AGIPVELYGYGSPR.V**
79	***Hsp70***	**67300**	**5.28**	***Chaperone, protein export, Splisosome, Endocytosis, subunit in human GR receptor***	**1.1**	**222**	**gi|45356863**	**K.FELTGIPPAPR.G, K.SETFSTYSDNQPGVLIQVYEGER.A**
148	**Conserved hypothetical protein**	**18188**	**5.13**	**-**	**1.8**	**778**	**gi|70983267**	**R.DFTSELNNAR.G, R.DFDAQVEFFFR.D, R.AEISLGWVETYVR.E, R.VRDFDAQVEFFFR.D, R.EGQVVNLDEAGKPTFLPFK.D, K.GAADADFQALQAEEGPFVSGER.A, R.QPNVSEEEFYQWVTEQHAAR.A**
139	**Conserved hypothetical protein**	**18188**	**5.13**	**-**	**1.4**	**708**	**gi|70983267**	**R.DFTSELNNAR.G, R.DFDAQVEFFFR.D, R.AEISLGWVETYVR.E, R.EGQVVNLDEAGKPTFLPFK.D, K.GAADADFQALQAEEGPFVSGER.A, R.QPNVSEEEFYQWVTEQHAAR.A**
3	**Catalase**	**79924**	**5.65**	**Tryptophan metabolism, Methane metabolism, Peroxisome**	**1.5**	**604**	**gi|1857716**	**R.VPVHNNNR.D, K.QFVIDAIR.F, K.NNVIIQLNR.I, R.QDLHDAIEAGR.Y, R.LFSYLDTQLNR.H, R.GVDFTEDPLLQGR.L, R.AVSSSFEDVWSQPR.L, R.HGGPNFEQLPINQPR.V**
9	**Amino-peptidase**	**72919**	**5.93**	**Glutathione metabolism, Arginine and proline metabolism(lap-3)**	**2.1**	**63**	**gi|70998210**	**K.THHFFENDEFTR.S**
18-i	**Aldehyde dehydrogenase +**	**61477**	**6.30**	**Glycolysis / Gluconeogenesis , Pentose and glucuronate interconversions ,Ascorbate and aldarate metabolism,Fatty acid metabolism, Valine, leucine and isoleucine degradation, Lysine degradation, Arginine and proline metabolism, Histidine metabolism,Tryptophan**	**1.9**	**559**	**gi|70992355**	**K.VAFTGSTLVGR.N, R.ILVQESIYEEFLAR.F, K.EAGFPPGVINIISGFGR.T, K.YEQPLGLFINNEFVK.G, K.IHGQTIDVNPETLTYTR.H, K.VGDPFDPQTFQGPQVSQLQFDR.I**

				metabolism, beta-Alanine metabolism, Glycerolipid metabolism, Pyruvate metabolism, Propanoate metabolism, Biosynthesis of secondary metabolites				
18-ii	ATP citrate lyase subunit	52999	5.88	Citrate cycle (TCA cycle), Biosynthesis of secondary metabolites	1.9	175	gi|70992211	K.GVHNVLVDFISR.L, R.EVAPVLNEHNVQIWVR.R, R.APVIKPTPLPPSSTHNPPPR.L
21	Prolidase pepP	52367	5.46	Dipeptidase, Proline dipeptidase, also acts as aminoacylhydroxyproline analogue	2.5	143	gi|71000810	R.NVLIDAGGEYR.T, R.LIEDNDEPAPFR.Q
24	Methylmalonate-semialdehyde dehydrogenase	64338	8.26	Valine, leucine and isoleucine degradation, beta-Alanine metabolism, Inositol phosphate metabolism, Propanoate metabolism	3.0	331	gi|70993396	K.ALNVNGGFEK.G, R.VPQSTDEELR.A, K.NHAAVLPDCNK.N, K.NEYGNGAAVFTCSGSTASR.F
64-i	D-xylose reductase+	35692	6.09	Found in no pathway in *A.fumigatus*, xylose metabolism	1.7	175	gi|70990764	K.LWNSFHDGDR.V, R.LAQNLDVTGWDLEASEIEAISALNR.N
64-ii	myo-inositol-phosphate synthase+	58806	5.82	Inositol phosphate metabolism, Biosynthesis of secondary metabolites	1.7	105	gi|70988765	K.ASWAHVER.I, R.YAYHTTDITR.T
64-iii	Nitrilase family protein +	31904	6.38	Tryptophan metabolism, Cyanoamino acid metabolism, Nitrogen metabolism,	1.7	59	gi|70992703	R.AVDNQIYVGLCSPAR.D
64iv	metallo-beta-lactamase domain protein	32432	6.14	No pathway matched-member of glyoxalase II family that catalyses the hydrolysis of S-D lactoyl-glutathione to form glutathione and D-lactic	1.7	53	gi|238487352	K.FTLQGTNTYLIGR.G
68-i	cell wall biogenesis protein /Glutathione transferase +	50603	8.51	Glutathione metabolism	1.7	378	gi|71002462	R.FATADEEVAGENVTPDPLHPDFTHLR.A, R.AIYFSNDPDYTGR.F, R.NVDLYPPALR.S, K.DANSPYFFGSSITEVDIR.L, R.LYWDVPAFR.E
68-ii	Serine protease Alp2	52779	5.81	Lysine degradation, Biotin metabolism	2.0	354	gi|70997972	R.AYFSNYGK.C, K.TIPTDDEDADGNGHGTHCSGTIAGR.K,

								K.YLYASEGGEGVDAYTIDTGINV DHVDFEGR.A
89	Spermidine synthase	33766	5.33	Cysteine and methionine metabolism, Arginine and proline metabolism, beta-Alanine metabolism, Glutathione metabolism	1.8	197	gi|70995928	R.YYNQDIHR.A, K.VLVIGGGDGGVLR.E, K.VHIGDGFQFLK.E
76	Autophagic serine protease Alp2	52779	5.81	Lysine degradation, Biotin metabolism	2.6	640	gi|70997972	R.AYFSNYGK.C, R.DSLSFGTFNK.Y, R.SSGSGTMSDVVAGVEWAVK.S, R.SSGSGTMSDVVAGVEWAVK.S, K.TIPTDDEDADGNGHGTHCSGT IAGR.K, K.YLYASEGGEGVDAYTIDTGINV DHVDFEGR.A
187-i	Allantoicase/	46472	6.02	Purine metabolism, nucleotide metabolism		113	gi|70999292	K.ADTEHVYEGAELAAGGEDTR.T
187-ii	aldehyde dehydrogenase /	54068	6.16	As in 18i		85	gi|70982606	K.EAGFPPGVINILSGFGR.V
187-iii	transketolase	73843	5.53	Pentose phosphate pathway, Biosynthesis of secondary metabolites		68	gi|18202255	K.YSHEQFGLNR.F

Table 3.2
Identified protein spots within various cellular activity categories.

Category	Example
Stress related	HSP70, catalase, spermidine synthase
Amino acid metabolism	Methylmalonate-semialdehyde dehydrogenase, Nitrilase family protein, methyltransferase SriN-like
Gluconeogenesis	Fru-Bp-aldolase Class II, Aldehyde dehydrogenase
Protease	Aminopeptidase, prolidase pepP, serine protease Alp2
Other	Lipase, conserved hypothetical proteins, transketolase, peptidyl-prolyl cis-trans isomerase-CPr-7

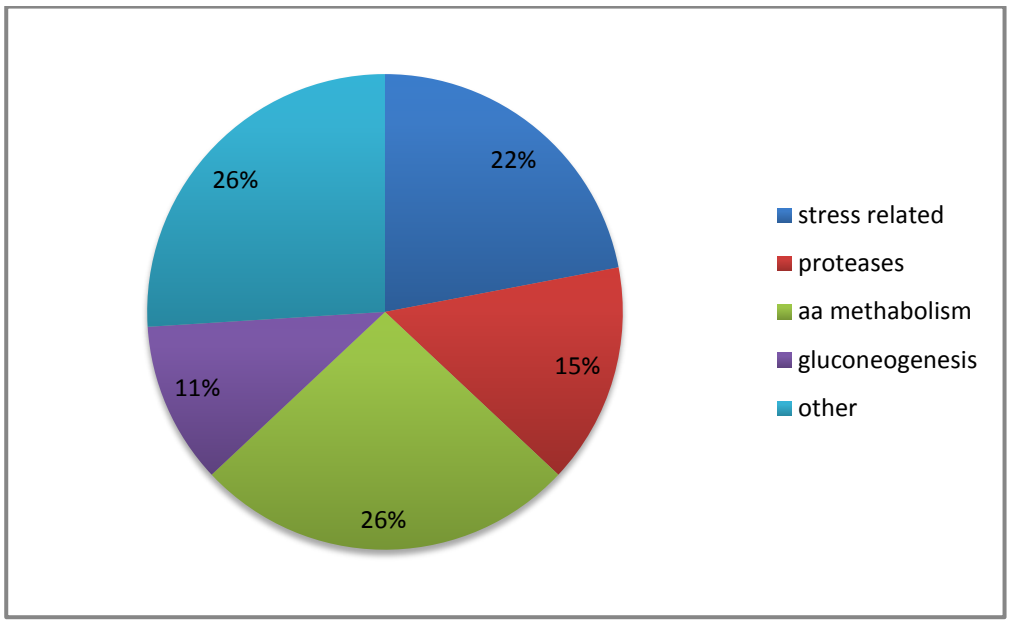


Figure 3.25
Functional grouping of the *A. fumigatus* AF-10 proteins in the cytosolic fraction affected by hydrocortisone treatment.

3.5 Transcriptomic studies

In order to further explore the molecular responses to hydrocortisone treatment in *A. fumigatus* AF-10 transcriptomic experiments were carried out. Changes in the mRNA expression levels of some of the genes encoding for the proteins involved in the growth and pathogenicity of this fungus were investigated. To this end, *A. fumigatus* was grown in a medium supplemented with or without hydrocortisone sodium phosphate. RNA was then extracted from both test (with hydrocortisone) and control (without hydrocortisone) samples (Figure 3.26). Following RNA purification cDNA synthesis was carried out to create a cDNA library of all the expressed genes for both test and control samples. The generated cDNA was then used as DNA template in subsequent PCR reactions to investigate the expression of the desired genes at mRNA level. Both A260 to A280 ratio ($A_{260/A280}$) (measure of nucleic acid purity) and the concentration of the purified RNA and generated cDNA in each experiment were quantified using the Nano Drop equipment before gel electrophoresis. Genes of interest in this study initially consisted of *HSP70, MMSAD, FBA, Alp-2, gliZ, gliP* and prolidase *pepP*, for which specific primers were designed and employed in PCR and qRT-PCR experiments (Table 3.3). PCR reactions were run on a 1.5% agarose gel to confirm that the product was unique and of the correct size. However not all the designed primers performed as expected. Due to the time constrains, after troubleshooting, complete analysis was only performed on the following remaining genes: *hsp70*, *Alp2*, *gliZ*, TEF-1 and *MMSAD*. In order to further confirm the specificity of the designed primers, sequence analysis was also performed on all generated PCR products by the UCL DNA sequencing service at the Wolfson Institute for Biomedical Research, London, UK and sequence homology of the PCR products to *A. fumigatus* target genes at nucleotide level was performed by the input of the obtained DNA sequences into nucleotide BLAST.

Analysis of the level of gene expression in the test and control samples was then performed by (quantitative real-time PCR) qRT-PCR technique. qRT-PCR experiments were also performed for the expression analysis of the reference gene (*TEF-1*). RNA quantitation was performed according to the 2ΔΔct method. qRT-PCR results were normalized against (TEF-1) gene and compared to the expression of the same gene in control condition.

A negative control (reaction mixture without template) was included in all experiments including cDNA synthesis (control with no RT enzyme), PCR and RT-PCR reactions to ensure there was no DNA contamination. All the genes used in this study are listed below.

Table 3.3
Table demonstrates a list of all the genes for which primers were designed in order to explore in the RNA quantitation studies.

Shortened Name	Full name and function
HSP70	Heat-shock protein 70- chaperon
MMSAD	Methylmalonate-semialdehyde dehydrogenase-involved in amino acid degradation, propanoate and inositol phosphate metabolism
FBA	Fructose-BP-aldolase,Class II- involved in Glycolysis / Gluconeogenesis, Pentose phosphate pathway, Fructose and mannose metabolism, Biosynthesis of secondary metabolites
ALP2	Serine protease Alp2- involved in growth and sporulation in *A. fumigatus,* lysine degradation
gliZ	A transcriptional regulator of gliotoxin biosynthesis pathway contribute to *A. fumigatus* virulence, gliZ is encoding a putative Zn_2Cys_6 binuclear transcription factor
gliP	Non-ribosomal peptide synthase involved in the biosynthesis of gliotoxin and virulence in *A. fumigatus*
Prolidase pepP	Prolidase pepP- dipeptidase, involved in amino acid degradation
TEF-1	Encoding translation elongation factor-1- Endogenous reference gene
Tub-1	Encoding β-tubulin- Endogenous reference gene

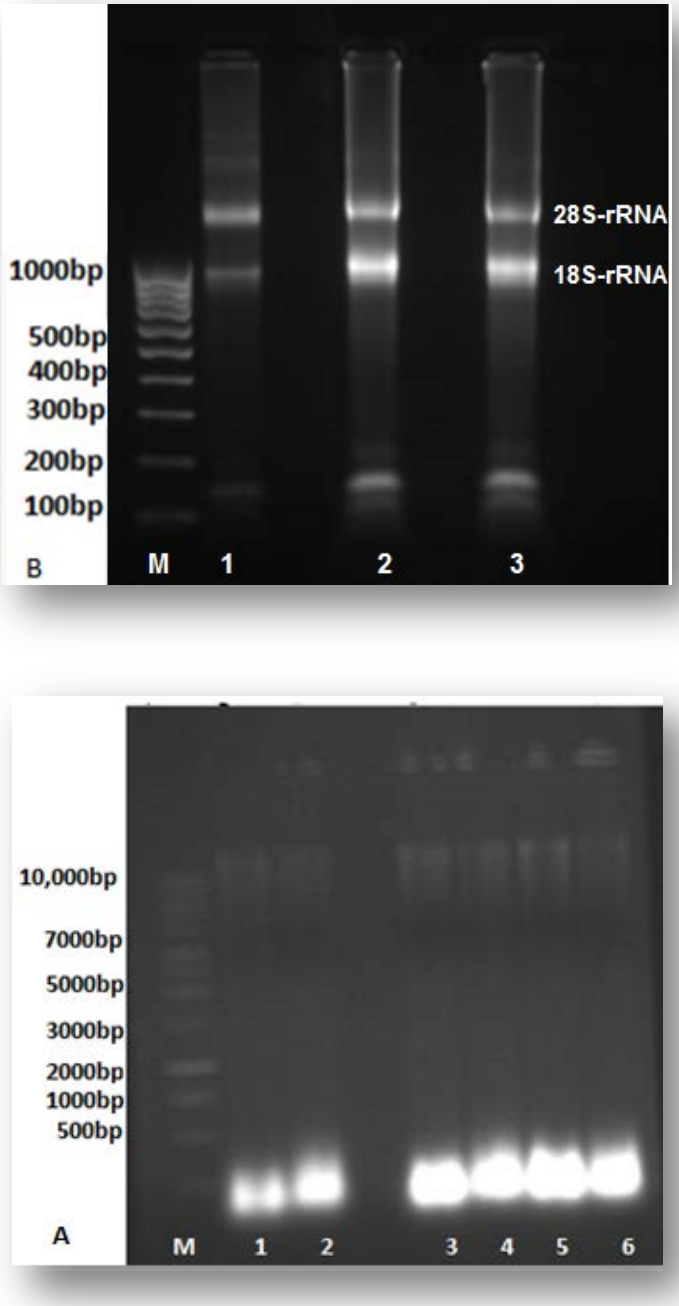


Figure 3.26
Ethidium bromide stained agarose gel showing total RNA purified from *A. fumigatus* AF-10 strain. RNA was isolated from *A. fumigatus* cells grown with (test) and without (control) hydrocortisone sodium phosphate. RNeasy Plant Mini kit was used for RNA extraction following the disruption of cells in a homogenizer. The purified RNA (10µl) from each sample was loaded onto an agarose gel stained with Ethidium bromide to visualize the 28S and 18S RNA species integrity and overall RNA quality. Panel A shows the purified RNA from 48h culture and panel B shows the RNA extracted from 24h old culture. In panel A the first lane from left is the 1kb DNA ladder, lanes 1-3 total RNA from control culture, and lanes 4-6 total RNA from test cultures.

Figure 3.26 shows the total RNA from *A. fumigatus* electrophoresed on a 1.5% agarose gel. Total RNA was purified from both 48h old culture (panel A) and 24h old cultures (panel B). The RNA gel of the 24h culture shows the two ribosomal RNA subunits (28S rRNA and 18SrRNA). However, the RNA extracted from the 48h culture suggests that the distinct ribosomal RNA subunits are no longer visible. This may be due to an increased expression of tRNA. This pattern can be compared with the growth curve of the *A. fumigatus* in Vogel's medium (the same medium was used here). Figures 3.7 suggest that at around 48h the fungus is at the stationary phase of growth where tRNA is mainly produced and rRNA genes are potentially less active. A similar case has been reported in yeast where a Rpd3p gene-encoded protein resulted in rRNA inactivation in the stationary phase in yeast cells. This same gene is also reported to be present in *A. fumigatus* encoding the histone deacetylase protein (Moss, 2004; Miskei *et al.*, 2009). In contrast, the 24h fungal cells are still in their growth phase. This stage is associated with rRNA synthesis (Wagner, 2002). Moreover, mRNA generally accounts only for 1-5% of the total RNA and majority of the RNA molecules are tRNAs and rRNAs which depending on the cell type and physiological state either of them can be in abundance (Lodish *et al.*, 2000). This could also be another reason for the results observed. Total RNA samples from all the three independent experiments at 48h had A260/A280 purity ratio of between 1.8 and 2 (as measured by NanoDrop). Subsequent cDNA generation and PCR and qRT-PCR reactions resulted in specific products of the expected size. Sequence analysis further confirmed the generated PCR products.

3.5.1 The heat-shock protein 70 (*hsp70*) mRNA expression in *A. fumigatus*

Following cDNA biosynthesis from the extracted mRNA, PCR was carried out to examine the expression of the *hsp*70 gene in the mRNA of *A. fumigatus* AF-10. Figure 3.27 represents the result of the PCR reaction after running of the samples on a 1.5% agarose gel and staining by ethidium bromide.

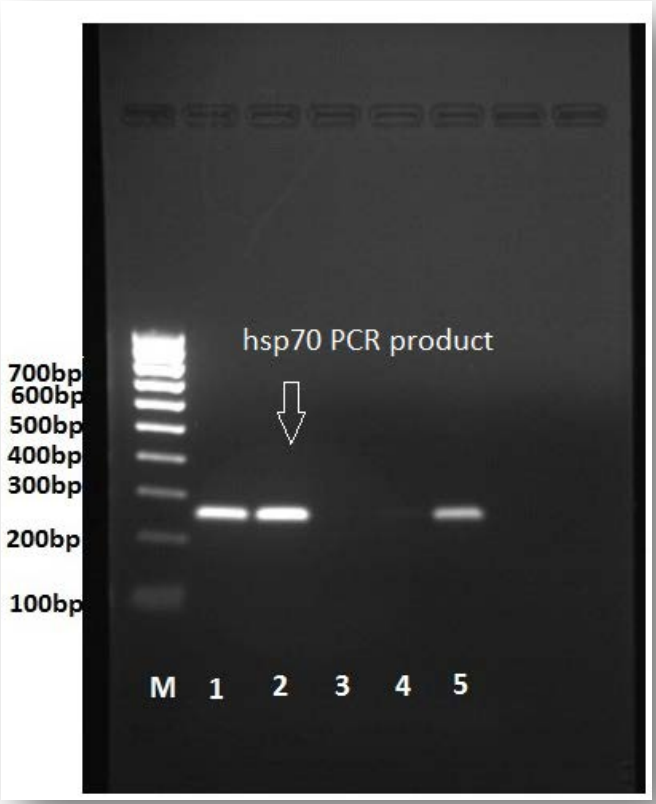


Figure 3.27
Ethidium bromide stained agarose gel (1.5% w/v) showing mRNA expression of the *hsp70* gene from the template cDNA of *A. fumigatus.* M: Molecular ladder (hyper ladder IV) 1Kb, lanes 1, 2, and 5 show the hsp70 PCR product and lane 3 indicates the negative control.

*hsp*70 was chosen as one of the genes used in qRT-PCR studies because it was identified from various protein spots in the 2-D gel electrophoresis and an up-regulation in its protein expression was observed after hydrocortisone treatment. Figure 3.26 demonstrates the PCR carried out for mRNA expression of *hsp*70 in *A. fumigatus* cells grown for 48h. The expected 261bp product observed in Figure 3.27 confirmed the *hsp*70 mRNA expression for the *hsp*70 peptide. Furthermore,

observation of only one band with the expected product size also confirms the specificity of the designed primer pairs for this gene and an efficient amplification of the *hsp*70 mRNA which was further confirmed by sequence analysis.

