

Transcriptomic and Proteomic Profile of *Aspergillus fumigatus* on Exposure to Artemisinin

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Abstract Artemisinin, an antimalarial drug, and its derivatives are reported to have antifungal activity against some fungi. We report its antifungal activity against *Aspergillus fumigatus* (*A. fumigatus*), a pathogenic filamentous fungus responsible for allergic and invasive aspergillosis in humans, and its synergistic effect in combination with itraconazole (ITC), an available antifungal drug. In order to identify its molecular targets, we further analyzed transcript and proteomic profiles of the fungus on exposure to the artemisinin. In transcriptomic analysis, a total of 745

genes were observed to be modulated on exposure to artemisinin, and some of them were confirmed by real-time polymerase chain reaction analysis. Proteomic profiles of *A. fumigatus* treated with artemisinin showed modulation of 175 proteins (66 upregulated and 109 downregulated) as compared to the control. Peptide mass fingerprinting led to the identification of 85 proteins—29 upregulated and 56 downregulated, 65 of which were unique proteins. Consistent with earlier reports of molecular mechanisms of artemisinin and that of other antifungal drugs, we believe that oxidative phosphorylation pathway (64 kDa mitochondrial NADH dehydrogenase), cell wall-associated proteins and enzymes (conidial hydrophobin B protein, cell wall phiA protein, extracellular thaumatin domain protein, 1,3-beta-glucanotransferase Gel2) and genes involved in ergosterol

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biosynthesis (ERG6 and coproporphyrinogen III oxidase, HEM13) are potential targets of artemisinin for further investigations.

Keywords *Aspergillus fumigatus* · Artemisinin · Microarray · Proteomics

Introduction

Aspergillus fumigatus (*A. fumigatus*) is a human pathogen causing allergic or invasive aspergillosis depending upon the immune status of the host. Invasive aspergillosis, caused primarily by *A. fumigatus*, has emerged as the leading cause of mortality among immunocompromised patients with underlying hematological diseases or bone marrow transplantation [1, 2]. The therapeutic outcome of the available antifungal drugs belonging to polyenes, azoles or echinocandins or their derivatives is limited by their toxicity (nephrotoxicity, cytotoxicity and hepatotoxicity, etc.) in the host or resistance developed in the pathogen [3, 4]. Hence, new antifungals are needed for treatment of invasive aspergillosis.

Artemisinin is a standard antimalarial drug, however, it has also been reported to have antifungal activity against *A. flavus*, *A. niger*, *C. albicans* and *C. neoformans* [5–7]. The mechanism of action of artemisinin for its antimalarial activity is partly understood, whereas the specific mechanism of its antifungal action has not been studied.

Artemisinin is reported to inhibit hemoglobin degradation, by acting on proteases in the food vacuole of the parasite, and heme polymerization which causes accumulation of heme as hemozoin, the product of heme polymerization. In addition, the endoperoxide bridge of artemisinin initiates breakdown of the malarial pigment, hemozoin. Heme-catalyzed cleavage of endoperoxide bridge forms free oxygen radicals followed by specific and selective alkylation of some malarial proteins leading to death of the parasite [8–16]. Eckstein-Ludwig et al. [17] suggested that artemisinin acts more specifically by irreversibly inhibiting a critical parasite enzyme, PfATP6 (*Plasmodium falciparum* endoplasmic reticulum Ca^{2+} -dependent ATPase). Li et al. [18] have demonstrated that active artemisinin locally depolarizes the mitochondrial membrane in yeast model. Recently, Wang et al. [19] reported that the artemisinin and its

homologs exhibit correlated activities against yeast and malaria but not against mammalian mitochondria, with peroxide playing a key role for the inhibitory action in both organisms.

The present antifungal drugs have a serious limitation as most of them target cell wall or ergosterol biosynthesis pathway, and drug resistance develops fast even if they are used in combination. From the studies in yeast and *Plasmodium*, it has been inferred that artemisinin targets oxidative phosphorylation [18, 19]. Hence, artemisinin, if targeting pathways different than the available antifungals, may be synergistic in combination with the available antifungal drugs, leading to improved efficacy and slower development of resistance. With this hypothesis in focus, we studied the interaction of artemisinin with an available antifungal drug, itraconazole (ITC) using in vitro assay, and explored the mechanism of its antifungal action against *A. fumigatus* using transcriptomic and proteomic approach.

Materials and Methods

Aspergillus fumigatus Strain

Aspergillus fumigatus strain Af293, isolated from the lungs of an invasive aspergillosis patient, was kindly provided by D. W. Denning (School of Medicine, University of Manchester, Manchester, United Kingdom). The Af293 strain was used for a whole-genome sequencing project by the Sanger Institute (Hinxton, Cambridge, United Kingdom) and the J. Craig Venter Institute (Rockville, MD).