3.5.1.1 Sequence analysis data for the A. fumigatus hsp70

3.5.1.1.1 *hsp70* mRNA forward sequence

CCTGACCATTGAGGAGGGTATCTTCGAGGTCAAGGCTACCGCTGGTGACACTCACCTGGGTGG
TGAGGACTTCGACAACCGTCTTGTTAACCACTTTGTTAATGAATTCAAGAGAAAGCACAAGAA
GGTATGTAGATCCCTTTCCTATGGTATCTCTATTGACAGTGGCTAACCCTGTTCACAGGATCTA

- Accession number: XM_745397
- Length of sequence: 190 nucleotides
- Identities: 128/128(100%)
- Sequence location on *A. fumigatus* mRNA: 621 to 748

3.5.1.1.2 *hsp70* mRNA reverse sequence

TAGGAAGGGATCTACATACCTTCTTGTGCTTTCTCTTGAATTCATTAACAAAGTGGTTAACAAG
ACGGTTGTCGAAGTCCTCACCACCCAGGTGAGTGTCACCAGCGGTAGCCTTGACCTCGAAGAT
ACCCTCCTCAATGGTCAGGAGGGAAACATCGAAAGTACCACCACCAAGATCGAAGATCAGGAC
GTTACGCTCGCCCT

- Accession number: XM_745397
- Length of sequence: 204 nucleotides
- Identities: 186/186(100%)
- Sequence location on *A. fumigatus* mRNA: 563 to 748

3.5.2 The translation elongation factor-1 (*TEF*-1) mRNA expression in *A. fumigatus*

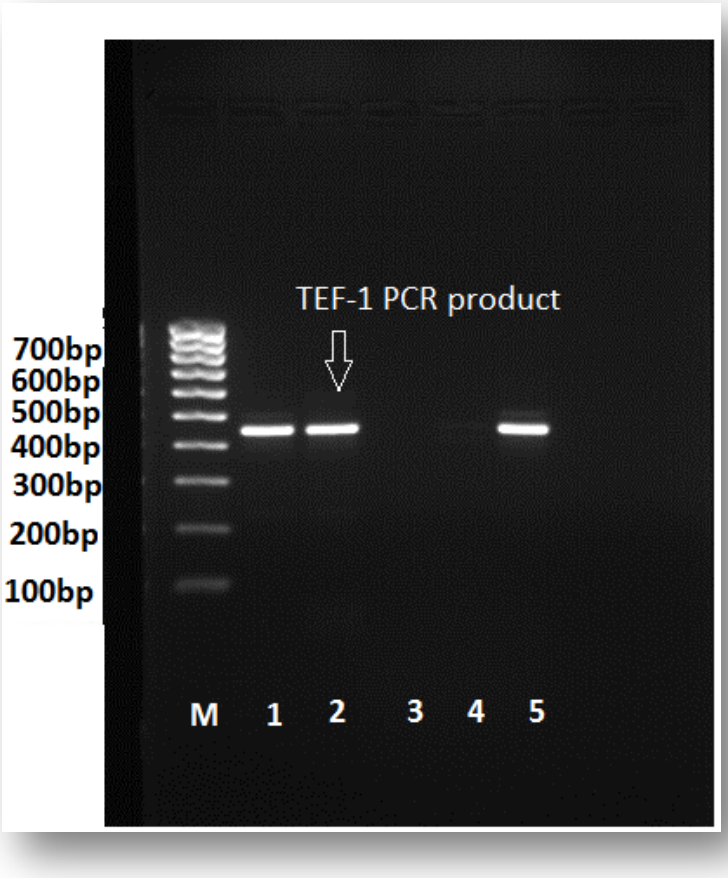


Figure 3.28
Expression of *TEF*-1 mRNA by *A. fumigatus* on a 1.5% (w/v) agarose gel stained with ethidium bromide. M: Molecular ladder (hyper ladder IV) 1Kb, lanes 1, 2, and 5 show the *TEF*-1 PCR product and lane 3 indicates the negative control and line 4 is empty.

TEF-1 was selected as the reference gene for qRT-PCR studies since it has been used for the same purpose in other studies on *A. fumigatus* (Sheppard *et al.*, 2005; Gravelat *et al.*, 2008; Al Abdallah *et al.*, 2012). Following PCR using *A. fumigatus* cDNA template and designed gene specific primers agarose gel electrophoresis was performed and an expected PCR product of 449 bp was visualized after ethidium bromide staining. Figure 3.28 shows that using these primers at annealing temperature of 56°C results in formation of a single specific amplicon for *TEF-1*. The specificity of the amplicon was further confirmed by sequence analysis.

3.5.2.1 Sequence analysis data for the A. fumigatus TEF-1

3.5.2.1.1 *TEF*-1 mRNA forward sequence

ACGTCTGCGGTGACTCCAAGAACGATCCCCCTCAGGGTGCTGCCTCCTTCAACGCCCAGGTCAT
CGTCCTCAACCACCCCGGTCAGGTCGGCGCTGGTTACGCCCCCGTCCTCGACTGCCACACTGCC
CACATTGCTTGCAAGTTCTCTGAGCTGCTTGAGAAGATTGACCGCCGTACCGGCAAGTCTGTTG
AGAACAACCCCAAGTTCATCAAGTCCGGTGATGCCGCCATCGTGAAGATGGTTCCTTCCAAGCC
CATGTGTGTCGAGTCCTTCACTGACTACCCCCCTCTGGGTCGTTTCGCCGTCCGTGACATGCGC
CAGACTGTTGCTGTTGGTGTCGTCAAGTCCGTCGAGAAGGCCGCCAGCGGTGCCGTAAAGGTC
ACCCAACAGCAACAGTCTGGCGCATGTCACGGACGGCGAAACGACCCAGAGGGGGGTAGTCA
GTGAAGGACTCGACACACATGGGCTTGGAAGGAACCATCTTCACGATGG

- Accession number: XM_745295
- Length of sequence(range1): 384 nucleotides
- Identities: 384/386(99%)
- Sequence location on *A. fumigatus* mRNA: 1070-1455
- Length of sequence(range2): 109 nucleotides
- Identities: 109/109(100%)
- Sequence location on *A. fumigatus* mRNA: 1298 to 1406

3.5.2.1.2 *TEF*-1 mRNA reverse sequence

CCACAGCAACAGTCTGGCGCATGTCACGGACGGCGAAACGACCCAGAGGGGGGTAGTCAGTG
AAGGACTCGACACACATGGGCTTGGAAGGAACCATCTTCACGATGGCGGCATCACCGGACTTG
ATGAACTTGGGGTTGTTCTCAACAGACTTGCCGGTACGGCGGTCAATCTTCTCAAGCAGCTCAG
AGAACTTGCAAGCAATGTGGGCAGTGTGGCAGTCGAGGACGGGGGCGTAACCAGCGCCGAC
CTGACCGGGGTGGTTGAGGACGATGACCTGGGCGTTGAAGGAGGCAGCACCCTGAGGGGGA
TCGTTCTTGGAGTCACCGCAGACGTTACCACGGCGGACTTCCTTGACGGAAACGTTCTTGACGT
TGAAACCGACGTTGC

- Accession number: XM_745295
- Length of sequence: 387 nucleotides
- Identities: 387/387(100%)
- Sequence location on *A. fumigatus* mRNA: 1017 to 1403

3.5.3 The methylemalonate-semialdehyde dehydrogenase (*MMSAD*) mRNA expression in *A. fumigatus* AF-10 cells

Figure 3.29
Expression of *MMSAD* mRNA by *A. fumigatus* AF-10 on a 1.5% (w/v) agarose gel stained with ethidium bromide. M: Molecular ladder (hyper ladder IV) 1Kb, lanes 1-5 show the *MMSAD* PCR amplicon and lane 6 indicates the negative control.

The reason for selection of this gene for qRT-PCR analysis was also, similar to hsp70, due to the observation of up-regulation of the corresponding protein in earlier proteomic studies following hydrocortisone treatment. Methylmalonate-semialdehyde dehydrogenase is one of the genes involved in amino acid metabolism and might contribute to the virulence of *A. fumigatus*. Following PCR using *A. fumigatus* cDNA template and gene specific primers agarose gel electrophoresis was performed and an expected PCR product of 500 bp was visualized after ethidium bromide staining. Figure 3.29 shows that using these primers at the annealing temperature of 56°C results in formation of a single specific amplicon for *MMSAD*. The specificity of the amplicon was further confirmed by sequence analysis.

3.5.3.1 Sequence analysis data for the A. fumigatus MMSAD

3.5.3.1.1 *MMSAD* mRNA forward sequence

TTGATCGCCAGTGCTGAACAGGAGGGTGCCACCATCCTTCTGGATGGCAGGGGCTATAAGCCT
GAGAAGTATCCCAACGGCAACTTTGTCGGCCCAACTATCATCACCAACGTGACGCCTGACATG
AAATGCTACAAGGAGGAAATCTTCGGCCCTGTCCTCATCTGCCTCAACGTCCCCACGCTGGACG
ACGCCATTGAACTCATCAACAAGAACGAGTATGGTAACGGAGCCGCCGTCTTCACTTGCTCCG
GCTCGACCGCCTCGCGCTTCCAGAAGGACATTGAGGCCGGCCAGGTCGGCATCAATGTCCCCA
TCCCCGTCCCCCTACCCATGTTCTCCTTCACCGGCAACAAGAAGAGTATTGCCGGTGGCGGTGC
CAACACCTTTTACGGCAAGCCCGGCCTGCAATTCTACACCCAGCAGAAGACCGTGACC

- Accession number: XM_746452
- Length of sequence: 438 nucleotides
- Identities: 438/438(100%)
- Sequence location on *A. fumigatus* mRNA: 1306 to 1743

3.5.3.1.2 *MMSAD* mRNA reverse sequence

GGCTTGCCGTAAAGGTGTTGGCACCGCCACCGGCAATACTCTTCTTGTTGCCGGTGAAGGAGA
ACATGGGTAGGGGGACGGGGATGGGGACATTGATGCCGACCTGGCCGGCCTCAATGTCCTTC
TGGAAGCGCGAGGCGGTCGAGCCGGAGCAAGTGAAGACGGCGGCTCCGTTACCATACTCGTT
CTTGTTGATGAGTTCAATGGCGTCGTCCAGCGTGGGGACGTTGAGGCAGATGAGGACAGGGC
CGAAGATTTCCTCCTTGTAGCATTTCATGTCAGGCGTCACGTTGGTGATGATAGTTGGGCCGAC
AAAGTTGCCGTTGGGATACTTCTCAGGCTTATAGCCCCTGCCATCCAGAAGGATGGTGGCACC
CTCCTGTTCAGCACTGGCGATCAAGTCTTCGATGCGCTTCTTGCTCTCGGGGCTGATGACAGGG
CCGAGATCGGCAAAGAA

- Accession number: XM_746452
- Length of sequence: 449 nucleotides
- Identities: 452/453(99%)
- Sequence location on *A. fumigatus* mRNA: 1254 to 1706

3.5.4 The serine protease (*Alp*-2) mRNA expression in *A. fumigatus* AF-10 cells

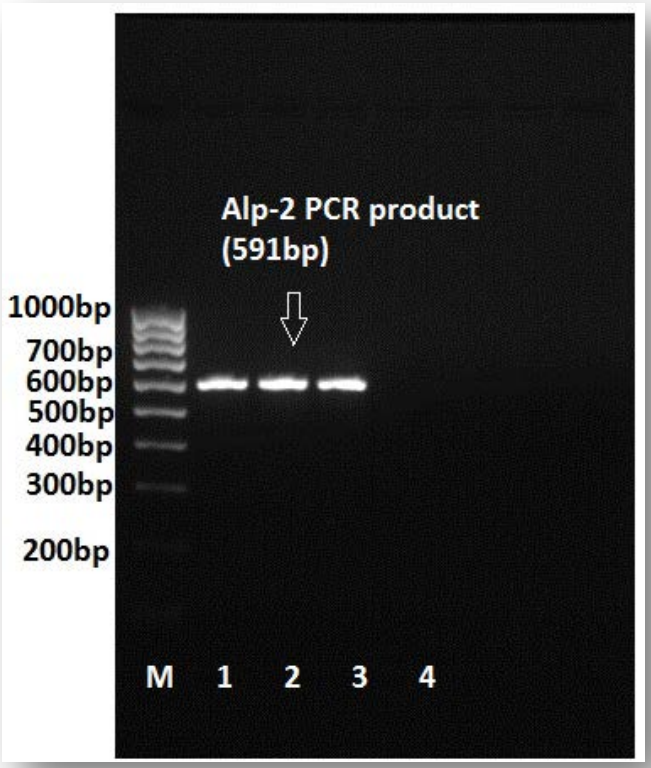


Figure 3.30
Expression of *Alp*-2 mRNA by *A. fumigatus* AF-10 on a 1.5% (w/v) agarose gel stained with ethidium bromide. M: Molecular ladder (hyper ladder IV) 1Kb, lanes 1-3 show the Alp-2 gradient PCR product and lane 4 indicates the negative control. Annealing temperatures for each lane are as follow: lane1= 56°C, lane 2= 58°C, and lane 3= 60°C.

The initially designed primers for *Alp-2* gave rise to an extra nonspecific product band. Consequently, primers for this gene were re-ordered based on a similar study using the same gene (Reichard *et al.*, 2000). In order to obtain the best annealing temperature a gradient PCR was carried out using three different annealing temperatures. Figure 3.30 shows that all the three different temperature resulted in a single specific product band hence for an efficient amplification of *Alp-2* gene, annealing temperatures of 56°C, 58°C, and 60°C could be used. The specificity of the amplicon was also further confirmed by sequence analysis.

3.5.4.1 Sequence analysis data for the A. fumigatus Alp-2

3.5.4.1.1 *Alp-2* mRNA forward sequence

GAGAAACAAAGTCGATTCGAGGTCGTGCCCAGTGGGGCAAGACCATTCCTACCGATGATGAG
GACGCTGATGGCAATGGTCATGGGACGCACTGCTCTGGAACCATTGCCGGCCGGAAGTATGG
TGTCGCTAAGAAGGCCAACCTCTACGCCGTCAAGGTCCTCAGATCAAGTGGCTCTGGTACCAT
GTCCGATGTTGTTGCTGGCGTCGAGTGGGCTGTCAAGTCTCATCTCAAGAAAGTTAAGGATGC
CAAAGACGGTAAGATCAAGGGATTCAAGGGCAGTGTCGCCAACATGAGTCTTGGTGGTGGCA
AATCCAGGACGCTTGAAGCTGCTGTCAATGCTGGTGTTGAAGCTGGTCTTCACTTCGCTGTTGC
TGCCGGTAACGACAATGCCGATGCCTGCAACTACTCTCCAGCAGCTGCCGAGAATCCCATTACT
GTTGGTGCCTCAACACTCCAGGATGAGCGTGCCTACTTCTCCAACTATGGAAAGTGCACTGATA
TCTTCGCTCCTGGTCTCAACATCCTCTCCACTTGGATTGGCTCTAAGCAG

- Accession number: XM_748625
- Length of sequence: 538 nucleotides
- Identities: 543/545(99%)
- Sequence location on *A. fumigatus* mRNA: 568 to 1112

3.5.4.1.2 *Alp-2* mRNA reverse sequence

AACGTGAAGCTGTTGAGACAGGAGCGAGATATCAGTGCACTTTCCATAGTTGGAGAAGTAGG
CACGCTCATCCTGGAGTGTTGAGGCACCAACAGTAATGGGATTCTCGGCAGCTGCTGGAGAGT
AGTTGCAGGCATCGGCATTGTCGTTACCGGCAGCAACAGCGAAGTGAAGACCAGCTTCAACAC
CAGCATTGACAGCAGCTTCAAGCGTCCTGGATTTGCCACCACCAAGACTCATGTTGGCGACACT
GCCCTTGAATCCCTTGATCTTACCGTCTTTGGCATCCTTAACTTTCTTGAGATGAGACTTGACAG
CCCACTCGACGCCAGCAACAACATCGGACATGGTACCAGAGCCACTTGATCTGAGGACCTTGA
CGGCGTAGAGGTTGGCCTTCTTAGCGACACCATACTTCCGGCCGGCAATGGTTCCAGAGCAGT
GCGTCCCATGACCATTGCCATCAGCGTCCTCATCATCGGTAGGAATGGTCTTGCCCCACTGGGC
ACGACCTTCGAAATCGACATGGTCGACATTGATGCCAGTGTCATGGGGGGTAGGCCATTCAAT
GGCCGACCATGGTCGATTTCGAAGGTCGTGCGCAGTGGGGGCAAGACCATTCCTACCGATGAT
GAGGACGCTGATGGCAATGGTCATGGGGACGCACTGCTCTGGAACATTGCCGGCCGGAAGTA
TGGTGTCGCTAAGAAGGCAACCTCTACGCCGTCAAGGTCCTCAGATCAGTGGCTCTGGTACAT
GTCGATGTTGTTGCTGGCGTCAGTGGGCTGTCAGGTCTCACTCAGAAAGTTAAGGATGCAAAG
ACGGTTAAGATCAGGGATTCAAGGGCGTGTTGTCACATGAGTCTTGGTGGTGGCAATCGGAC
GCTGGAAGCTGCTGTCATGCTGGTGTTGGAAGCTGGTCTTCTTCCTGTTGCTGCGGTAGACAT
GCGATGCTGCACTACTCGTCCACTGCCGAGAGACCCATTCTACTTTGGGGTCGCCCCACACTCG
CGAGAGACCGCCTCCTCTCTCACTCACTTGCAAAGGGAGAGGAGAGAGATTGTCGCTCTGGGG

- Accession number:
- Length of sequence(range1): 535 nucleotide
- Identities: 543/546(99%)
- Sequence location on *A. fumigatus* mRNA: 539 to 1084

3.5.5 The mRNA expression of one of the transcriptional regulators of gliotoxin biosynthesis (*gliZ*) in *A. fumigatus* cells

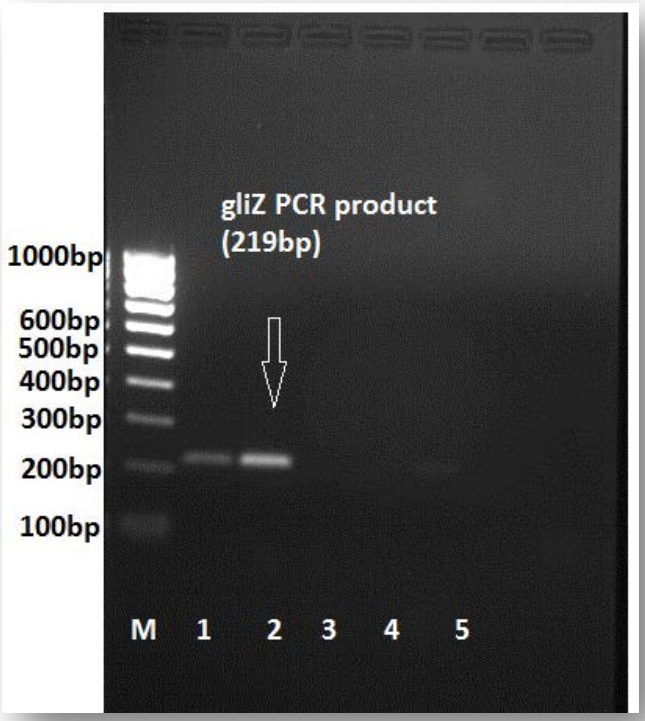


Figure 3.31
Expression of *gliZ* mRNA by *A. fumigatus* on a 1.5% (w/v) agarose gel stained with ethidium bromide. M: Molecular ladder (hyper ladder IV) 1Kb, lanes 1, 2, and 5 show the Alp-2 gradient PCR product and lane 3 indicates the negative control. (1-50˚C, 2-54˚C, 3-negative control, 4-60˚C, 5-64˚C).

The *gliZ* gene was selected for qRT-PCR studies as a result of the cell stimulation studies using wild type and gliotoxin knockout strains of *A. fumigatus* grown with or without hydrocortisone in the medium (Figure 3.19). *gliZ* encodes a transcriptional regulator in the gliotoxin biosynthesis gene cluster. Following PCR using *A. fumigatus* cDNA template and gene specific primers agarose gel electrophoresis was performed and an expected PCR product of 219 bp was visualized after ethidium bromide staining. Figure 3.31 shows that using these primers at the annealing temperature of 54°C results in formation of a single specific amplicon for *gliZ*. The specificity of the amplicon was further confirmed by sequence analysis.

3.5.5.1 Sequence analysis data for the A. fumigatus gliZ

3.5.5.1.1 *gliZ* mRNA forward sequence

CCGCCGATTTGATGCCACCTTTGTCTTTGTGCGGAACTATGCGCGCGCATTTTCCGCCTTCTACC
ACTGTTCCCACTGTCCGAAAGATGCCGGCAGTATTAGCATGGCCGTGACCGCGCTGCAGCTGG
CCACGACCGCGCTGGAAAAGACCGCCACCGCCCAGAGATGTCCGCCGATTTGATGCCCCCCTT
TGTCTTTGTGCGGAACTATGCGCGCGCATTTTCCGCCTTCTACCACTG

- Accession number: XM_745759
- Length of sequence (range 1): 160neucleotides
- Identities: 160/160(100%)
- Sequence location on *A. fumigatus* mRNA: 840 to 999
- Length of sequence (range 2): 80 nucleotides
- Identities: 80/85(94%)
- Sequence location on *A. fumigatus* mRNA: 826 to 908

3.5.5.1.2 *gliZ* mRNA reverse sequence

GCCATGCTAATACTGCCGGCATCTTTCGGACAGTGGGAACAGTGGTAGAAGGCGGAAAATGC
GCGCGCATAGTTCCGCACAAAGACAAAGGTGGCATCAAATCGGCGGATATCTCGAGCGGTTA
GGCTGCTGAGGGTCTGGAGGCATTTGCAGGAGCGCAGCAGCTATGACCGCTCGAGATATCAC
GAATGATGCCACCTTTGTCTTTGTGCGGAACTATGCGCGCGCATTTTCCGCCTTCT

- Accession number: XM_745759
- Length of sequence (range 1): 166
- Identities: 166/166(100%)
- Sequence location on *A. fumigatus* mRNA: 782 to 947
- Length of sequence (range 2): 80
- Identities: 80/88(91%)
- Sequence location on *A. fumigatus* mRNA: 816 to 901

3.5.6 The mRNA expression of gliotoxin peptide synthase (*gliP*), Fructose-BP-aldolase classII (FBA) and prolidase pepP in *A. fumigatus* AF-10

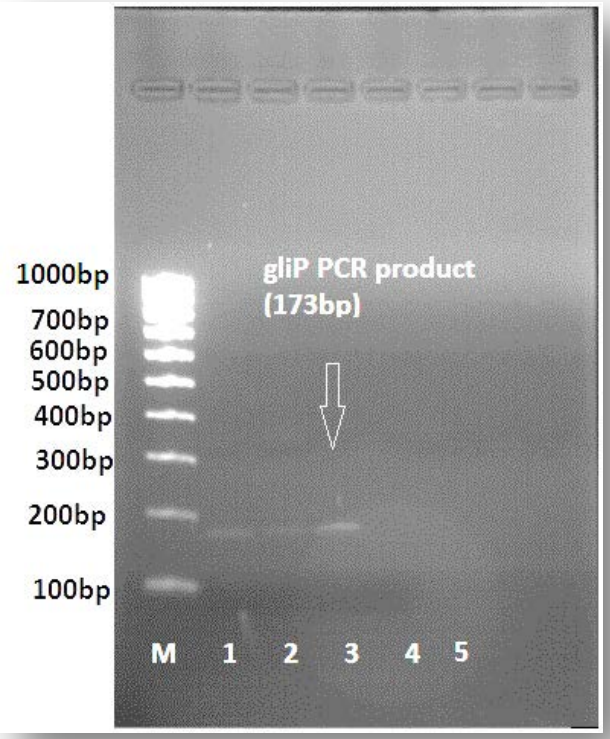


Figure 3.32
Expression of *gli*P mRNA by *A. fumigatus* on a 1.5% (w/v) agarose gel stained with ethidium bromide. M: Molecular ladder (hyper ladder IV) 1Kb, lanes 1-4 show the Alp-2 gradient PCR product and lane 5 indicates the negative control. Annealing temperatures for each lane are as follow: lane1= 48°C, lane 2= 50°C, lane 3= 56°C, and lane 4= 60°C.

gliP is one of the main genes involved in the biosynthesis of gliotoxin in the *A. fumigatus* genome encoding a non-ribosomal peptide synthase protein. Similar to *Alp*-1, the initial primers designed for this gene resulted in either extra nonspecific bands or no band at all. So they were not suitable for qRT-PCT experiments. Other primers were ordered for this gene based on a pre-designed primer-set from a similar study (Gardiner and Howlett, 2005). In order to obtain the best annealing temperature a gradient PCR was carried out using three different annealing temperatures. Using the latter primers resulted in the generation of a single band especially at 56°C and a specific product which was consequently reconfirmed by sequencing analysis. However, the expression level of the genes from the samples appeared to be low in the mRNA of *A. fumigatus* extracted from culture grown in

Vogel's medium (as it can be observed from the gel picture in Figure 3.32). This makes it less suitable for comparative studies of the expression levels of the genes between different samples. Besides *gliP* gene, the primers designed for Fructose-BP-aldolase Class II (*FBA*) and Prolidase *pep*P genes also gave rise to more than one amplicon. Although three primer sets were designed for them and a range of annealing temperatures were tested using gradient PCR, they were still unsuitable for the qRT-PCR experiment. Therefore, further testing of those genes along with *gliP* in the qRT-PCR experiment was discontinued. However among the produced amplicons by these genes the one that was matching the right product size was sequenced. The sequences for these genes are stated in sections 3.5.6.1, 3.5.6.2, and 3.5.6.3.

3.5.6.1 Sequence analysis data for the A. fumigatus gliP

3.5.6.1.1 *gliP* mRNA forward sequence

CACTTTGCGTCTACGCGTGCAGCGAGCCAGGCAGCCGTCTGCAATTCCATTCCATTCGAGCAG
GTCCTGAACCTCCTCCACCTGCCGCGGACCATCCGGCAACACCCGCTGTTCGAAGCCATGGTCA
CCTTTCATCTCAAGGGGAATGGTTACCGGACCCGGGGGTTGCTGAATACGCGCTTCTGTGGAG
GATGTTCAGGACTGTTCAGAGTGATGGAATTTCCAAACCGAGCACGCTGTTGGCCACACTGCC
CTTGAATCTGTTGATACTGCCGTCCTTGGCTTACTTAACTTTCTTGAGATGAGACTTGACAGCCC
ACTCGACGCCCGCAACAACATCGGACATGGTACGAGCGCCACTTGTTCTGAAGACCTTGACGG
CGTAGAGGTTGGCCTTCTTCCGACACCATACTTCCGGCCGGCAATGGTTCCAGAGGAGGGCGT
CCCATGACCATTGCCATCAGCGTCCTCATCATCCGTGGAATGGTCTTGCCCCACTGGGCACGAC
CTTTCAAATCGACTGGCCTACATTGATGCCAGTGTCAGGTCCTAAGGCAAAGGGGAAGTGTGC
CAATCTATCTCCGAACGGGATC

- Accession number: XM_745762
- Length of sequence: 593 nucleotides
- Identities: 139/140(99%)
- Sequence location on *A. fumigatus* mRNA: 5747 to 5886

3.5.6.1.2 *gliP* mRNA reverse sequence

GGTCCCCCGCGGGTGTTGCGGAGGTCCGCGGCAGGTGGAGGAGGTTCAGGACCTGCTCGAAT
GGAATGGAATTGCAGACGGCTGCCTGGCTCGCTGCACGCGTAGACTGCAGGATGGTGGCGCA
GTCTGCATTCACAGGGGTTTAACCCACTTCCAACCATCAACTGAGTATGGGAGTCTCAAGATGG
CGTGATCACCATCCAGTGCACTGGCTTCCCACATCGAACCTGTGTTGCGCTCCCTGCCCTTGGA
CTCTTCAGTGAAGTGTCTTTTCCTCTACTATGCTTTTTGCTTCCATAACCTCTGACCCCGTTTTTG
CTGCCCAAGTTGTTCTTCAAACTGAGCGTCACCGGTGCTGACTGCTTGACCTACTCACGTTGGT
CTGGAAACCACTGTTAAGCTTGGCAGCACATGATTGAGAAGCTGCTCCACATGACAATTGCATC
GTGCCTACTCCTCCAGCTTCGAGTGCTCT

- Accession number: XM_745762
- Length of sequence: 475 nucleotides
- Identities: 136/138(99%)
- Sequence location on *A. fumigatus* mRNA: 5714 to 5851

3.5.6.2 Sequence analysis data for the A. fumigatus FBA

3.5.6.2.1 *FBA* mRNA forward sequence

TCTCTTCGAGTACGCTCAGGAGAAGAACTTTGCGATTCCCGCCGTTAACGTCACCTCTTCCTCCA
CCGTCGTCGCTTGCCTTGAGGCCGCTCGCGACCAGAACTGCCCCATCATTCTCCAGGTCTCTCA
GGGTGGTGCTGCCTACTTTGCCGGAAAGGGTGTCAGCAACGATGGCCAGAAGGCCTCCATCG
CTGGTTCTATCGCCGCTGCCCACTACATCCGCAGCATTGCTCCCTCCTACGGCATTCCCGTCGTC
CTCCACACCGACC

- Accession number: XM_749359
- Length of sequence: 269 nucleotides
- Identities: 269/269(100%)
- Sequence location on *A. fumigatus* mRNA: 63 to 331

3.5.6.2.2 *FBA* mRNA reverse sequence

TGTAGTGGGCAGCGGCGATAGAACCAGCGATGGAGGCCTTCTGGCCATCGTTGCTGACACCCT
TTCCGGCAAAGTAGGCAGCACCACCCTGAGAGACCTGGAGAATGATGGGGCAGTTCTGGTCG
CGAGCGGCCTCAAGGCAAGCGACGACGGTGGAGGAAGAGGTGACGTTAACGGCGGGAATCG
CAAAGTTCTTCTCCTGAGCGTACTCGAAGAGACGGAGGACGTCATCGCCGACAATGACGCCGG
ACTT

- Accession number: XM_749359
- Length of sequence: 253 nucleotides
- Identities: 253/253(100%)
- Sequence location on *A. fumigatus* mRNA: 28 to 280

3.5.6.3 Sequence analysis data for the A. fumigatus **prolidase** *(pepP)*

3.5.6.3.1 Prolidas (*pepP)* mRNA forward sequence

ACAAATACATCGATACCAACGTCCTCGACCGTTATTGGAGGGTTGGGGGTGTCCGCATAACG

- Accession number: XM_749993
- Length of sequence: 58 nucleotides
- Identities: 58/58(100%)
- Sequence location on *A. fumigatus* mRNA: 1256 to 1313

3.5.6.3.2 Prolidas (*pepP)* mRNA reverse sequence

CGATGTATTTGTTTGACTCGGGGGACTTGATGTAAGGCTCGATGATAAACCGACAGAAATATA
CGCCTGGCTCGACGGTGAAAAACG

- Accession number: XM_749993
- Length of sequence: 81 nucleotides
- Identities: 81/81(100%)
- Sequence location on *A. fumigatus* mRNA: 1187 to 1267

3.5.7 qPCR studies for analysis of the relative gene expression of *hsp70*, *MMSAD*, *gliZ*, and *Alp-2* genes in *A. fumigatus* AF-10 by $2^{-\Delta\Delta C}$q method

This section covers the result of experiments that aimed to investigate the relative expression of *gliZ*, *hsp70*, *MMSAD*, and *Alp-2* genes by qPCR in order to determine any changes in their relative expression following treatment with hydrocortisone sodium phosphate. The cell death studies (section 3.2.3.1) showed an increase in the number of apoptotic cells due to gliotoxin production following treatment of *A. fumigatus* cells with hydrocortisone and addition of culture filtrate of the wild type fungus to A549 cells. This increase was particularly significant in the test samples when lung epithelium cells were treated with the Aspergillus culture broth for longer period. Likewise, proteomic experiments demonstrated an increased abundance in *Hsp70, MMSAD,* and *Alp-2* along with some other proteins as described in section 3.4. Therefore, in order to further explore the responses of these affected genes during growth of *A. fumigatus* to the presence of hydrocortisone in the medium, qPCR experiments were conducted to compare the relative expression of these genes when the fungus was grown with hydrocortisone (test) with the control (no hydrocortisone present in the medium).

For qPCR studies, *A. fumigatus* AF-10 was cultivated in Vogel's modified medium with (test) or without (control) 10^{-6}M and 10^{-5}M hydrocortisone sodium phosphate (as described in section 2.3.6). Total RNA was extracted from the test and the control cultures and subjected to qPCR experiments to determine the effect of hydrocortisone treatment on the relative expression of *gliZ, hsp70, MMSAD,* and *Alp-2* mRNA. qPCR experiments were conducted using a two-step qRT-PCR process as detailed in section 2.5.8 and the relative quantification of these genes (*gliZ, hsp70, MMSAD,* and *Alp-2*) was performed using comparative cq (or $2^{-\Delta\Delta cq}$) method of analysis where expression changes are expressed as fold changes relative to the calibrator (untreated sample that all others are compared to). All qPCR experiments shown in this thesis employed SYBER Green for amplicon detection. SYBER Green is a fluorescent dye that is able to bind to double stranded DNA; this includes amplicons, primer dimers, and any contaminating DNA.

Three different experiments (n=3) were performed for the reference gene (*TEF-1*) and each one of the genes of interest (*gliZ, hsp70, MMSAD,* and *Alp-2*). For each experiment (n=1), qPCR in the control and treated samples was performed in triplicates and three different control and treated RNA preparations were used. All genes of interest were normalized against the endogenous reference gene as well as the calibrator. Relative expression using the $2^{-\Delta\Delta cq}$ method was calculated according to the following Equations:

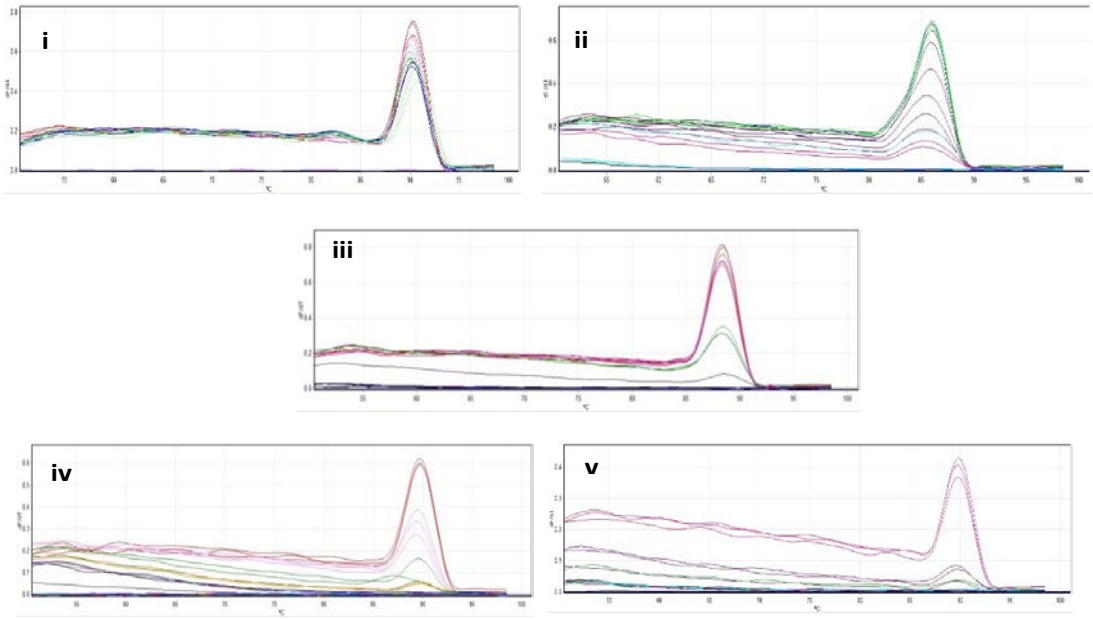
Equation 1: Δcq= average cq (gene of interest) - average cq (reference gene)

Equation 2: ΔΔcq= Δcq gene of interest -Δcq calibrator

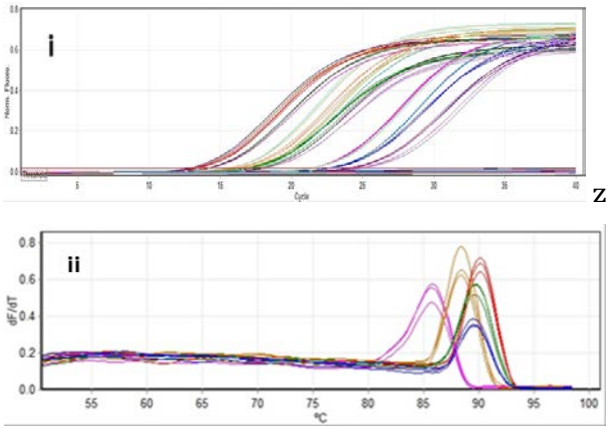
Equation 3: Fold change= $2^{-\Delta\Delta cq}$

If the calculated value for the ΔΔcq is positive, it results in a $2^{-\Delta\Delta cq}$ value that is negative, indicating a reduction in the expression levels. On the contrary, if ΔΔcq is a negative value, it leads to the generation of a positive $2^{-\Delta\Delta cq}$ value implying an increase in the expression of the target gene as a result of the treatment (Schmittgen and Livak, 2008). All calculations for the relative expression analysis using $2^{-\Delta\Delta cq}$ were performed using Microsoft Excel 2010 (Microsoft, USA).

In order to test the specificity and efficiency of the primers used in this study a standard calibration curve of different concentrations (2.5- 25000pg/reaction) of the template cDNA was generated by running real time PCR reactions. Each of these was followed by a melt curve for all individual genes (Figure 3.33A). Melt curve analysis was also performed following the qPCR reaction for all test and control samples (Figure 3.33B-ii). The presence of one single curve is indicative of the specificity of the primers used resulting in the generation of only one amplicon. If more than one amplicon (hence more than one curve) is formed, the generated data from the qPCR experiment (using ct values) would not be a true representative of the expression level of the desired product. Figure 3.33B-i shows the real time amplification curves generated in each experiment for the control and both tests (10^{-6}M and 10^{-5}M hydrocortisone treatment); comparing the relative mRNA expression of genes of interest. The amount of fluorescence emission as a result of binding of SYBER Green to the produced amplicon for each gene is proportional to the amount of PCR product generated.



A



B

Figure 3.33

Section-A Real time melt curves (dissociation curves) for the amplification of the individual genes tested within the standard range of cDNA template (2.5pg to 25000pg/reaction). The graph was generated by plotting temperature (˚C) of the X axis against the first derivative of the measured fluorescence [d (f)] on the Y axis. Panel-i: Dissociation curve for the amplicon of *TEF-1* gene; panel-ii: dissociation curve for the amplicon of *hsp70* gene; panel iii: dissociation curve for the amplicon of *Alp-2* gene; panel-iv: dissociation curve for the amplicon of *MMSAD* gene; and panel-v: dissociation curve for the amplicon of *gliZ* gene. **Section-B** Panel-i: real time amplification curves of the genes (*hsp70, MMSAD, Alp-2, gliZ* and *TEF-1*) with and without hydrocortisone treatment. Horizontal axis indicates the cycle number and is plotted against the relative fluorescence (Norm Fluoro) on the vertical axis (linear scale). The amount of fluorescence is proportional to amount of PCR product generated. Panel-ii melt curve analysis for the amplification of all the genes tested for their mRNA expression level with and without hydrocortisone treatment. Each of the colours is representative of one gene of interest as well as the endogenous reference gene. Red: TEF-1, Pink: hsp70, Green: MMSAD, Orange: Alp-2, and Blue: gliZ

3.5.7.1 Determination of the relative expression of *hsp70* in *A. fumigatus* following treatment with hydrocortisone sodium phosphate

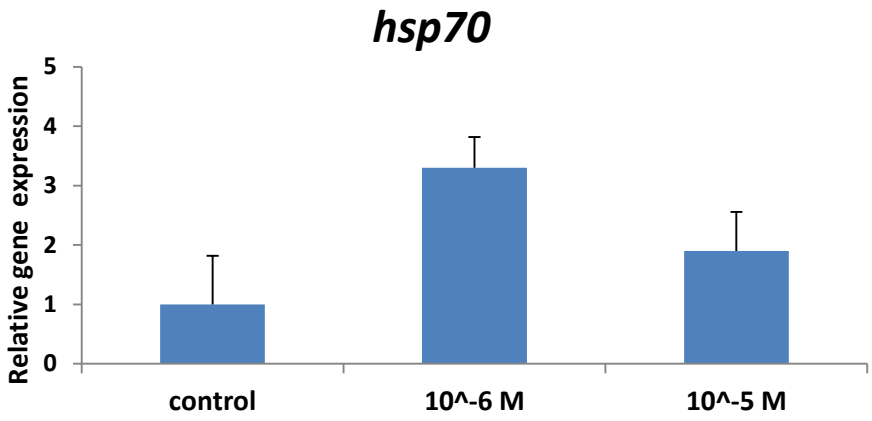


Figure 3.34
Expression level of *hsp*70 mRNA in *A. fumigatus* AF-10 following stimulation with hydrocortisone sodium phosphate. *Aspergillus* was grown for 48 in medium containing 10^{-6}M and 10^{-5}M hydrocortsisone (test) and without and hydrocortisone in the medium (control). The expression levels of all genes were normalized to the expression level of the endogenous control gene *TEF1.* The Cq values used for calculations represented mean±SEM of three independent experiments in which control, Test (10^{-6}M) and Test (10^{-5}M) were performed in triplicates.

Analysis of the qPCR experiments for the relative expression of *hsp70* mRNA in *A. fumigatus* AF-10 grown with (10^{-6}M and 10^{-5}M) and without hydrocortisone sodium phosphate, was determined using a comparative cq method. The student unpaired T-test was used for statistical analysis of the qPCR data. As shown in Figure 3.34, the presence of hydrocortisone in the growth medium of the fungus significantly ($P<0.05$) enhances the expression of the hsp70 mRNA in both tests (up to 3 fold increase in the relative mRNA expression in the test cultures treated with 10^{-6}M hydrcortisone, compared to the control (no hydrocortisone added to the medium). This data is also in agreement with the results obtained for hsp70 protein expression level following hydrocortisone treatment as observed earlier in the proteomic studies.

3.5.7.2 Determination of the relative expression of *MMSAD* in *A. fumigatus* following treatment with hydrocortisone sodium phosphate

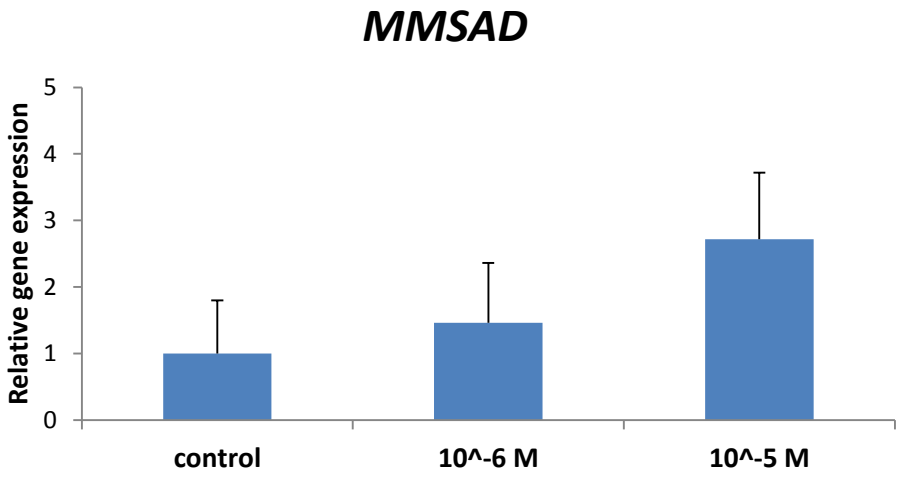


Figure 3.35
Expression level of *MMSAD* mRNA in *A. fumigatus* following stimulation with hydrocortisone sodium phosphate. Aspergillus was grown for 48 in medium containing 10^{-6}M and 10^{-5}M hydrocortsisone (test) and without ant hydrocortisone in the medium (control). The expression levels of all genes were normalized to the expression level of the endogenous control gene *TEF1.* The Cq values used for calculations represented mean±SEM of three independent experiments in which control, Test (10^{-6}M) and Test (10^{-5}M) were performed in triplicates.