Spore Harvesting

Aspergillus fumigatus culture was maintained on Sabouraud dextrose agar (SDA) (4.7 g/l; Himedia, Mumbai, India) slants, and spores were harvested using a method described previously [20].

Determination of MICs of Artemisinin and Itraconazole

Aspergillus fumigatus spores (1×10^6 spores/ml) were incubated in RPMI 1640 medium (with L-glutamine and sodium bicarbonate), pH 7.4 (Sigma, St. Louis, MO), with solvent control, dimethyl

sulfoxide (DMSO) (Sigma, Saint Louis, Missouri, USA) or artemisinin (50–250 µg/ml) or itraconazole (ITC) (0.032–0.5 µg/ml) (Sigma, Saint Louis, Missouri, USA) at 37°C for 24 h in a radiation-sterilized 96-well flat-bottom microtiter plate (Tarsons, Kolkata, India) [21]. MIC₉₀ and MIC₅₀ were determined as described earlier [20]. The experiment was performed thrice, and the mean MIC₉₀ and MIC₅₀ value was considered.

Interaction of Artemisinin and ITC In Vitro

Drug interactions were assessed by broth microdilution method that employs the dye MTT. *A. fumigatus* spores (1×10^6 spores/ml) were incubated in RPMI 1640 medium (with L-glutamine and sodium bicarbonate), pH 7.4 (Sigma, St. Louis, MO) with DMSO or various concentrations of ITC (0.032–0.5 µg/ml) along with artemisinin (31.25–250 µg/ml) at 37°C for 24 h in a radiation-sterilized 96-well flat-bottom microtiter plate. An MTT assay was performed to study the inhibition of *A. fumigatus* growth [21]. Growth percentages were calculated by dividing average OD of each well by average OD of solvent control well and the percentages span a range of 0–100%. The combination effect was expressed as fractional inhibitory concentration (FIC) index. A FIC index of <1 indicates synergy, and FIC index between 1 and 2 indicates additive effect [22]. MIC₉₀ for artemisinin and MIC₉₅ for ITC were considered to calculate FIC.

Large-Scale Culture for Total RNA and Protein Extraction

For transcriptomic, proteomic and real-time PCR analysis, the fungal spores were cultured for 48 h (late exponential phase) and then treated with solvent control or artemisinin for 3 h. The fungal culture thus analyzed was homogenous containing mycelia. Culture flasks containing 100 ml of RPMI 1640 medium (with L-glutamine and sodium bicarbonate) (Sigma, St. Louis, MO) were inoculated with *A. fumigatus* spores (1×10^6 /ml) and incubated at 37°C for 48 h. Solvent control or artemisinin at MIC₅₀ concentration (as determined above) was added to separate flasks and incubated at 37°C for 3 h. *A. fumigatus* was scraped from the culture flasks and pelleted at 4°C by centrifugation at 18,000 rpm for 10 min. The pellets

were quick chilled with liquid nitrogen and kept at –70°C. Transcriptomic, proteomic and real-time PCR analysis were carried out with two biological replicates obtained from the above-mentioned experiments.

Microarray Analysis

Isolation of Total RNA from A. fumigatus

DNA-free total RNA was isolated from *A. fumigatus* by using RNeasy plant minikits (Qiagen, GmbH, Hilden, Germany) as per the protocol described previously [20].

Fluorescence Labeling

Total RNA (7 µg) from *A. fumigatus* treated with solvent control or artemisinin was reverse transcribed separately using fluorescein-labeled dCTP or biotin-labeled dCTP from Micromax TSA labeling kits (Perkin Elmer, Wellesley, MA). Labeled cDNA was purified by isopropanol precipitation as per the protocol of the Micromax TSA labeling kit. Each of the purified labeled cDNAs was dissolved in 20 µl of hybridization buffer [50% formamide, 5× sodium citrate-sodium chloride (SSC) containing 0.75 M NaCl plus 0.075 M sodium citrate, 0.1% SDS, and 300 µg salmon sperm DNA]. Purified labeled cDNA probes were mixed for hybridization process. A dye swap experiment with two biological replicates was performed to ensure the reproducibility of each gene expression pattern.

Prehybridization, Hybridization and Scanning of DNA Slides

The whole-genome *A. fumigatus* microarray slides (version 2) were obtained from the J. Craig Venter Institute (Rockville, MD). Each of the slides was prehybridized and then subjected to a hybridization process with labeled cDNA probe mix (<http://pfgrc.jcvi.org/index.php/microarray/protocols.html>). After hybridization, the slides were processed as per the protocol of the Micromax TSA labeling kit. Briefly, fluorescein or biotin-labeled cDNA is sequentially detected with anti-fluorescein HRP antibody enzyme conjugate that catalyzes Cy3 tyramide and then with streptavidin HRP that catalyzes Cy5 tyramide amplification. The slide is

scanned in both the Cy3 and the Cy5 channels with a model 4200 Axon Instruments scanner with a 10- μ m resolution. Each of the signals was converted into a resolution of 16 bits per pixel.