Analysis of the qPCR experiments for the relative expression of *MMSAD* mRNA in *A. fumigatus* AF-10 grown with (10^{-6}M and 10^{-5}M) and without hydrocortisone sodium phosphate was similarly determined by employing the equations described in section 3.5.7. Figure 3.35 shows that presence of both concentrations of hydrocortisone affect the expression of *MMSAD* mRNA by 1.5 fold and 3 fold in cultures containing 10^{-6}M and 10^{-5}M hydrocortisone respectively, compared to the control cultures. The fold increase in the test cultures containing 10^{-5}M hydrocortisone apeared to be statistically significant ($p<0.05$). The fold increase in the expression level of this gene in cultures containing 10^{-6}M hydrocortisone was statistically insignificant when compared to the control ($P>0.05$).

3.5.7.3 *Determination of the relative expression of gliZ in A. fumigatus AF-10 following treatment with hydrocortisone sodium phosphate*

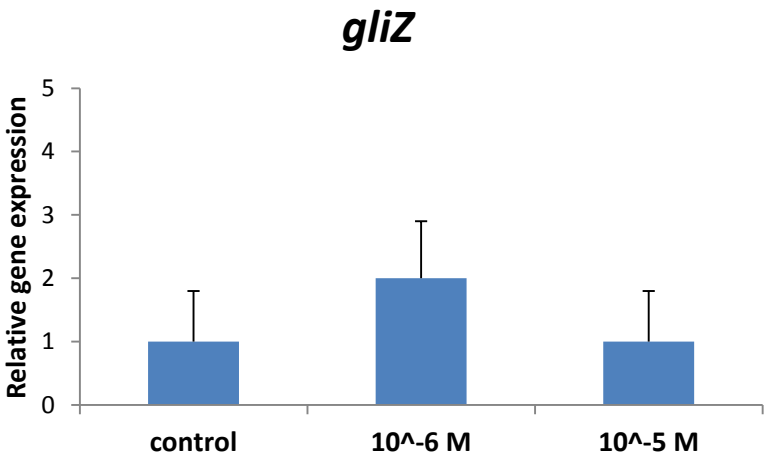


Figure 3.36
Expression level of *gliZ* mRNA in *A. fumigatus* following stimulation with hydrocortisone sodium phosphate. Aspergillus was grown for 48 in medium containing 10^{-6}M and 10^{-5}M hydrocortsisone (test) and without any hydrocortisone in the medium (control). The expression levels of all genes were normalized to the expression level of the endogenous control gene *TEF1.* The Cq values used for calculations represented mean±SEM of three independent experiments in which control, Test (10^{-6}M) and test (10^{-5}M) were performed in triplicates.

The relative quantification of *gliZ* mRNA, (Figure 3.36) demonstrates an increase up to 2 fold change in gliZ with the culture supplemented with 10^{-6}M. This is due to growth of the fungal cells in the presence of hydrocortisone in their growth medium relative to the control. However, there was no statistically significant change in the gliZ mRNA expression level in cultures supplemented with 10^{-5}M and 10^{-6}M hydrocortisone (*P>0.05*).

3.5.7.4 Determination of the relative expression of *Alp-2* in *A. fumigatus* following treatment with hydrocortisone sodium phosphate

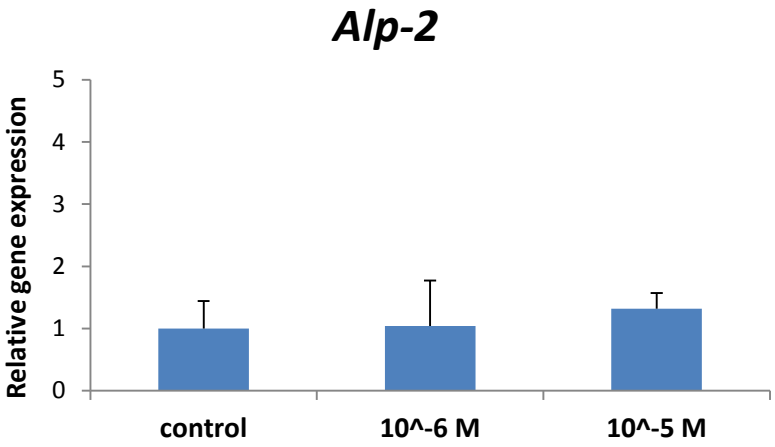


Figure 3.37
Expression level of *Alp-2* mRNA in *A. fumigatus* following stimulation with hydrocortisone sodium phosphate. Aspergillus was grown for 48 in medium containing 10^{-6}M and 10^{-5}M hydrocortsisone (test) and without ant hydrocortisone in the medium (control). The expression levels of all genes were normalized to the expression level of the endogenous control gene *TEF1.* The Cq values used for calculations represented mean±SEM of three independent experiments in which the control and the two tests (10^{-6}M and 10^{-5}M) were performed in triplicates.

Analysis of the qPCR experiments for the relative expression of *Alp-2* mRNA in *A. fumigatus* AF-10 growing with (10^{-6}M and 10^{-5}M) and without hydrocortisone sodium phosphate, is demonstrated in Figure 3.37. Stimulation of the cells with different concentrations of hydrocortisone did not significantly change the expression of *Alp-2* mRNA when compared with the control.

It can be concluded from all the above data that stimulation of the fungal cells with hydrocortisone results in an increased fold change ($P<0.05$) in *hsp70* and *MMSAD* mRNA's of the cells with both hydrocortisone sodium phosphate tested concentrations. Also an increased fold change in *gliZ* mRNA as a result of stimulation of the cells with 10^{-6}M hydrocortisone was observed. However, no statistical significant change was observed in the mRNA expression of *gliZ* when stimulated with 10^{-6}M, and 10^{-5}M hydrocortisone, nor was it observed in the *Alp-2* mRNA following stimulation with both hydrocortisone concentrations.

Chapter IV: Discussion

4 Discussion

The host pathogen interaction during an infection is very complex. It comprises interplay of environmental factors, signal molecules, and cellular processes generated/possessed by the interacting organisms to increase their chance of survival. For pathogens the understanding/ sensing of their environmental signals (e.g. sense that they are within the host environment) is crucial for their initial colonization. This will help them to express the appropriate set of genes essential for their survival and adaptation to the new environment.

The cell to cell communication between microorganisms, via inter bacterial/fungal species, has been shown, in many studies, to be through quorum sensing signals. Likewise, Human (eukaryotic) cell to cell signalling is known to be via hormones (Sperandio *et al.*, 2003). Considering the above, it is possible to speculate that in a host pathogen relationship; both (the host and the pathogen) can cross communicate/interact through these signalling systems as well. Hence an understanding of the interplay of these signals on both host and the pathogen can enhance the understanding of the host pathogen relationships. Consequently, this also increases the possibility of finding new mechanisms of overcoming these infections. Unravelling the mechanisms of host-pathogen communication will provide a better insight into ways to combat infections.

In this research project various approaches have been used in order to further elucidate the effect of compounds produced by *A. fumigatus*, reported to be involved in the pathogenicity of this fungus, on host cells (mainly lung epithelial cells) (at the site of infection). Additionally, the effect of host hormones/signals on the pathogen (*A. fumigatus*) was also investigated. In this chapter a further detailed discussion of the outcome of the experiments are presented.

4.1 Studies of the effect of the secondary metabolites produced by *Aspergillus* sp.

4.1.1 Butyrolactone I; investigating quorum sensing in *A. terreus* and *A. fumigatus*

Butyrolactone I has been reported in previous studies as a putative quorum sensing signal in the environmental strains of *A. terreus* (Raina, 2008). Quorum sensing is a means of cell to cell communication in microorganisms via hormone-like signals or auto-inducers. In brief, the regulation of gene expression is affected once the auto-inducer concentration reaches a critical density in the extracellular environment where the cells colonise. This includes genes involved in regulation of adopting new modes of growth, such as biofilm formation and sporulation which in turn may have a protective role for the cells in the environment. Expression of many virulence factor genes is reported to be cell density dependent (de Kievit and Iglewski, 2000). This phenomenon has been widely studied in bacteria (Further details can be found in section 1.9 of this thesis). However data is more limited about quorum sensing in the fungal species particularity in filamentous fungi. Understanding quorum sensing in pathogenic organisms is particularly important as it can help to control the infection by interfering with putative quorum sensing signal production and consequently virulence gene regulation.

For a substance to qualify as a quorum sensing molecule it should satisfy some critical characteristics such as being produced during the growth of the organism and exerting a response in the producing organism (such as secondary metabolite production, sporulation, virulence determination). Another characteristic is that the exogenous addition of the quorum sensing molecules to the low cell density cultures should result in a similar response as it would do endogenously by the organism at its critical density. Thus, this study investigated whether butyrolactone I meets these criteria in clinical strains of *Aspergillus* (*A. terreus* and *A. fumigatus*).

4.1.1.1.1 Exogenous addition of the quorum sensing molecules to the low cell density cultures

Exogenous addition of 100 nM butyrolactone I to the low density cultures of the environmental strain of *A. terreus,* increased its own production as well as the production of lovastatin (Raina, 2008). Confirming the same property in the clinical isolates of *A. terreus*, and *A. fumigatus*, would provide a basic understanding of the role of quorum sensing in pathogenic fungi during the infection they cause.

However, the present work did not confirm the observation of the previous study when the same approach was adopted for studies with the clinical strains of *A. terreus*. Addition of butyrolactone I (100 nM) to the test cultures of the clinical isolates of *Aspergillus* grown in DMEM and complex medium did not significantly (P>0.05) change the level of this putative signal molecule (Figures 3.2 and 3.3). Nor did it change the cell dry weight of test cultures compared to the control cultures (where there was no addition of butyrolactone I). However, the pattern and levels of butyrolactone I production was very similar to the previous studies.

The level of butyrolactone I production in the complex medium was higher than butyrolactone I concentrations produced in animal cell culture medium (DMEM). Compared to DMEM, complex medium is rich, containing higher concentrations of hexoses. Hence, it encourages the growth and secondary metabolite production. This is in accordance with earlier reports showing that different levels of metabolic activity correlate with growth-rates in different media(Meletiadis *et al.*, 2001). In DMEM, the medium composition contains low concentration of sugars. Nonetheless due to having protein hydrolysate, it is closer to the nutritional environment encountered by the fungus in the lung (Latgé, 1999) and hence DMEM was used to test the level of butyrolactone I production.

4.1.1.1.2 Stimulating a response in the producing organism (effect on secondary metabolite production) and production of a signal molecule during the growth

In this study the effect of signal molecule (butyrolactone I) addition on the gliotoxin production (as secondary metabolite and virulence factor) was not investigated. This was due to two reasons: a) the initial clinical isolates of both *A. terreus* and *A. fumigatus* were not gliotoxin producers, and b) *A. fumigatus* clinical isolate did not produce notable amounts of butyrolactone I (the highest level was 0.4 µM at 72 h post inoculation) (Figure 8.3 in Appendix); lessening the possibility of this compound being a signal molecule in *A. fumigatus* clinical isolates.

4.1.1.1.3 Stimulating a specific response in the host

According to the literature in this field, and considering the results obtained, it seems that nano-molar concentrations of butyrolactone I do not have a notable effect on the butyrolactone I production and cell biomass in clinical isolates of *Aspergillus*. For a molecule to be considered as a QS molecule it should exert a specific response in the host. Concentrations of butyrolactone I that have effects on the human cells as well as on the secondary metabolite production in *A. terreus,* are reported to be in the range of 50 to 500 µM (Schimmel *et al*., 1998). In another study, addition of similar concentrations of Butyrolactone I (100 µM) was shown to inhibit the eukaryotic cyclin-dependent kinases (cdk1 and cdk2) and non cyclin-dependent kinases of mammalian cells. In their work, Kitagawa and colleagues screened cdc2 kinase inhibitors from the cultures of microorganisms such as *Aspergillus* species F-25799. They found a selective inhibitor of both cdc2 and cdk2 kinases that showed little or no effect on other protein kinases in the cultures of *Aspergillus terreus* strain F-25799. They identified the molecule as Butyrolactone I (Kitagawa *et al.*, 1993; Nishio *et al.*, 1996). This information, together with the fact that *A. fumigatus,* which is the main cause of *aspergillosis,* is unable to produce high enough concentrations of butyrolactone I (as above) to

have physiological effects on mammalian cells suggest that butyrolactone I is less likely to be a signal in the fungal-host cell communication. Therefore, this part of the research was discontinued. However based on other criteria mentioned in the literature for a molecule to be considered as a quorum sensing molecule, more investigations are needed in this area. The results obtained so far do not provide enough evidence to accept or reject the potential role of a quorum sensing molecule in pathogenicity and aspergillosis.

One interesting area to look into, and possibly link it to research on lactone containing molecules as quorum sensing molecules in filamentous fungi, is the extensive and on-going research about lipid signalling. There is evidence of fatty acids and other lipid compounds acting as signalling molecules in different pathogenic and non-pathogenic fungi (Rhome and Del Poeta, 2009). Considering quorum sensing phenomenon in Gram negative bacteria, they use acylated homoserine lactones (AHL) as quorum sensing molecules (Parsek *et al.*, 1999; Miller and Bassler, 2001). The main common structure of these compounds is an acyl chain that is bound to a lactone ring. In filamentous fungi the involvement of lactone containing structures such as γ-butyrolactone and butyrolactone I as potential QS molecules has been shown. Furthermore, during the GC-MS analysis of the culture extracts of *Penicillium sclerotiorum*, the presence of the oxylipin molecules such as linoleic acid has been noted along with the lactone containing structure (Raina *et al*., 2010). Taken together, these findings suggest that similar to AHL structure in Gram negative bacteria, it might be possible that the presence of both lactone containing structures and fatty acid structures are necessary for the signalling in filamentous fungi. Therefore, further investigations in this area are recommended.

4.1.2 Gliotoxin production by *A. fumigatus* clinical strains and its effect on A459 cells (MTT assay)

Gliotxin, as described earlier in section 1.3.2.1 of introduction chapter, is a secondary metabolite of *A. fumigatus*. The role of gliotoxin as a virulence factor has been investigated in earlier studies and it has been shown that it exerts several immunosuppressive properties. During aspergillosis, lung is the major site of infection. It has been reported that, when conidia of *Aspergillus* adhere to the alveolar cells, one third of them are endocytosed. The rest germinate externally on lung alveolar cells, penetrating and damaging the cells via production of metabolites such as toxins and enzymes (Paris *et al.*, 1997; Osherov, 2012). Among the endocytosed conidia, some are able to escape phagocytosis. They germinate inside pneumocytes and cause damage to the cells via their produced metabolites (Osherov, 2012). Therefore, investigating the time and level of gliotoxin production is important to provide an insight into the damage to the lung during the infection.

The results of the current study show (section 3.1.2) that there is no gliotoxin production during the early growth phase (the first 24 hours) of *A. fumigatus* (Figure 3.6). Hence, indicating that no gliotoxin is produced during the early hours of infection. Gliotoxin was detected in the culture filtrates from 48 h post inoculation and reached a peak at 72 h post inoculation. This is a typical pattern of secondary metabolite production, where production (expression of the related genes) starts towards the end–of logarithmic phase and starting of the stationary phase of growth (Belkacemi *et al.*, 1999). The observed gliotoxin production pattern also correlates with the growth pattern of the *A. fumigatus* (Figure 3.7). Furthermore, maximum fungal biomass was also observed at 72 h which relates to the maximum gliotoxin production at the same time.

The time of gliotoxin detection also affects the amount of gliotoxin detected in the culture medium. In this study gliotoxin concentration in

culture medium increased, together with fungal biomass, from a mean of 12 ± 6 µg/ml at 48h to approximately 24 ±12 µg/ml by 72h, in various strains, where it reached the peak concentration. Thereafter, a decline was observed in gliotoxin production. A similar pattern of gliotoxin production was observed in the cultures of different strains of *Trichoderma* (plant pathogen), where optimum gliotoxin production was between 48 h to 64 h and a decline in the toxin production was observed after 96 h (Harcz, 2004).

Screening of the different clinical isolates of *A. fumigatus*, for gliotoxin detection allowed discrimination between the isolates with minimal gliotoxin production and the ones that produced larger amounts of gliotoxin (Figure 3.8). Similar reports on quantification of gliotoxin production in various strains of *Aspergillus* confirm the results of this study; however, the generated data with regards to the time of gliotoxin production is inconsistent in different studies. For instance, in a study by Lewis *et al.*, the gliotoxin is reported to have been detected in the culture broth from 24 h post-inoculation with maximum amount by 96 h, and maximum fungal biomass at 48 h (Lewis *et al.*, 2005). In another study, Kupfahl *et al.*, adopting a similar experiment reported the gliotoxin detection after 5 days of fungal cultivation (Kupfahl *et al.*, 2008). Similar to the data presented here, a study by Kosalec *et al*., has also reported the gliotoxin detection after 3days from *Aspergillus fumigatus* cultures (Kosalec *et al.*, 2005). Presence of different culture conditions might be a reason for the variability observed in the gliotoxin production in different reports. Several studies have investigated the effect of various culture conditions such as aeration rate and agitation speed, medium, pH, and temperature on gliotoxin production in *A. fumigatus* (Belkacemi *et al.*, 1999; Watanabe *et al.*, 2004; Kosalec *et al.*, 2005). In the first two studies (Lewis *et al.*, 2006 and Kupfahl *et al*, 2008) the culture growth conditions were very similar to the culture conditions used in the present work (RPM1640 Medium, pH 7, 37° C, and 160-180 rpm) with

the only difference being supplementation of the RPMI medium with 5% FCS in this study. This provides an explanation for the discrepancies observed in different studies. However, comparing the two above studies mentioned earlier together (Lewis et al., 2006 and Kupfhal *et al*, 2008) although culture condition were the same yet they have reported slightly different outcomes.

Similar to the presented study, the work carried out elsewhere also indicates that the amount of gliotoxin production varies in different *Aspergillus* strains (Lewis *et al.*, 2005; Kupfahl *et al.*, 2008). This might pose the question: is the burden caused by gliotoxin in the host tissue dependent on the spores of the *Aspergillus* strain inhaled? Answer to this question is highlighted in the discussion of the results obtained from *in vitro* stimulation studies (section 4.1.3).

In this project, investigation of the effect of gliotoxin on the lung alveolar cells was initiated by examining the influence of different gliotoxin concentrations on A549 cells. Commercially available gliotoxin was added to the cells in concentrations ranging from 0.05 to 20 μM. The results showed that reduction in the viability of A549 cells was dose dependent with a minimum growth inhibitory concentration of 1.0 μM (Figure 3.9). In this study human epithelial cells have shown to be very sensitive to low concentrations of this mycotoxin at both sub-confluent and confluent cell concentrations (with 5μM minimum inhibitory effect on confluent cells). This could be particularly due to the different levels of gene expression in cells at sub-confluent and confluent stages i.e. in different stages of growth such as lag phase and log phase (Chapman *et al.*, 1984; Owens *et al.*, 1986). The results confirm the data from similar experiments on the lung cells in the literature (Peterson *et al.*, 2010). A related study by Pan and Harday has also demonstrated a similar response associated with 1 to 5 μM concentrations of this toxin on different cancer cell lines (Pan and Harday, 2007).

4.1.3 Effect of gliotoxin in causing apoptosis and necrosis in A549 cells measured by flow-cytometry (in vitro stimulation studies)

Gliotoxin has been reported to possess immunosuppressive activities including inhibition of superoxide release, migration of leukocytes, and cytokine release by leukocytes, microbicidal activity, and T-lymphocyte mediated cytotoxicity (Kamei and Watanabe, 2005). Besides, it is shown that gliotoxin induces apoptosis in immune cell lines such as monocytes and macrophages by encouraging activation of cytochrome release from mitochondria and stimulation of caspase cleavage (Baust *et al.*, 2003; Martins *et al.*, 2004; Stanzani *et al.*, 2005). These studies have mostly targeted the effect of gliotoxin in the cells of the immune system. However, majority of the patients who develop aspergillosis infection are in an immunosuppressed state (as a result of extensive immunosuppressive therapy). Hence, there are not many responses from the immune system due to the inhibitory effect of the glucocorticoids on immune response. The inhibitory effects of corticosteroids on macrophages, cytokine release, and activation of monocyte antigens has been demonstrated in several studies (Galanaud *et al.*, 1983; Gerrard *et al.*, 1984; Wilkinson *et al.*, 1991; Ruud *et al.*, 2013) and also described earlier in more detail (section 1.6 of the introduction chapter). Hence, one of the main targets of *Aspergillus* toxins such as gliotoxin would be cells of the lung epithelium (which are the first colonization site). Perhaps inducing apoptosis in these cells would also help the fungus to breach this barrier and create a quicker transition path to the underlying blood vessels and becoming invasive.

In this research, in order to further investigate the mechanism of cell death on lung alveolar cells, a series of cell death investigations were carried out using flow-cytometry. In these studies a gliotoxin knockout strain of *A. fumigatus* was also used in parallel to ensure that the effect observed on the cells was mainly due to the presence of gliotoxin in the culture filtrates.

The lack of gliotoxin in the culture filtrate of the knockout strain was initially examined by high performance liquid chromatography. No gliotoxin was detected from the cultures of this strain as shown in Figure 3.8. In addition, the inability of the same strain used in this study in gliotoxin production has also been reported in a study by Cramer and colleagues (Cramer *et al.*, 2006).

As shown in section 3.1.2.2 of the result chapter, gliotoxin induced mainly apoptosis and to a notably lesser extent necrosis in the lung alveolar cells (Figures 3.13 and 3.15). It can be seen from the results that induction of apoptosis in A549 cells started from the very early hours of incubation of the cells with culture filtrates of the *A. fumigatus*. Both high and low gliotoxin producer strains, resulted in high number of apoptotic cells (with slightly higher number in AF-293 than AF-10). This indicates the potency of the toxin in causing cell death. This also confirmed the earlier experiments that showed even very low concentration of gliotoxin (1μM) reduces significantly the viability of the A549 cells (Figure 3.9 and 3.10).

Earlier in this chapter (section 4.1.2 of this chapter) a question was raised about whether the burden caused by gliotoxin in the host tissue is dependent on the spores of the *Aspergillus* strains inhaled. The above results indicate that the gliotoxin produced by both wild type strains results in significant cell death in lung epithelial cells from early hours of incubation. In addition, the earlier cell viability assay (Figure 3.10) shows that 1μM concentration of gliotoxin reduces the viability of lung cells to more than 50 percent. Therefore, it can be concluded that provided *A. fumigatus* is able to produce gliotoxin, the extent of the damage caused to the lung cells would be very similar, irrespective of the strain inhaled.

With regards to the gliotoxin knockout strain, the results of this study showed that incubation of pneumocytes with culture filtrates from the *A. fumigatus* (*Δglip*) hardly caused any apoptosis to A549 cells

(Figure 3.13). The response was very similar to the control where no fungal culture filtrates was added to the cells. However, from 8 h of incubation onwards some morphological changes in the cells treated with the knockout strain was observed (Figure 3.14). This indicates that other factors in the culture broth such as the presence of other toxins or proteases, to a lesser extent, cause damage to the A549 cells. It can be observed from the results in Figure 3.14 that from 8 h incubation onwards cells started to show changes in morphology indicating a possible initiation of necrosis (as cells seem to have moved upwards along the PI axis in the histograms). General information about the changes happening during necrosis is described in section 1.8.2 of the introduction chapter.

Similar to the observed results of this study, *in vitro* studies have shown that *ΔgliP* failed to cause apoptosis in EL4 thymoma cells. Also, during *in vivo* studies *ΔgliP* strains of *A. fumigatus* are shown to be significantly less virulent compared to the wild type strain in an animal infection model (two different mouse strains, 129/Sv and BALB/c). Therefore an important role as a virulence factor has been suggested for gliotoxin (Sugui *et al.*, 2007). In contrast, Cramer has shown in his study that there is no difference in the mortality rate of the mice infected with gliotoxin knockout strain (*ΔgliP*) and mice infected with wild type strain of *A. fumigatus* (AF-293). He suggested that gliotoxin is not required for virulence in this fungus (Cramer, 2006). Thus, due to the discrepancies observed in the literature, the role of this toxin as a virulence factor in this fungus is still subject of many debates. However, according to the results of this study gliotoxin is a very potent toxin with significant damaging effect on the lung cells. Therefore, its role as a virulence factor is supported by the data obtained in this study.

Moreover, similar to the control (A549 cells without any fungal culture filtratate addition), addition of culture filtrates of *A. fumigatus* (both wild type strains as well as the knockout strain) harvested earlier than 48h at 36-38h post inoculation failed to cause any significant cell death

in the lung epithelial cells. This also further confirmed the earlier results shown in Figure 3.6, indicating that no gliotoxin production was observed before 48 h post inoculation. Lastly, although induction of apoptosis in lung alveolar cells by gliotoxin has been demonstrated in this study, further research is required to unravel the molecular mechanisms through which gliotoxin causes apoptosis in these cells.

4.2 Investigation of the effect of hydrocortisone treatment on enhancing growth and toxin production in *A. fumigatus*

It has been suggested that in the host-pathogen interaction, host signals can be exploited by pathogens to activate their virulence genes. Recent reports show that although bacterial cells do not express adrenergic receptors, they respond to adrenaline and /or noradrenaline (Hughes and Sperandio, 2008). It has also been shown that noradrenaline induces bacterial growth, production of an auto-inducer, fimbriae and toxin expression in pathogenic *Escherichia coli* (Lyte and Ernst, 1992; Burton *et al.*, 2002). Furthermore there are reports indicating noradrenaline induces the expression of virulence genes in *Pseudomonas aeruginosa*. The same hormone together with adrenaline has been shown to induce the expression of flagella and the type III secretion system in entrohaemorrhagic *E. coli* (Sperandio *et al.*, 2003; Hughes and Sperandio, 2008). This cross-kingdom signalling is a new field of research that has evolved from the initial observations of cell to cell signalling between microorganisms. The realization that in the bacterial world host hormones may regulate bacterial gene expression (Sperandio *et al.*, 2003), has opened a new insight into the host-pathogen interaction in aspergillosis in this study.

Invasive aspergillosis is often associated with corticosteroid therapy. Predisposition towards developing fungal infections is among the side effects of this type of therapy in immunocompromised patients (Palmer *et al.*, 1991; Leav *et al.*, 2000; Lionakis and Kontoyiannis, 2003; Ruud *et al.*, 2013). On this basis, apart from the effect of

hydrocortisone on the immune system, it was important to investigate its direct effect on *A. fumigatus*.

The effect of cortisol acetate on the growth rate of *A. fumigatus* has been reported both in *vivo* and in *vitro*. Lehman and White, 1975 have shown that cortisone acetate-treated mice were more susceptible to infection with *A. fumigatus*. *A. fumigatus* mycelium grew more rapidly in cortisone treated mice compared to the control (untreated mice) (Lehmann and White, 1975). It is also reported that addition of pharmacological doses of hydrocortisone to the cultures of *A. fumigatus* and *A. flavus* enhances the growth rate of these fungi by 30-40 percent. Therefore a ligand receptor role for these compounds and *Aspergillus* has been suggested (Ng *et al.*, 1994). Lipid-based hormones such as cortisol are able to freely penetrate through the cell membrane and interact with members of the nuclear receptor family to modify transcriptional regulation in eukaryotes (Downward, 2001). This provides an explanation for a possible similar signalling system in primary eukaryotes such as *Aspergillus* and a reason for the above mentioned observations.

Interactions are also reported between other human steroid hormones and fungi. Among those some have been proven to be important in the pathogenesis of certain fungal diseases. One example is the effect of progesterone and 17β-oestradiol on increasing the growth rate and endospore release in *Coccidioides immitis* (Powell *et al.*, 1983). This has been shown to be related to the increased incidence of coccidiomycosis during pregnancy (Powell *et al.*, 1983). In addition, specific binding proteins for steroid hormones have been found in various fungi such as corticosterone-binding protein in *Candida albicans* (Loose *et al.*, 1981). Ergostrol has been reported to increase the growth rate of *A. fumigatus* with maximum increase in the specific growth-rate at 1μg/ml (2.5×10^{-6} M); this concentration is equivalent to the concentration of ergostrol that is associated with an increase in the protein kinase activity in *S. cerevisiae.* Similar

concentrations of cortisol have also been shown to be most effective on the growth of *A. fumigatus* (Ng *et al.*, 1994). All these observations highlighted the lack of a more detailed examination of the effect of hydrocortisone in *A. fumigatus*. Hence part of this research was allocated to this investigation.

4.2.1 Changes in morphology during growth

Hydrocortisone treatment of *A. fumigatus* AF-10 strain (addition of hydrocortisone in 10^{-5} M and 10^{-6} M concentrations), caused an early onset of sporulation (Figure 3.17 of the result chapter). This is the first report on the effect of hydrocortisone on the morphology (sporulation) of the fungal cells. Earlier studies on the same strain (AF-10), reported an increased growth rate based on the measurement of the hyphae length on agar plate (Vogel's medium) as well as the rate of incorporation of N-acetylglucosamine (Ng *et al.*, 1994). In the present work unlike the earlier observations by (Ng *et al.*, 1994), no significant change in the growth-rate of the *A. fumigatus* treated with hydrocortisone was observed (Figure 3.18). Although the same medium (however in liquid form) and same strain of A. *fumigatus* was used. Solid medium was used initially in this study, however due to the filamentous nature of the fungus and variability/errors in measurement of the hyphae length during growth, studies were continued in liquid medium for more reliable assessment.

The exact reason behind observed morphological changes is not completely clear. However, proteomic results of this study have demonstrated an up-regulation of the serine protease following hydrocortisone treatment. This might be a possible reason for this observation. Serine protease Alp-2 is a vacuolar enzyme (Reichard *et al.*, 2000). Disruption of serine proteases has been reported to effect the growth and morphology of *Aspergillus* (Monod *et al.*, 1993; Monod *et al.*, 2009). Disruption of *Alp*2 in *A. fumigatus* resulted in slightly reduced growth-rate, smaller sized conidiophores and a decrease in

the number of conidia (Reichard *et al.*, 2000). In another study, increase in the conidial discharge has been shown to be related to an increase in the level of serine proteases in fungi (Phadatare *et al.*, 1989). Involvement of serine proteases in sporulation and germination of spores has been reported in both bacteria and fungi in various studies (Leighton and Stock, 1970; Dancer and Mandelstam, 1975; Rao *et al.*, 1998). Taken together, the above reports indicate the involvement of serine proteases in conidiation of *A. fumigatus* and therefore might provide a basis for the morphological changes observed after hydrocortisone treatment.

4.2.2 Effect of hydrocortisone on gliotoxin production

There was a slight increase in the level of gliotoxin production in the wild type culture (AF-293) after hydrocortisone treatment. This was particularly pronounced after prolonged treatment of the cells with culture filtrates of *A. fumigatus* (Figures 3.19 and 3.21). Thus, suggesting that hydrocortisone treatment appears to effect the gliotoxin production. Further to these observations, the mRNA expression of two of the main genes involved in gliotoxin production (details of the gliotoxin gene cluster is described in section 1.3.2.1 of introduction chapter) was also studied under similar conditions. This was done to see if there are any changes at mRNA expression level when *A. fumigatus* was grown in the presence of hydrocortisone in the culture. The *gliZ* gene showed an enhanced expression as a result of hydrocortisone treatment; however this was not significant considering the data from different independent experiments. g*liZ* is a transcription factor, controlling the regulation of gliotoxin gene cluster.

A point to consider here, is the variation in the ability of *A. fumigatus* for gliotoxin production in different media and hence the expression of genes encoding for this toxin. Several studies have reported the dependence of gliotoxin production on culture medium and have shown that minimal medium does not support gliotoxin production (Belkacemi *et al.*, 1999; Cramer Jr *et al.*, 2006; Gravelat *et al.*, 2008).

The culture used in real time PCR experiments was grown in a minimal medium. Minimal medium is usually used in molecular biology because growth conditions can be more accurately defined in this medium. Furthermore Vogel's minimal medium is the medium that has been used frequently for molecular studies of *A. fumigatus* genes.

In addition, the results obtained from PCR analysis of *gliZ* (Figure 3.31) and *gliP* genes (Figure 3.32) show the presence of faded product bands (especially for *gliP*). This also confirms the above mentioned point (less/lack of gliotoxin production in minimal medium). It is important to mention that the specificity of the primers used for these genes was confirmed by sequencing analysis (sections 3.5.5.1 and 3.5.6.1 in the result chapter). Therefore, performing the same experiment using a more favourable medium for gliotoxin production is suggested and would probably lead to more convincing results.

Finally, the effect of hydrocortisone treated *ΔgliP* mutant of *A. fumigatus* on A549 cells also showed that long term incubation increased the morphological changes (initiation of necrosis) in lung epithelial cells (Figures 3.20). This observation can be correlated to the result of proteomic studies where addition of hydrocortisone to the culture broth of the fungus enhanced the abundance of several proteins including different proteases (Table 3.1). Proteases have been shown to contribute to tissue damage in the lung during aspergillosis (Robinson *et al.*, 1990). For instance, a study on the effect of elastases on the lung tissue burden during aspergillosis, has shown that lungs of mice exposed to elastase-producing strains of *A. fumigatus* undergo necrosis of the alveoli (Kothary *et al.*, 1984).

Alkaline proteases are among the identified proteases in the present study. These proteases degrade collagen, fibrin, fibrinogen, and elastin (Tomee and Kauffman, 2000). Similarly serine proteases with elastinolytic activity can cause tissue damage in the lung during aspergillosis (Kolattukudy *et al.*, 1993). Hence the observed changes in

A549 cells caused by gliotoxin deficient strain can be due to the effect of the proteases. More interestingly, the proteolytic activity of *A. fumigatus* seems to be enhanced in the presence of hydrocortisone. This suggests that a similar effect may occur on the fungus during aspergillosis infection in immunosuppressed patients.

4.3 Proteomic studies

The discussion of the identified proteins that showed up-regulation in hydrocortisone containing cultures is classified into three main sections based on the role of the identified proteins. This includes proteins involved in proteolytic activity and amino acid metabolism (e.g. proteases), proteins involved in carbohydrate metabolism, and stress related proteins (e.g. catalases and the heat-shock proteins). Where relevant, the results obtained from real time PCR experiments have also been discussed in this section.

Production and secretion of hydrolytic enzymes such as proteases and lipases are of importance for virulence of *A. fumigatus* as these are among various virulence factors produced by pathogenic fungi (Monod *et al.*, 2009). These enzymes are also involved in other processes/interactions including the nutritional demands of the organism, dissemination within the host, tissue damage, and overcoming the host immune system (Karkowska-Kuleta *et al.*, 2009). Furthermore, fungi are exposed to reactive oxygen species (ROS) produced by the host immune system while growing within the host. In response, these organisms produce enzymes such as catalases and the heat-shock proteins which help them overcome the host defence mechanism (Brown *et al.*, 2007; Karkowska-Kuleta *et al.*, 2009).

4.3.1 Proteolytic activity in *A. fumigatus*

Proteases are essential for the growth of the fungus in a protein medium or a protein rich environment. They cleave proteins to smaller peptides and amino acids that can be easily absorbed by the fungus. Furthermore, intracellular proteases play an important role in creating a pool of amino acids for the biosynthesis of necessary proteins for the growth and development of the fungus.

In terms of virulence of *A. fumigatus*, proteases have been shown to play a role in the pathogenicity of the fungus by helping the invasion of the host tissue during the infection. Several studies have reported the

identification of *A. fumigatus* proteolytic enzymes. These include exoproteases like serine protease and endoproteases such as alkaline proteases and metalloproteases, both *in vitro* and *in vivo*. They have therefore, suggested a role for *A. fumigatus* proteases in invading the host tissue (Reichard *et al.*, 1990a; Moutaouakil *et al.*, 1993a; Markaryan *et al.*, 1994). Furthermore, it has been hypothesized that specific host factors are able to induce the production of proteases (Latgé, 1999). This hypothesis was based on the observations of a study showing that a double mutant strain of *A. fumigatus* (*Δalp, Δmep*), lacking total extracellular proteolytic activity *in vitro*, was still able to infect and kill immunosuppressed mice *in vivo* similar to the wild type strain (Jaton-Ogay *et al.*, 1994). This statement supports the hypothesis of this research. As demonstrated in this study, hydrocortisone as a host factor plays a role in the induction of some proteases in *A. fumigatus*. This in turn can contribute to the pathogenicity of this fungus in immunocompromised patients who develop aspergillosis.

Secreted proteases are among different virulence factors in *A. fumigatus* and have been the subject of several studies. Amino acids are known to be the main nutritional source for *A. fumigatus* during aspergillosis. The environment encountered by the fungus is more likely to be rich in proteins with amino acids being the primary nutritional source (Latgé, 1999; Krappmann, 2009). Furthermore, it has been reported that *Aspergillus* grows well in a medium containing protein as nitrogen and carbon source and is able to produce both endo and exo proteases (Reichard *et al.*, 1990a; Monod *et al.*, 2005). Proteases contain around 1% of the total proteins being synthesized by *A. fumigatus* and uptill now an overall of 111 proteases together with 26 non-protease homologues have been verified in the *A. fumigatus* genome (Monod *et al.*, 2009).

Peptidases are responsible for a variety of physiological functions and play an important role in several physiological and pathological processes such as cell growth, protein catabolism, morphogenesis and

development. They are also involved in degradation of many regulatory proteins that control functions like the heath shock response and response to DNA damage (Gottesman and Maurizi, 1992; Rao *et al.*, 1998). Proteases contribute to the protein turnover in the cell (intracellular proteases), and hydrolysis of large proteins to smaller molecules facilitating their absorption by the cell (secreted proteases) (Rao *et al.*, 1998).

This study shows that addition of hydrocortisone sodium succinate to the culture medium of *A. fumigatus* significantly affects the abundance of some proteases compared to the control cultures (Figure 3.23 and Table 3.1). The up-regulated proteases include serine protease Alp2, and autophagic serine protease Alp2, Peptidase, aminopeptidase, and prolinedipeptidase (prolidasepepP).

Serine endoproteases use host factors such as extracellular matrix proteins (e.g. actin fibre in mice pneumocyte cytoskeleton) and serum proteins as their substrate (Kogan *et al.*, 2004; Monod *et al.*, 2009). Serine proteases have elastinolytic activity and having considered that lung tissue is rich in elastin, producing these enzymes can greatly contribute to the lung tissue colonization of the *A. fumigatus* during pulmonary infection (Karkowska-Kuleta *et al.*, 2009).

The proteomic results showed that serine protease Alp2 overexpressed significantly (2.0 fold increase) at 48 h post inoculation in the cultures containing 10^{-6} M hydrocortisone in their medium. *A. fumigatus* serine proteases, also called subtilisin, include two types: Alp1 and Alp2. Alp1, characterized in 1990, acts by cleaving elastin and collagen. Alp2 is a vacuolar enzyme. Both Alp-1 and Alp-2 are synthesized as preproteins (Monod *et al.*, 2009). Apart from the earlier mentioned role of Alp2 in conidiation, *A. fumigatus* Alp2 has been reported to be involved in the general supply of amino acids for the synthesis of developmental proteins during conidiophore development. Given this, it can be suggested that the presence of hydrocortisone

might play a role in the general cell homeostasis in *A. fumigatus*. This role is also observed in higher eukaryotes. (Further explanations of the role of hydrocortisone in regulating homeostasis including the relevant references are explained in section 1.6 of the introduction chapter). Despite the proteomic observations of Alp2 up-regulation, no significant change in mRNA expression of this gene was observed following hydrocortisone treatment (as shown in qRT-PCR studies in Figure 3.37). This suggests that probably *de novo* mRNA synthesis of Alp-2 is not required. Thus, the increased abundance of the Alp-2 protein may reflect the excess of this protein in vacuoles within the cell which is released when required.

Among other up-regulated proteins under the influence of hydrocortisone are also two exo-peptidases; amino-peptidase and proline dipeptidase (prolidase pepP). Four genes in the genome of *A. fumigatus* have been recognized that encode for amino-peptidases including Lap1 and Lap2, Xp-749158, and MER050730 (Monod *et al.*, 2009). These enzymes are metalloproteases, containing two zinc binding sites. Amino-peptidases act on the free N-terminal of the peptides (Rao *et al.*, 1998). They are also reported to contribute to the protein turnover in the cell by helping to provide a pool of amino acids for protein synthesis (Rao *et al.*, 1998; Monod *et al.*, 2009). Proteomic results show that under the influence of hydrocortisone (present in the environment) amino-peptidase expression increases by 2.1 fold. This again suggests that cortisol contributes to the generation of amino acids necessary for the synthesis of developmental proteins. This indicates that hydrocortisone triggers a similar response in *A. fumigatus* cells in regulating homeostasis, as it does in higher eukaryotes.

Prolidase is another exo-peptidase in *A. fumigatus* that its expression has been up-regulated by 2.5 fold under the effect of hydrocortisone treatment. Prolidase belongs to the dipeptidyl-peptidase group of *A. fumigatus* proteases (Monod *et al.*, 2009). There are two main

dipeptidases in *A. fumigatus* dipeptydyl-peptidaseIV (DppIV) and dipeptydyl-peptidaseV (DppV) (Beauvais *et al.*, 1997). Landis and colleagues reported that DppIV contributes to lowering the bronchial inflammation by degrading unknown peptide substrates released by histamine in response to allergens in the lower respiratory tract (Landis *et al.*, 2008). Furthermore, it is reported that *A. fumigatus* DppIV has been used to cleave pro-inflammatory peptides in animal models suffering from rhinosinusitis and asthma (Grouzmann *et al.*, 2002; Landis *et al.*, 2008; Monod *et al.*, 2009). Given that cortisol supresses the immune response. It may be suggested that during aspergillosis infection in immunocompromised hosts, *A. fumigatus* is able to sense/respond to the hydrocortisone in the environment and this eventually results in over-expression of genes involved in the degradation of pro-inflamatory proteins. This may create a favourable environment for the growth of the fungus and its development in the lung. However, further studies are needed to confirm this hypothesis.

4.3.2 Proteins involved in carbohydrate metabolism

The carbohydrate metabolism enzymes such as Fructose-BisPhosphate-aldolase Class II (FBA-II), Aldehyde dehydrogenase and Methylmalonate-semialdehyde dehydrogenase are among the identified proteins in this study. The protein abundance of these enzymes has shown an increase in response to the presence of hydrocortisone in the medium. Fru-BP-aldolase Class II (FBA-II) and aldehyde dehydrogenase are among the proteins involved in gluconeogenesis process. Methylmalonate-semialdehyde dehydrogenase is involved in metabolic processes such as degradation of many amino acids including Valine, leucine and isoleucine, beta-Alanine metabolism, and Inositol phosphate metabolism. It seems that the main action of these proteins is facilitating the protein degradation and providing necessary substrates for Tri-Carboxylic acid (TCA) and gluconeogenesis pathways. This seems to be a feasible response at this stage (early stationary) of the growth of *A. fumigatus.* Considering that a minimal medium has been used for the growth of the fungus in these

experiments and cultures were 48 hours old *Aspergillus* cells are expected to provide the necessary carbon and energy sources by breaking down lipids and proteins and gluconeogenesis. However, an interesting point that has been revealed in this study is the over-expression of these proteins in the presence of hydrocortisone in the culture medium in comparison to the control where no hydrocortisone was added. This may suggest a similar signalling role for hydrocortisone in *A. fumigatus* to the one generally observed in other eukaryotic cells, in facilitating and stimulating gluconeogenesis during stress and starvation conditions (Pilkis and Granner, 1992; Larson *et al.*, 2003). A study by Liu and colleagues has demonstrated that regulation of gluconeogenesis in *A. fumigatus* is controlled by a zinc clustered transcription factor (AcuM). It is also demonstrated that this transcription factor cluster is necessary for virulence of this fungus during invasive aspergillosis in mice (Liu *et al.*, 2010). Moreover it has been reported that this transcript is up-regulated in the conidia exposed to neutrophils (Sugui, 2008). Further studies investigating the effect of hydrocortisone on the expression of AcuM in *A. fumigatus* are necessary to confirm the possibility of the effect of this hormone on gluconeogenesis regulation.

Fructose bisphosphate aldolase (FBA) is another enzyme whose abundance increased during proteomic studies. FBA is a heat-stable enzyme involved in both glycolysis and gluconeogenesis pathways. This enzyme catalyses a reversible reaction where it splits the aldehyde/aldole, fructose 1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Considering the conditions used for proteomic studies (minimal medium, and 48h harvest), presence of this enzyme is most probably related to its involvement in the gluconeogenesis pathway. Furthermore, since *A. fumigatus* can grow at higher temperatures (even in fever temperatures and higher) which is normally the case in patients developing aspergillosis, and given that a) FBA is heat-stable, and b)

there are nutritional limitations in the host, it can be suggested that hydrocortisone induction of this enzyme may enhance the survival of this organism in the host environment during the infection (via gluconeogenesis) (Rhodes, 2006; Albrecht *et al.*, 2010; Say and Fuchs, 2010).

Among the above mentioned enzymes, fructose bis phosphate aldolase (FBA) and Methylmalonate-semialdehyde dehydrogenase (MMSAD) were selected to investigate their mRNA expression in the presence of hydrocortisone in the medium. qRT-PCR analysis of the mRNA expression of MMSAD (Figure 3.35) showed a significant increase (up to 3 fold) in *Aspergillus* cultures where hydrocortisone was present in the medium. This confirms the earlier proteomic observations. However, the investigation of FBA was incomplete due to the problems with experimental optimization of this gene and lack of time. Therefore, a thorough investigation of the mRNA expression of this gene is suggested.

4.3.3 Stress related proteins

Spermidine synthase, catalase, and the heat-shock protein 70 (Hsp70), all stress response related proteins are among the highly abundant proteins identified from the 2-D gels of the cultures treated with hydrocortisone (Table 3.1).

Spermidine synthases are involved in the biosynthesis of polyamines, which play a vital role in defence against environmental stress. In *Cryptococcus neoformance* spermidine synthase mutants, deletion of the spe3 gene, showed a reduction in capsule formation, melanin production (both virulence factors) and growth-rate (Kingsbury *et al.*, 2004). In addition, deletion of spermidine synthase in *A. nidulance* has been associated with defects in transitions from germ tube to hyphae, hyphal growth, and secondary metabolite production (Jin *et al.*, 2002). Therefore, the increase in the abundance of this protein in hydrocortisone-treated cultures appears to be involved in a stress response mechanism helping the fungus to cope with environmental

stress conditions such as nutrient limitation and/or oxidative stress. This is another evidence for the reception of hydrocortisone by this fungus that could play a role in the fungus' virulence during aspergillosis.

Catalases are among the stress response proteins responsible for protecting *A. fumigatus* against oxidative stress. They have been shown to play a role in the pathogenesis of this fungus (Paris *et al.*, 2003; Chauhan *et al.*, 2006; Thön *et al.*, 2007; Askew and Rhodes, 2009). The presence of hydrocortisone in the culture medium increased the abundance of catalase (1.5 fold) during proteomic studies. Interestingly, similarly, another study investigating the effect of the treatment of *A. fumigatus* with different concentrations of dexamethasone has also shown an increase in the expression of *cat-A* gene (encoding for a spore-specific catalase in this fungus) (Xu *et al.*, 2011). The reported increase was concurrent with increase in the treatment time of the fungus with this steroid (particularly after 7 h and 16 h treatment). This is in agreement with the results of this work, indicating the involvement of glucocorticoids in regulation of this gene in *A. fumigatus*. This also provides a possible explanation for the ability of the fungus to survive the oxidative stress during infection in immunocompromised hosts.

Increased synthesis of heat-shock proteins, particularly in pathogens involved in direct interaction with host phagocytes has been suggested to play a role in their survival in the host environment, protecting them from host-derived defence mechanisms (Kaufmann, 1990; Young and Garbe, 1991; Zügel and Kaufmann, 1999).

Heat-shock proteins were first identified as proteins that their synthesis is up-regulated in response to high but sub-lethal temperatures (the heat-shock). They help the cell to survive subsequent exposures, minimize the accumulation of other denatured cellular proteins, and facilitate restoring their native conformation

(Hartl, 1996; Plesofsky, 2010). However, later studies revealed that apart from the increased synthesis of heat-shock proteins under heat stress, the synthesis of these molecular chaperones also increases under other physical and chemical stresses (e.g. pharmacological reagents, oxidative stress, infection and inflammation) (Hahn and Thiele, 2004; Plesofsky, 2010). Hsp70 is a member of the heat-shock family of proteins related to DnaK protein of *E. coli* with 50% homology. Phosphorylation is a common post translational modification of Hsp70 and contributes to its activation (Plesofsky, 2010). Transcription of the heat-shock protein synthesis is regulated by heat-shock transcription factor (HSF). HSF is reported to play an important role in the cellular homeostasis in response to stress (Hahn and Thiele, 2004). Interestingly it has been reported that in yeast, other than heat stress, other stress conditions such as oxidative stress and glucose starvation also induce the heat-shock transcription factor (Hahn and Thiele, 2004; Yamamoto *et al.*, 2007; Plesofsky, 2010). Therefore the initial assumption for the results observed in this study, i.e. identification of multiple spots as hsp70, could be due to the induction of the HSF transcription factor in *A. fumigatus* due to glucose starvation (considering that cultures were grown in Vogel's minimal medium for 48 h). However, the question is why the expression of hsp70 proteins in the cultures from the hydrocortisone containing medium is considerably higher compared to the control cultures? (Figure 3.23 and Table 3.1). qRT-PCR experiments have also revealed that expression of Hsp70 mRNA is significantly increased when the fungus is grown in the presence of 10^{-6} M and 10^{-5} M hydrocortisone sodium succinate (Figure 3.34).

With regards to the regulatory effect of steroid hormones on the heat-shock protein/gene expression in mammalian cells, it has been reported that treatment of the mice L929 cells with dexamethasone (glucocorticoid agonist) has inhibitory effects on the heat-shock induced expression of many heat-shock proteins including Hsp 90, Hsp

70 and Hsp 110. Hence, it has been suggested that dexamethasone inhibits the heat-shock transcription factor (Wadekar *et al.*, 2001). On the other hand, in another report by Knowlton and Sun, it has been reported that dexamethasone and some other steroid hormones activate the heat-shock protein transcription factor HSF-1 and increase the synthesis of Hsp72 (the inducible form of Hsp70 in heart) by 60% in adult cardiac myocytes. So it was suggested that Hsps have a role in protection against heart injuries (Sun *et al.*, 2000; Knowlton and Sun, 2001). Moreover in a study by Kainuma and co-workers it has been reported that hydrocortisone treatment increases the level of Hsp70 mRNA in mice (Kainuma *et al.*, 2009). Therefore the latter studies are in agreement with the results observed in this study, indicating a similar regulatory effect for hydrocortisone in regulating the expression of Hsp-70 in *A. fumigatus*. The heat-shock proteins are highly conserved proteins across species. The bacterial heat-shock proteins for instance have been reported to share considerable similarities with the eukaryotic ones (Neckers and Tatu, 2008).

Several reports have described the involvement of molecular chaperons such as hsp70 in pathogen virulence (Neckers and Tatu, 2008). Once entered the host, most pathogens encounter an extreme change in the environment such a change in the temperature, oxidative burst in response to infection, and other host environmental factors (e.g. steroid hormones). Therefore, coping with and adapting to these environmental conditions is essential for their establishment inside the host. This is usually achieved through different adaptation strategies in pathogens. Synthesis of the heat-shock proteins has appeared as a common survival strategy when pathogens are inside the host environment (Maresca and Kobayashi, 1994b; Neckers and Tatu, 2008). Induction of the heat-shock protein genes during bacterial, parasites, and viral infections has been reported in several studies. Also, their role as virulence factors that help pathogens to adapt to, or overcome the unfavourable conditions in the host are described

(Maresca and Kobayashi, 1994b; Kamiya *et al.*, 1998; Kalayoglu *et al.*, 2000; Kumar and Mitra, 2005). Pathogens have been shown to not only utilise their endogenous Hsps during infection but also to exploit the host heat-shock proteins too (Neckers and Tatu, 2008; Jelenska *et al.*, 2010). Bacterial heat-shock protein synthesis is shown to be induced inside phagosome. This helps bacterial pathogens to cope with oxidative, acidifications, and in general stressful environment inside the host. Interestingly DnaK (hsp70) was among the up-regulated heat-shock proteins under such conditions (Hosogi and Duncan, 2005). Deletion of DnaK gene in *Brucella suis*, and *Salmonella entrica* has resulted in failure of these bacteria to colonize inside the host and survival in the macrophages (Köhler *et al.*, 1996; Takaya *et al.*, 2004). Many studies have reported that *Aspergillus* conidia are able to survive inside macrophages, escape the phagosomes, and germinate inside the acidic organelles (Wasylnka and Moore, 2003; Osherov, 2012). However, the mechanism of this process is not clear. Therefore, it would be interesting to look into the expression level of this protein (Hsp70) inside macrophages during aspergillosis infection in immunocompromised hosts.

Likewise, several studies have reported the importance of different classes of the heat-shock proteins in the virulence of human protozoan parasites (Neckers and Tatu, 2008). For instance, it has been shown that Hsp70 plays an important role in the virulence of *Toxoplasma gondii* by helping this parasite to escape proinflammatory responses of the host (Dobbin *et al.*, 2002). In the case of viral pathogens, although most of them are unable to synthesise the heat-shock proteins, there are evidences showing their ability to exploit the host heat-shock proteins to establish their infection. The human immunodeficiency virus (HIV), for example has been shown to use host Hsp70 and incorporate it in the formation of virion core, reviewed in (Neckers and Tatu, 2008).

Taken together, the heat-shock proteins and particularly Hsp70 seem to play an important role in the virulence of various pathogens. However, its role in the pathogenicity of the fungal pathogens is not investigated sufficiently. Therefore, it would be interesting to examine the importance of this molecular chaperone in the colonization and survival of *A. fumigatus* inside the host. It has been reported that expression of hsp70 is induced under various stress conditions in this fungus (Paul *et al.*, 2012). Therefore, it would be possible to assume that *A. fumigatus* is able to perceive hydrocortisone as a stress signal (again, similar to its role in other eukaryotes) in addition to other stress factors in the host. This results in over-expression of Hsp-70. Hence, a role is suggested for this protein in helping the fungus to survive inside the host, especially immunosuppressed hosts suffering from prolonged hypercortisolemia.

Another interesting role for the heat-shock proteins in fungal pathogenesis is their involvement in drug resistance; which is one of the main problems in this field of research. In a series of studies by Cowen and Lindquist, the heat-shock protein 90 has been related to the development of resistance to azole antifungals in S*accharomyces cerevisiae*. They suggested that inhibition of the heat-shock proteins (using natural inhibitors of the heat-shock proteins) is a possible way to overcome antifungal resistance. Azole antifungals target the lanestrol 14-alpha demethylase enzyme in the ergosterol biosynthesis pathway. Inhibition of this enzyme leads to the lack/impairment of ergosterol synthesis, build-up of toxic intermediate metabolites, and stress to the fungal membrane. However, it has been shown that in an engineered strain of *S. cerevisae,* high concentrations of hsp90 and its function has contributed to the development of the drug resistance (Cowen and Lindquist, 2005; Cowen, 2009). In another very recent study, development of resistance to fluconazole has been shown in human pathogen *Candida albicans* to be due to Msi3P (a member of the Hsp70 family). Also, the role of this chaperone in growth-rate and

establishing the virulence of this fungus has been verified (Nagao *et al.*, 2012). Likewise, in another study the role of Ssa-1 (another member of Hsp70 family in *Candida*) in the virulence of this pathogen has been demonstrated. An attenuated virulence in this pathogen has been shown based on the defective binding to endothelial and epithelial cell receptors in Ssa-1 mutants in a murine model of infection (Sun *et al.*, 2010).

All the above examples of the involvement of molecular chaperones like hsp70 in virulence, survival of pathogens inside the host, and their involvement in developing drug resistance, provides a compelling case/argument for the involvement of molecular chaperones in pathogenesis of pathogens. Consequently, their role in the pathogenesis of *A. fumigatus* is also probable. This however requires detailed investigation of their function in surviving the killing by macrophages and neutrophils as well as possibility of their involvement in developing drug resistance.

Furthermore, this study shows that presence of hydrocortisone further induces the biosynthesis of an important molecular chaperone (Hsp70). Therefore, it is conceivable to suggest that *A. fumigatus* is able to sense this hormone in the environment in a manner that helps it to cope better with the stressful host environment and possibly increasing its survival in the host. In majority of organisms many genes encoding for Hsp70, have been identified. These genes are known to be induced by different factors, among them are stress factors (Maresca and Kobayashi, 1994b). Hence, a precise understanding of the regulatory mechanisms of these genes both at transcriptional and structural levels is required to make an accurate assumption into their role in the host pathogen relationship during aspergillosis.

Additionally, during infection the heat-shock proteins are among the mostly abundant proteins and play a role in both adaptations to the environment and as antigens. Taken together, further *in vivo* studies in

the hydrocortisone immunosuppressed animal models are required to see if a similar pattern is observed and to support this hypothesis.

4.4 Binding of *A. fumigatus* spores to lung epithelial cells after hydrocortisone pre-treatment of pneumocytes

This study was conducted to explore whether lungs of patients undergone immunosuppressive therapy by hydrocortisone were more liable than the normal lungs to allow the *Aspergillus* spores to adhere and colonize. Adherence to the host lung epithelial cells is one of the first and critical steps of pathogenesis of *A. fumigatus*. This study demonstrates for the first time that pre-treatment with physiological and pharmacological doses of hydrocortisone enhances the binding ability of *A. fumigatus* cells to the lung epithelial cells compared with the controlled untreated cells (Figure 3.22).

Generally, following inhalation of *Aspergillus* conidia, they reach the lung alveoli where they bind to the lung alveolar epithelial cells. Binding of conidia to alveolar cells is known to be via extracellular matrix proteins and glycolipids (Bromley and Donaldson, 1996a; DeHart *et al.*, 1997). In an early study by DeHart and colleagues, they have demonstrated that radiolabeled conidia of *Aspergillus fumigatus* bind to lactocylseramide and two other membrane glycolipids in A549 alveolar epithelial cells. They have therefore suggested a glycosphingolipid-mediated attachment of conidia to the lung alveolar epithelial cells (DeHart *et al.*, 1997). Glycosphigosines are glycolipids found in the plasma membrane of almost all cells and in the form of lipid rafts with some proteins (Glycosylphosphatidylinositol (GPI)-anchored proteins) anchored into them (Figure 4.1) (Schnaar *et al.*, 2009). Mediating cell-cell interaction is among the physiological activities of glycosphincosines (Schnaar *et al.*, 2009). Hence, it can be suggested that binding of conidia to these host cell glycolipids might be through glycan-glycanbinding proteins, or glycan-glycan interactions of host glycolipid rafts with glycan/glycoproteins of fungal cell wall.

Interestingly it has been demonstrated that glucocorticoid treatment can increase glycosphingolipids in some animal cells (Dawson and Kernes, 1979). Thus, this might provide a possible explanation for the increased binding of *Aspergillus* spores to pneumocytes observed in this study.

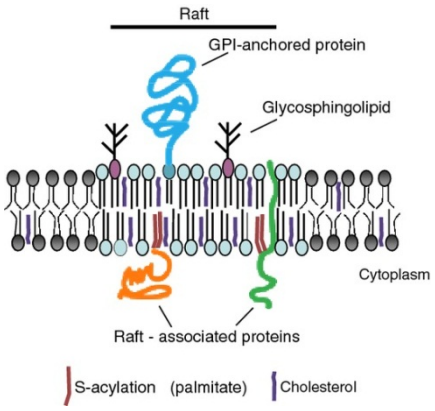


Figure 4.1
A schematic cell membrane structure in higher eukaryotic cells representing lipid rafts with glycophingolipid and GPI-anchored protein. Picture adopted from (Chichili and Rodgers, 2009).

Furthermore, epithelial cells synthesise and secret extracellular matrix (ECM) into the culture. The ECM contains glycoproteins such as collagen, fibronectin and laminin. These proteins have been shown to be involved in the binding of the conidia of *Aspergillus fumigatus* to the lung epithelial cells. *Aspergillus* conidia have been shown to adhere to fibronectin, laminin and Type I and IV collagen in ECM (Bromley and Donaldson, 1996b). In addition, Glucocorticoids have been shown to regulate extracellular matrix metabolism/synthesis in human cells. Dexamethasone enhances the synthesis of fibronectin and collagen Type IV however no effect was observed on the synthesis of laminin and Type I collagen (Zhou *et al.*, 1998). This provides another explanation for the observed results in this study.

Further investigations are required both on the lung epithelial cells and on the cell surface of *Aspergillus* conidia to unravel the exact mechanism behind this observation.

Chapter V: Conclusion

5. Conclusion

The aim of this project was to investigate the interaction of *Aspergillus fumigatus* and human alveolar epithelial cells (A549) through the produced metabolites and the environmental factors. *A. fumigatus* was chosen for this investigation as it is the main cause of aspergillosis.

Once conidia of *Aspergillus* adhere to the alveolar cells, and in the absence of immune response in immunosuppressed patients, they germinate on lung alveolar cells, penetrating and damaging the cells via production of metabolites such as toxins and enzymes (Paris *et al.*, 1997; Osherov, 2012).

Gliotoxin, a secondary metabolite of *A. fumigatus,* significantly reduced the viability of A549 cells at very low concentration (1µM) ($P<0.05$). This finding was consistent with observations made elsewhere. However there was a lack of knowledge on the type of cell death caused by this toxin in the lung alveolar epithelial cells. This study showed that gliotoxin produced in the culture broth of the wild type *A. fumigatus* strains have a profound damaging effect on the lung epithelial cells causing programmed apoptosis. Gliotoxin mutant strains on the other hand, failed to induce any notable apoptosis in lung alveolar cells. Gliotoxin production does not seem to start at the beginning of the infection, hence indicating the involvement of other metabolites and mechanisms at that stage.

Furthermore, a key risk factor for developing aspergillosis is corticosteroid therapy. Considering this, the effect of hydrocortisone on *A. fumigatus* was investigated. Molecular experiments (proteomic and/or quantitative real time PCR) showed an increase in the expression of some proteins involved in proteolytic activity and amino acid metabolism (e.g. proteases), in carbohydrate metabolism, and stress related proteins (e.g. catalases and the heat-shock proteins). Evidences provided in this study indicate the recognition of hydrocortisone by *A. fumigatus* and a role for this host factor in the regulation of cell haemostasis and stress response. This is similar to the regulatory role of this hormone in mammalian cells.

This study suggests that presence of hydrocortisone in the host environment may contribute to the pathogenicity and survival of this fungus during the infection. Further investigations could lead to identification of a glucocorticoid receptor in this fungus and better understanding of this signalling mechanism.

In addition, this study has provided evidence that hydrocortisone treatment of the pneumocytes enhances the binding of *A. fumigatus* spores to these cells. This is suggested/hypothesized to be through increasing the expression of extracellular matrix proteins in pneumocytes, hence enhancing their spore binding capacity. Further investigations in this area would promote the understanding of the spore binding mechanism. This could eventually lead to the discovery of new therapeutic interventions that might selectively block the mechanism of adherence of spores to the lung cells.

Lastly, the generated data in this study lay the foundations for further cellular and molecular analysis leading to a better understanding of interactions of *A. fumigatus* and host cells during pathogenicity of this fungus.

In conclusion, the new findings of this study provide opportunities for:

1- A better understanding of the host-pathogen relationship in aspergillosis leading to further knowledge of the mechanism(s) involved in these interactions.
2- Investigation of the important role of the fungal spores binding in development of aspergillosis particularly in immunosuppressed patients
3- As a longer term objective, development of new signalling based therapeutics
4- Hydrocortisone study leads to furthering the knowledge of the mechanism(s) involved in the interaction from a new perspective and through considering the effect of host factors (e.g. hydrocortisone) in this relation.

Chapter VI: Future Work

6 Future work

.There are several lines of research arising from this work, pursuit of which, could lead to further enhancements in understanding the mechanisms involved in the host pathogen relationship during aspergillosis

6.1 Investigating quorum sensing in clinical strains of *A. fumigatus*

A crucial point in the establishment of any infectious disease is the initial colonization of the host. One major contributor to this is the fungal cell to cell signalling, allowing them to regulate expression of genes that help them to overcome the defence mechanism of the host and colonize the host environment. Therefore, understanding the possible quorum sensing signalling pathways in filamentous fungi, particularly in opportunistic pathogens such as *Aspergillus,* would be an interesting line of research. Considering the discussion presented in section 4.1.1., investigation of the involvement of both lactone containing and fatty acid structures as signalling molecules in clinically important filamentous fungi is suggested. This eventually would lead to finding possibilities to interfere with the fungal signalling systems, blocking their successful colonization.

6.2 Investigation of molecular mechanisms underlying cell death caused by A. *fumigatus* culture filtrate on pneumocytes

This study has demonstrated that culture filtrates of wild type strains of *A. fumigatus* containing gliotoxin cause apoptosis in pneumocytes. However, the underlying mechanisms of induction of apoptosis process in lung cells are still to be elucidated.

Therefore, it would be of interest to investigate the underlying molecular mechanisms (whether it is death receptor or mitochondrial mediated) leading to apoptosis process in these cells.

To investigate the death receptor mediated mechanism, the study of the involvement of TNF-α death ligands in initiating the apoptotic process in these cells could be carried out. Gliotoxin induced apoptosis through caspases-3 activation (the key executioner of apoptosis) has been shown in monocytes (Stanzani *et al.*, 2005). Analysis of caspase-3 activation upon addition of *A. fumigatus* culture filtrates to pneumocytes could contribute in unravelling the molecular mechanism of apoptosis in these cells. The use of an optimized flow cytometric assay for the detection of activated caspase-3 is also suggested.

Alternatively western blot analysis using antibodies that recognize the cleaved caspases 9, 8, 7 and 3 in gliotoxin-treated pneumocytes would elucidate the involvement and activation of a broad range of caspases in gliotoxin-induced cell death.

Moreover, to examine the pro-apoptotic mechanisms induced by gliotoxin in lung alveolar cells, cytochrome c (Cyt c) release from mitochondria of the lung alveolar cells can be investigated. This could be done by western blot analysis of mitochondrial fraction of pneumocytes using Cyt c antibody.

In addition, gliotoxin has been shown to inhibit activation of the nuclear factor kappa B (NF-κB) in B and T lymphocytes (Pahl *et al.*, 1996b). Therefore, investigating whether induction of apoptosis in pneumocytes is NF-κB dependent would also further unravel the underlying mechanisms of gliotoxin- induced apoptosis in infected lung cells.

The outcome of these studies could contribute to the development of therapeutic agents that not only dampens the inflammatory response but also reduces apoptosis in lung alveolar cells.

6.3 Further investigation of the mechanism of glucocorticoid signalling in *A. fumigatus* by microarray analysis

The results presented in this work have already shown that the expression of several genes in *A. fumigatus* is affected by hydrocortisone supplementation. However, in order to further complete and confirm these

results, the use of whole-genome microarrays is suggested. This will offer the possibility to gain a picture of the global pattern of *A. fumigatus* gene expression affected by glucocorticoids.

In particular, the availability of the genome sequence of many *A. fumigatus* clinical isolates allows the design of genomic microarrays which could then be applied to determine the transcriptional response of *A. fumigatus* to the presence of hydrocortisone.

Comparative analysis could be undertaken with or without glucocorticoids supplementation of *A. fumigatus* cultures.

This experiment will further confirm the influence of hydrocortisone on the production of gliotoxin and other secondary metabolites produced by *A. fumigatus*, responsible for its virulence.

6.4 Identification of cortisol binding proteins in *A. fumigatus*

The mechanism(s) by which hydrocortisone affects gene expression in *A. fumigatus* is not clearly understood. However, it may involve binding to a glucocorticoid receptor. A similar mechanism has also been found in *Candida* and higher eukaryotes. Therefore, it would be interesting to look for a glucocorticoid binding protein in *A. fumigatus*. However, given that the experimental work carried out in this study (i.e. looking for cortisol binding protein in the cytosolic protein fraction of *A. fumigatus*), did not lead to identification of a specific cortisol binding protein, investigating the presence of this protein in other cellular fractions such as cell wall-membrane fraction or even nuclear fraction would be worthwhile. This is particularly suggested due to the understanding that some of mammalian steroid receptors are membrane bound (further details about membrane bound steroid receptors are presented in section 1.5 of introduction chapter). Affinity chromatography using hydrocortisone bound activated columns is suggested as a possible method to achieve this goal. The discovery of a cortisol binding protein in *A. fumigatus* can lead to important pathological implications. As such disruption of the ligand/receptor

interaction could have a major effect on the pathogenicity of this fungus. This could also lead to the development of novel therapeutic strategies against aspergillosis infection.

6.5 Investigating the role of heat shock protein 70 (Hsp70) in the pathogenicity of *A. fumigatus*

The expression of Hsp70 was shown to be significantly increased through proteomics as well as qRT-PCR studies.

The involvement of heat shock proteins (Hsps) and in particular Hsp70 in the pathogenicity of many pathogenic organisms has been demonstrated in many studies and is also discussed in the section 4.3.3 of the discussion chapter.

However, their involvement in the virulence of *A. fumigatus* and its survival inside the host is not clearly understood. Study of the role of Hsps in this fungus is particularly important, given that it has been reported that a) their biosynthesis increases under physical and chemical stresses (Plesofsky, 2010), b) they play a role in the cellular homeostasis in response to stress (Hahn and Thiele, 2004), c) they play a role as virulence factors that help pathogens to adapt to, or overcome the unfavourable conditions in the host (Maresca and Kobayashi, 1994a; Kalayoglu *et al.*, 2000; Neckers and Tatu, 2008), and d) they are involved in drug resistance of some fungal pathogenes (Cowen, 2009). All the above examples of the involvement of molecular chaperones like Hsp70 in virulence or enhancing fungus survival inside the host provide convincing evidences that their role in the pathogenesis and survival of *A. fumigatus* is also probable.

Many studies have reported that *Aspergillus* conidia are able to survive inside macrophages, escape the phagosomes, and germinate inside the acidic organelles (Wasylnka and Moore, 2003; Osherov, 2012). However, the mechanism of this process is not clear. Therefore, it would be interesting to look into the function of Hsps in helping the fungus to survive the killing by macrophages and neutrophils as well as possibility of

their involvement in developing drug resistance. This can be achieved through analysis of the mRNA or DNA expression of *hsp* genes using an in vivo model of *Aspergillus* infection, particularly in immunosuppressed hosts, given the observation of this study.

6.6 Further investigation of the binding of *A. fumigatus* spores to lung epithelial cells with and without hydrocortisone

Several reports studying the mechanism of binding of *Aspergillus fumigatus* spores to the lung epithelial cells have demonstrated that binding is mainly via adherence of the spores to the extracellular matrix proteins and glycolipids of the lung alveolar cells (Bromley and Donaldson, 1996a; DeHart *et al.*, 1997; Yang *et al.*, 2000). However exact mechanism of binding is not yet clear. An array of factors such as surface proteins of the spores, molecules that diffuse from the spore surface (also known as *Aspergillus fumigatus* diffusate (AfD)) lung surfactant proteins, extracellular matrix (ECM) proteins and lung surface glycolipids are suggested to be involved in the binding of spores to pneumocytes.

In this study it has been shown that pre-treatment of lung alveolar epithelial cells with hydrocortisone enhances the spore binding. The results of this experiment indicates that there is a potential in further investigation of the effect of hydrocortisone treatment on both the lung cells (i.e. to see the effect on surfactant proteins, lung extracellular matrix proteins and glycolipids) and on spore surface proteins (e.g. rodlet layer) and AfD. In addition it would be interesting to use fluorescent labelled ECM proteins and/or labelled lung surfactant proteins and infect them with Green fluorescent protein (GFP) expressing conidia of *A. fumigatus*, to visualize the binding prior to and after pre-treatment with hydrocortisone using confocal microscopy. This will indicate two points: 1) confirming that spores of *A. fumigatus* are binding to these proteins, and 2) establishing the effect of hydrocortisone treatment on the expression of ECM proteins and on increasing the spore binding capacity of lung cells. The latter

experiments will also help to confirm the hypothesis rose earlier from the observations of this study.

Alternatively, the effect of hydrocortisone treatment on expression of lung ECM proteins or surfactant proteins could be investigated at molecular level by looking into the changes in the expression level of the mRNA of the genes encoding for these proteins before and after treatment with hydrocortisone. Instead, to gain a more realistic insight, the expression level of these proteins in an *in vivo* immunosuppressed animal model can also be investigated.

Overall these experiments will further elucidate the mechanism of binding of spores to the lung cells and may provide a basis for the development of therapeutic agents able to interfere with/block this binding. This will reduce the spore adherence and consequently initiation of infection in immunosuppressed patients at high risk of developing aspergillosis.

Chapter VII: References

7 References

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Chapter VIII: Appendix

8 Appendix

8.1 Assays from the *A.terreus* cultures

8.1.1 Biomass assay

The biomass of the test and the control cultures was calculated as total cell dry weight at the end of fermentation as shown in (figure 8.3.) No statistically significant differences were observed bertween the test and the control cultures (P >0.05)

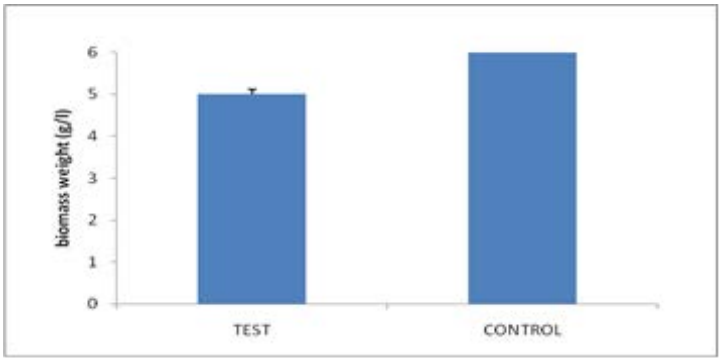


Figure 8.1
Total cell dry weight of test and control cultures of *A. terreus* culture . No statistically significant differences were observed bertween the test and the control cultures (P >0.05). The error bars indicate the standard error of the mean.

8.1.2 pH pattern from the *A. terreus* and *A. fumigatus* culture in complex medium

The pH profile of the samples taken from the shaken flask fermentation of *A. terreus* and *A. fumigatus* in complex medium is shown in figure s 8.4. and 8.5. the samples were taken from the test and the control cultures. Butyrolactone I was added at 24h to the final concentration of 100 nM to test flasks. Both the test and the control flasks showed a similar pattern and no significant differences were observed between the pH of the test and the control cultures (P>0.05).

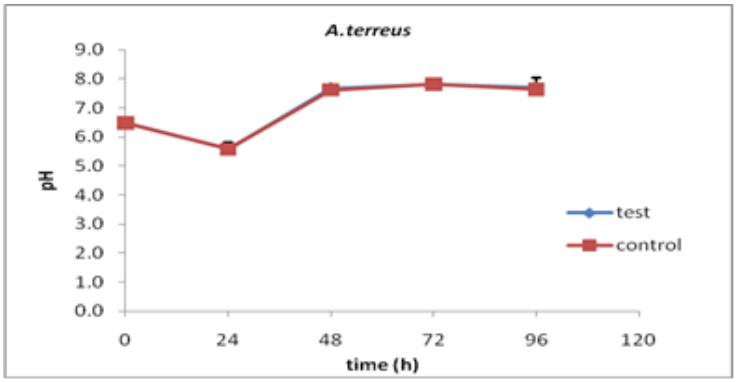


Figure 8.2
pH profile of the samples taken from the test and the control cultures of *A. terreus* in complex medium. Butyrolactone I was added at 24h post inoculation time. No significant difference was observed between the test and the control samples (P>0.05). Standard error of the mean of triplicate is represented by error bars

8.2 Butyrolactone I production in clinical strains of *A.fumigatus*

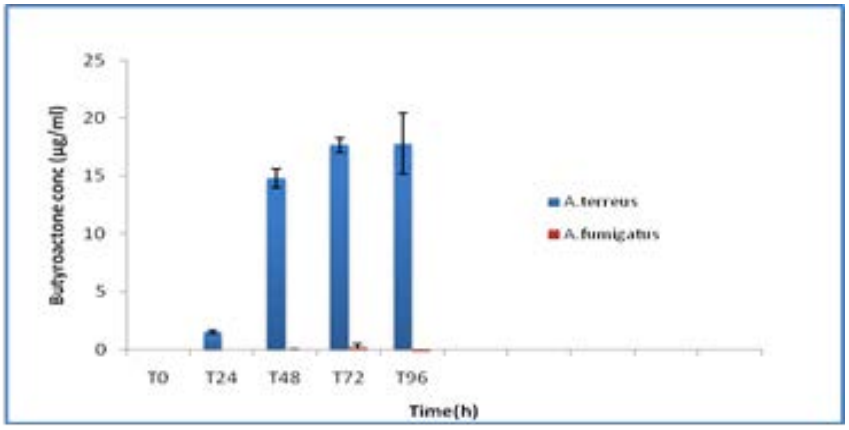


Figure 8.3
comparison between the level of butyrolactone I production between the clinical isolates of *A. terreus* and *A. fumigatus* cultures in complex medium. *A. fumigatus* shows a much lower level of butyrolactone production that starts after 24 hours post inoculation and reaches the highest level (0.4µg/ml) at 72 hours post inoculation. There is a significant different between the level of butyrolactone production between the two strains (P<0.05).

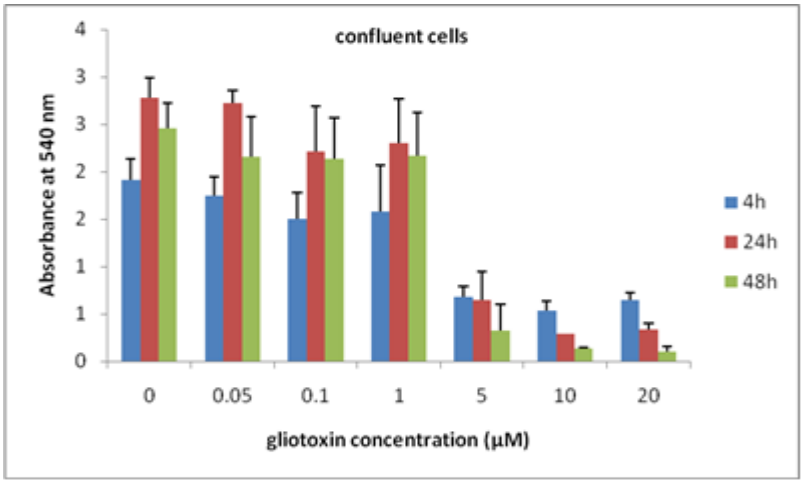


Figure 8.4
Effect of gliotoxin on the concentration of the A549 cells after 4h, 24h and 48h exposure. Cell concentration was measured by the measuring the absorbance at 540 nm. Up to 5µM gliotoxin concentration there is no statistically significant difference (P>0.05) between the control (0 concentration of toxin) and the tests wells whereas from 5µM gliotoxin concentration a significant decrease in the cell concentration observed in all three exposure time points (P<0.05). Error bars indicate the standard error of the mean between the two independent experiments.

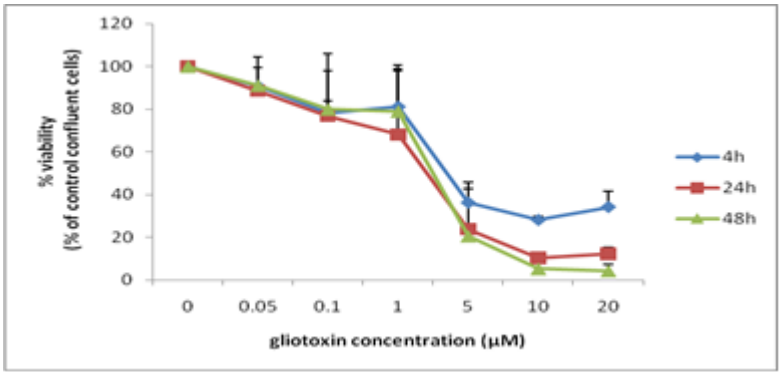


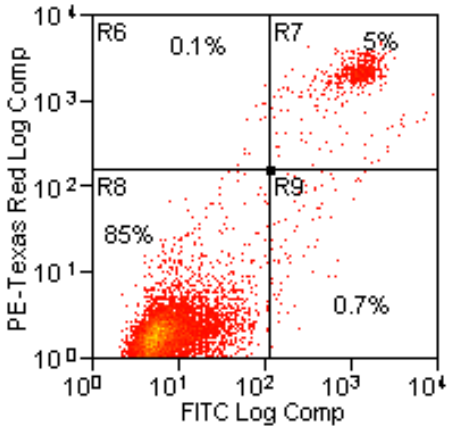
Figure 8.5
Response of A549 cells to gliotoxin quantified as metabolic activity after 4, 24 and 48 hour exposure to the toxin. Error bars indicate the standard error of the mean between the two independent experiments.

8.3 Flowcytometry

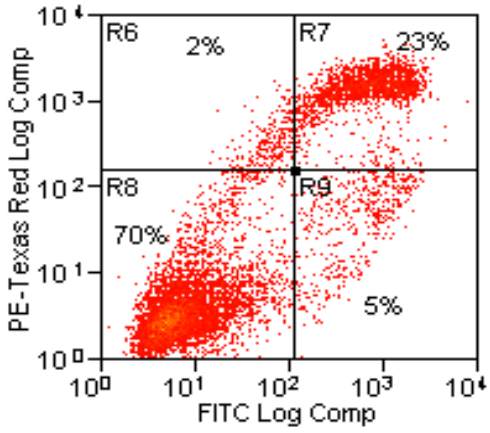
8.3.1.1 Flow-cytometric analysis of the effect of sterile filtered culture broth of A. fumigatus strains on A549 cells

After 4 h treatment

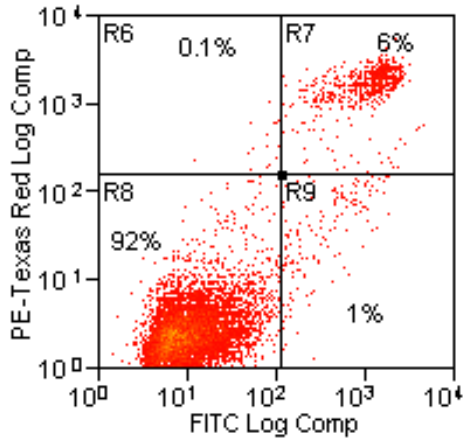
A- Control population producer)



B- AF -10 strain (low gliotoxin



C-ARC2 (*gliP* knockout strain)



D- AF-293 (high gliotoxin producer)

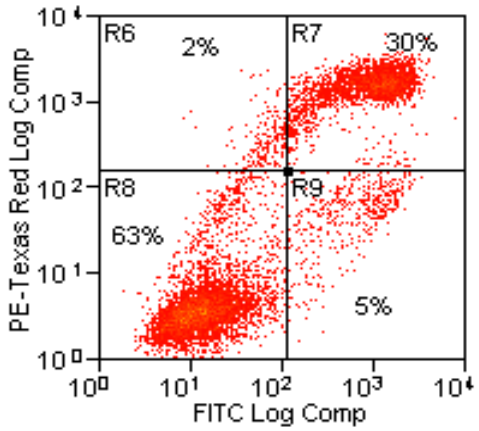
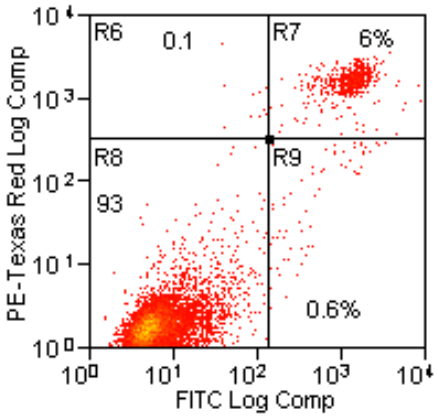


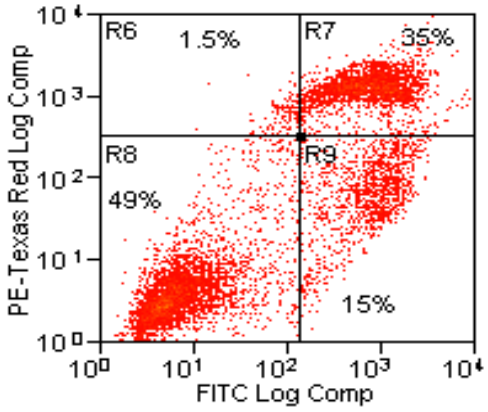
Figure 8.6
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of 48h old culture of *A. fumigatus* AF-10, AF-293 and ARC2 strains in RPMI medium after 4h treatment. Section (A) represents the control A549 cells without any treatment; (B) represents A549 cells treated with 40% culture broth from *A. fumigatus* AF-10 strain (wild type); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus gliP* knockout strain ARC2 (mutant-not able to produce gliotoxin); (D) representing A549 cells treated with 40% culture broth of *A. fumigatus* AF-293 strain (wild type). Results represent at least three repeated experiments.

After 8 h treatment

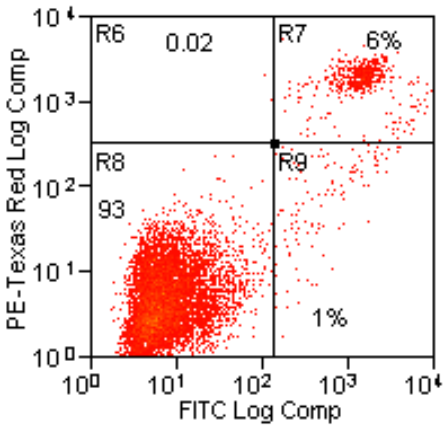
A-Control population



B- AF -10 strain (low gliotoxin producer)



C-ARC2 (*gliP* knockout strain)



D- AF-293 (high gliotoxin producer)

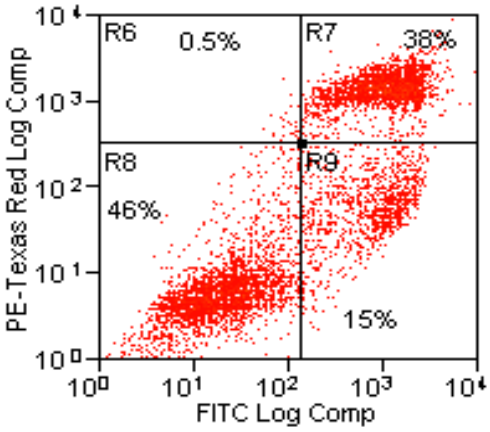
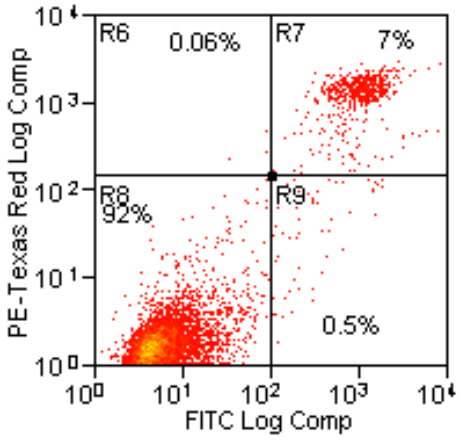


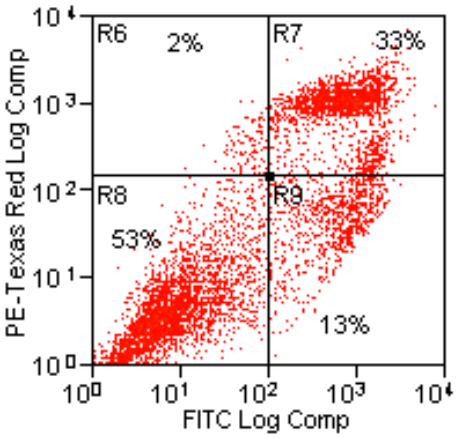
Figure 8.7
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of 48h old culture of *A. fumigatus* AF-10, AF-293 and ARC2 strains in RPMI medium after 8h treatment. Section (A) represents the control A549 cells without any treatment; (B) represents A549 cells treated with 40% culture broth from *A. fumigatus* AF-10 strain (wild type); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus gliP* knockout strain ARC2 (mutant-not able to produce gliotoxin); (D) representing A549 cells treated with 40% culture broth of *A. fumigatus* AF-293 strain (wild type). Results represent at least three repeated experiments

After 12 h treatment

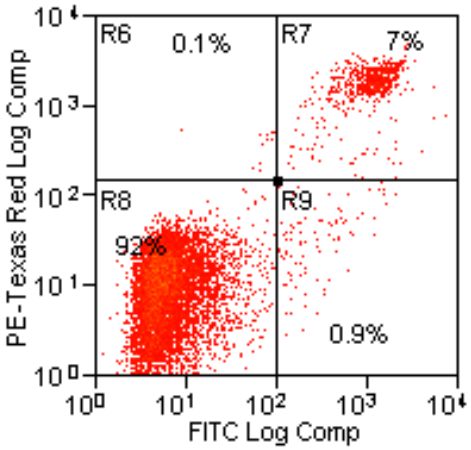
A-Control population



B- AF -10 strain (low gliotoxin producer)



C-ARC2 (*gliP* knockout strain)



D- AF-293 (high gliotoxin producer)

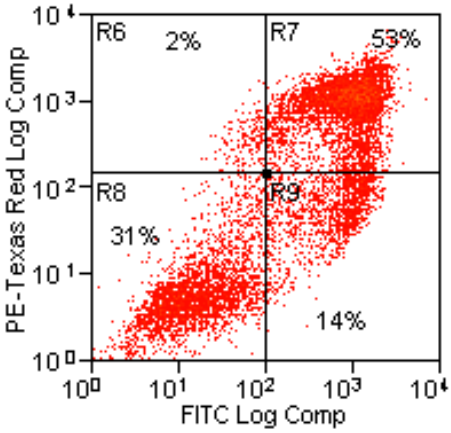
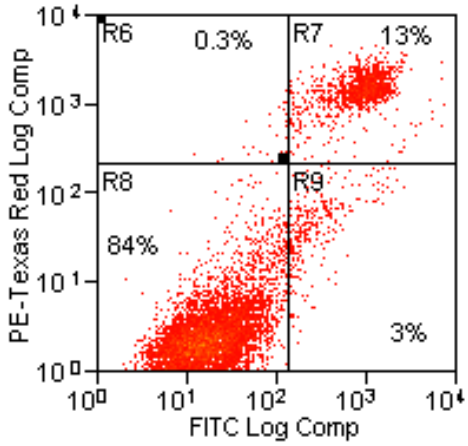


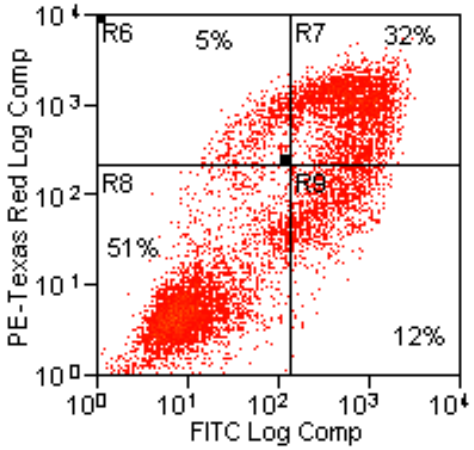
Figure 8.8
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of 48h old culture of *A. fumigatus* AF-10, AF-293 and ARC2 strains in RPMI medium after 12h treatment. Section (A) represents the control A549 cells without any treatment; (B) represents A549 cells treated with 40% culture broth from *A. fumigatus* AF-10 strain (wild type); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus gliP* knockout strain ARC2 (mutant-not able to produce gliotoxin); (D) representing A549 cells treated with 40% culture broth of *A. fumigatus* AF-293 strain (wild type). Results represent at least three repeated experiments.

After 24 h treatment

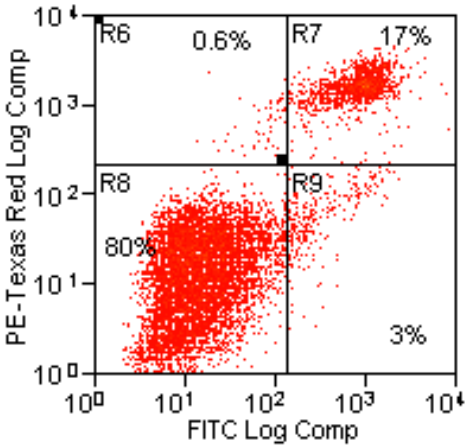
A-Control population



B- AF -10 strain (low gliotoxin producer)



C-ARC2 (*gliP* knockout strain)



D- AF-293 (high gliotoxin producer)

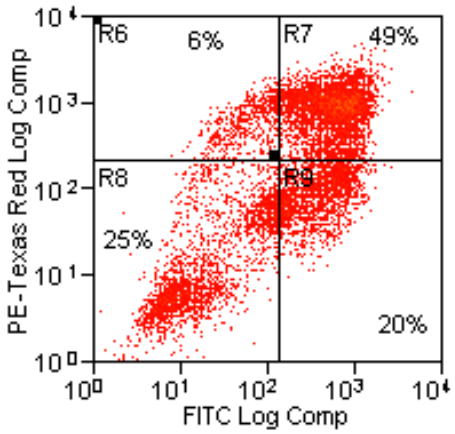
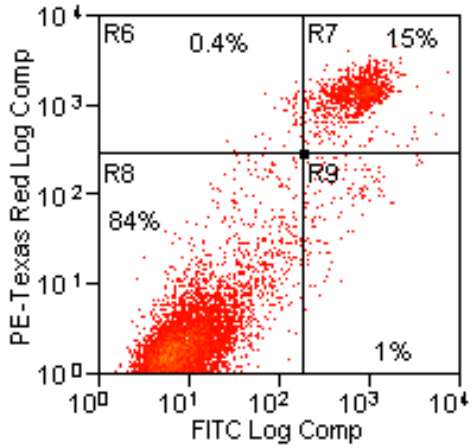


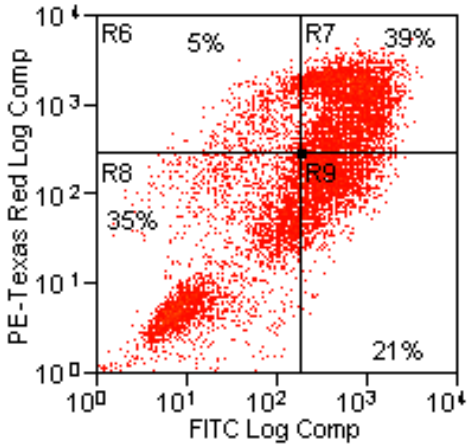
Figure 8.9
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of 48h old culture of *A. fumigatus* AF-10, AF-293 and ARC2 strains in RPMI medium after 24h treatment. Section (A) represents the control A549 cells without any treatment; (B) represents A549 cells treated with 40% culture broth from *A. fumigatus* AF-10 strain (wild type); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus gliP* knockout strain ARC2 (mutant-not able to produce gliotoxin); (D) representing A549 cells treated with 40% culture broth of *A. fumigatus* AF-293 strain (wild type). Results represent at least three repeated experiments.

After 48 h treatment

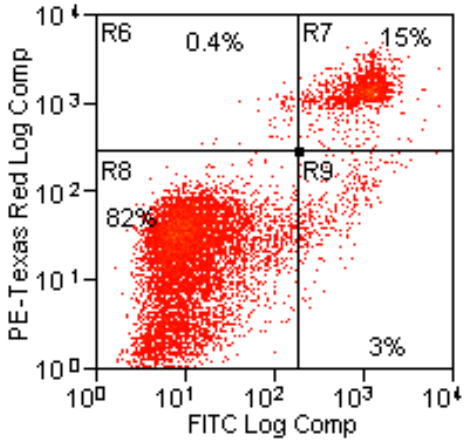
A-Control population



B- AF -10 strain (low gliotoxin producer)



C-ARC2 (*gliP* knockout strain)



D- AF-293 (high gliotoxin producer)

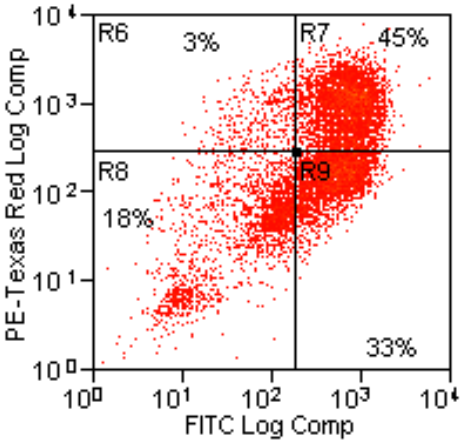


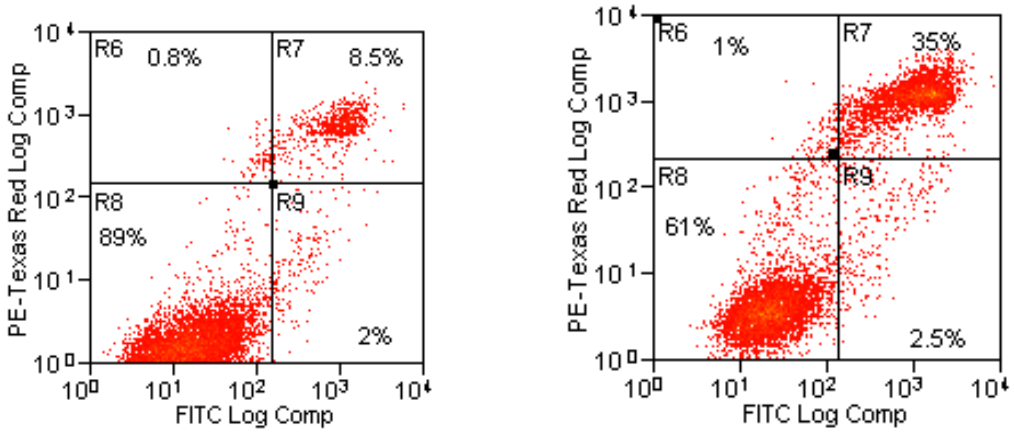
Figure 8.10
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of 48h old culture of *A. fumigatus* AF-10, AF-293 and ARC2 strains in RPMI medium after 48h treatment. Section (A) represents the control A549 cells without any treatment; (B) represents A549 cells treated with 40% culture broth from *A. fumigatus* AF-10 strain (wild type); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus gliP* knockout strain ARC2 (mutant-not able to produce gliotoxin); (D) representing A549 cells treated with 40% culture broth of *A. fumigatus* AF-293 strain (wild type). Results represent at least three repeated experiments.

8.3.1.2 Flow cytometric analysis of the effect of hydrocortisone treated culture broth of A. fumigatus on A549 cells

***Aspergillus fumigatus* AF-293**

After 4 h treatment

A-Control population **B- AF-293 without hydrocortisone treatment**



C-AF-293 with 10^{-6}M hydrocortisone treatment **D- AF-293 with 10^{-5}M hydrocortisone treatment**

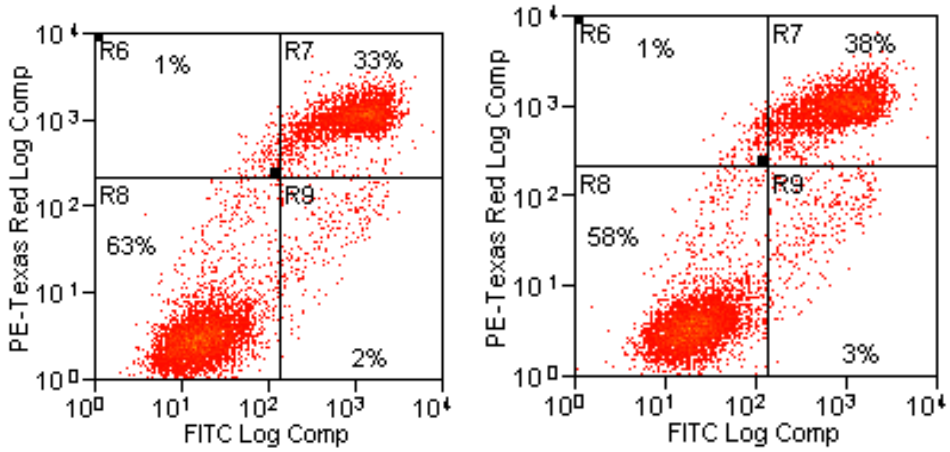
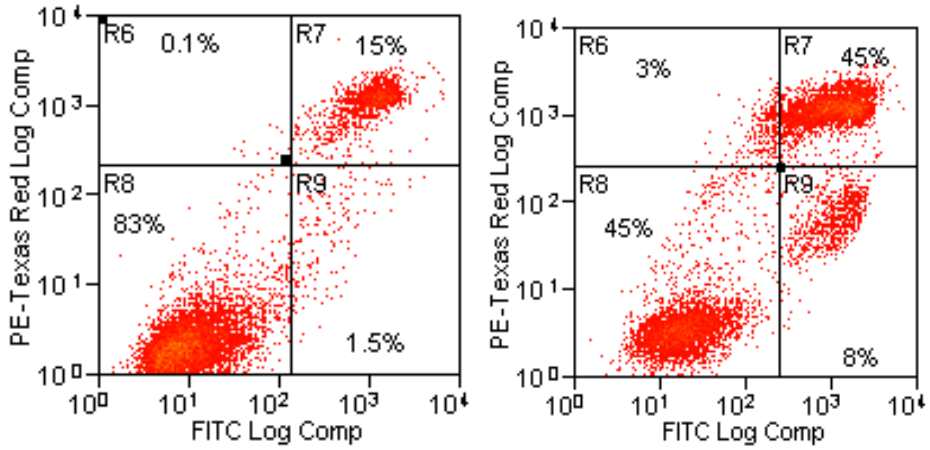


Figure 8.11
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of a 48h old culture of *A. fumigatus* AF-293 strain treated with or without hydrocortisone in RPMI medium after 4h treatment. Section (A) represents the control A549 cells without any treatment with Aspergillus culture broth; (B) represents A549 cells treated with 40% culture broth from *A fumigatus* AF-293 (without any hydrocortisone treatment); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus* AF-293 treated with 10^{-6}M hydrocortisone; (D) representing A549 cells treated with 40% culture broth of *A. fumigatus* AF-293 treated with 10^{-5}M hydrocortisone. Results represent at least two repeated experiments.

After 8 h treatment

A-Control population treatment

B- AF-293 without hydrocortisone



C-AF-293 with 10^{-6}M hydrocortisone treatment

D- AF-293 with 10^{-5}M hydrocortisone treatment

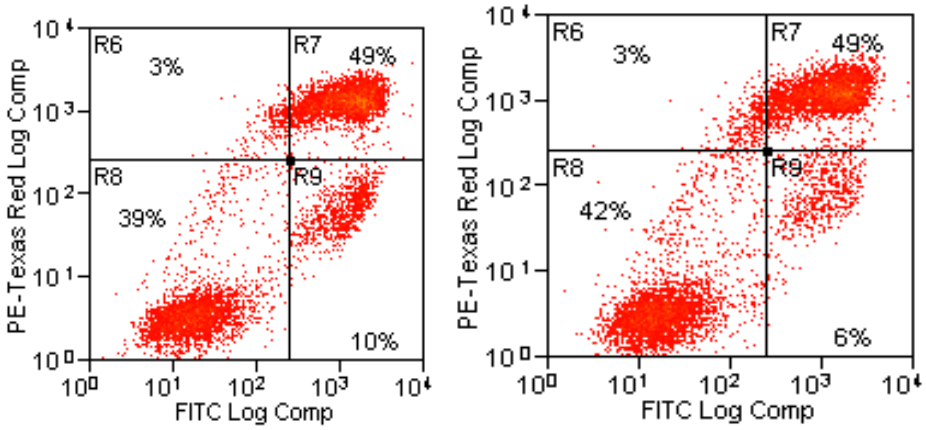
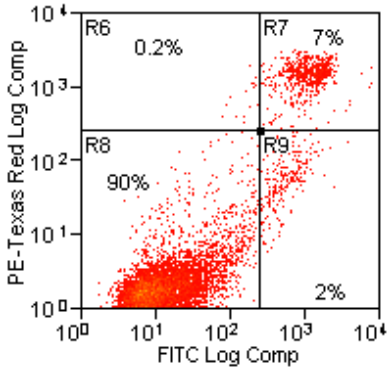


Figure 8.12
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of a 48h old culture of *A. fumigatus* AF-293 strain treated with or without hydrocortisone in RPMI medium after 8h treatment. Section (A) represents the control A549 cells without any treatment with Aspergillus culture broth; (B) represents A549 cells treated with 40% culture broth from *A fumigatus* AF-293 (without any hydrocortisone treatment); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus* AF-293 treated with 10^{-6}M hydrocortisone; (D) representing A549 cells treated with 40% culture broth of *A. fumigatus* AF-293 treated with 10^{-5}M hydrocortisone. Results represent at least two repeated experiments.

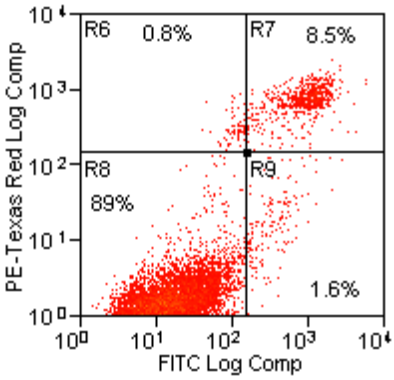
Aspergillus fumigatus ARC2 (*ΔgliP*)

After 4 h treatment

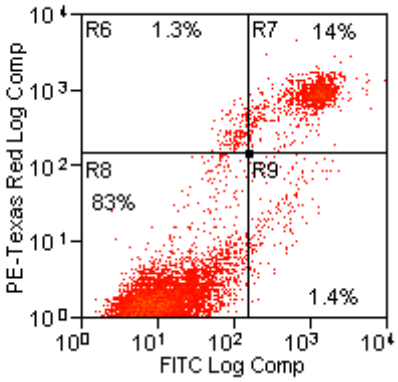
A-Control population



B- ΔgliP without hydrocortisone treatment



C-ΔgliP with 10^{-6}M hydrocortisone treatment



D-ΔgliP with 10^{-5}M hydrocortisone treatment

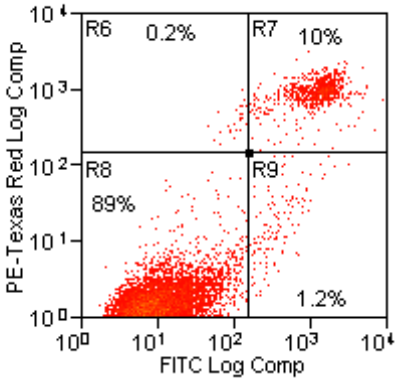


Figure 8.13
Diagrammatic representation of the flow-cytometric analysis of A549 cells showing the effect of treatment of the cells with 40% culture broth of 48h old culture of *A. fumigatus* ARC2 strain treated with or without hydrocortisone in RPMI medium after 4h treatment. Section (A) represents the control A549 cells without any treatment with Aspergillus culture broth; (B) represents A549 cells treated with 40% culture broth from *A fumigatus* ARC2 (without any hydrocortisone treatment); (C) shows A549 cells treated with 40% culture broth from *A. fumigatus gliP* knockout strain ARC2 treated with 10^{-6}M hydrocortisone; (D) representing A549 cells treated with 40% culture broth of *A. fumigatus* ARC2 treated with 10^{-5}M hydrocortisone. Results represent at least two repeated experiments.