Image Analysis

TIFF images were analyzed using GenePix Pro 6.0 software. The signal intensity value of each spot was reduced by the local background level for each spot. Diagnosis and normalization of microarray data (DNMAD) program were used for print-tip loess normalization of microarray data [23]. *M* values obtained from this program were converted to fold modulation. Genes having less than three spots in two dye swap slides were discarded and only those having their fold change values (from three or four replicates in two slides) within a coefficient of variation ± 0.5 were used for analysis. Gene expression ratios of $\geq$ twofold change in both replicates (dye swap) were considered to be differentially expressed genes. The function and functional category of each differentially expressed gene on exposure to artemisinin were assigned from the ExpASY database for biochemical pathways (<http://www.expasy.ch/tools/pathways/>).

Real-Time PCRs

Total RNA was extracted from *A. fumigatus* with solvent control or artemisinin as described above. cDNA was synthesized using total RNA after treatment with RNase-free DNase, using the Super Script III first-strand synthesis system for reverse transcription-PCR (Invitrogen, CA). Primers were designed using AmpliX version 1.4.0 software, with a preference for binding of at least one primer of a pair to the exon–exon junction, to amplify amplicons of approximately 100–150 bp for the respective genes (Table S1 in Supplemental material). The specificity of primers was examined by analyzing their cDNA amplification (PCR) product with agarose gel electrophoresis and later by analyzing the dissociation curve in the real-time RT-PCR. Real-time RT-PCRs were performed using the ABI 7900 HT Fast real-time PCR system (Perkin-Elmer Applied Biosystems). Sybr green ER quantitative PCR supermix (Invitrogen, CA) was used to perform real-time PCRs to study relative changes in mRNA expression profile. In the pilot experiments with real-time

RT-PCR for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin, and 18S rRNA genes in *A. fumigatus* treated with solvent control or artemisinin, 18S rRNA showed insignificant difference in expression and hence was included as a control. The results obtained for the target genes were normalized using the threshold cycles (CTs) obtained for the 18S rRNA (control gene) cDNA amplifications run on the same plate by using the $\Delta\Delta$ CT method [24]. The RT-PCR thermal cycling conditions consisted of an initial step at 50°C for 2 min, followed by 95°C for 10 min. The next stage involved 40 cycles as follows: 95°C for 15 s, 58°C for 30 s and 72°C for 30 s, followed by a dissociation step [25]. In all experiments, appropriate negative controls containing no template DNA or containing RNA were subjected to the same procedure to exclude or detect any possible contamination or carryover. The experiment was carried out with two biological replicates (performed in three technical replicates), and the average $\Delta\Delta$ CT values and standard deviations for the two biological replicates were calculated.

Nucleotide Sequence Accession Number

The microarray data have been made available in the public domain under GenBank accession number **GSE21297** (<http://www.ncbi.nlm.nih.gov/geo>).

Proteomic Analysis

Protein Extraction

Protein was extracted from *A. fumigatus* treated with solvent control or artemisinin (at MIC₅₀) using a method described earlier [20]. Protein was dissolved in rehydration buffer; 8 M Urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 25 mM DTT and estimated by Bradford's method [26].

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)

2-DE of proteins extracted from *A. fumigatus* was performed as described by Görg A et al. [27]. Proteins (600 μ g) solubilized in 300 μ l rehydration buffer with 0.2% bio-lyte with pI range 5–8 and 0.002% bromophenol blue) were used to perform

2-DE as described earlier [20]. Proteins were visualized by Coomassie Brilliant Blue R-250 staining (Sigma, Saint Louis, Missouri, USA).

Gel Image and Image Analysis

Images of Coomassie-stained 2-D gels were acquired using Fluor-S MultiImager (Bio-Rad, Hercules, California, USA) using a visible light source, and image analysis was carried out using PDQuest Image Analysis Software version 8.0. All images were taken under uniform settings, and three or four major spots in different parts of the gel were used for fixing the coordinates. 2-D gels were normalized for small variations in staining or protein loads by determining the total optical densities of the protein spots. The consensus protein spots present in 2-D gel replicates with spot intensity variations of 10% were considered for further analysis. The pooled spot intensities from the replicates were considered for determination of differentially expressed proteins (twofold up- or downregulated) [20]. These protein spots were then excised from the 2-D gel and subjected to trypsin digestion.

In Situ Tryptic Digestion of Proteins

Proteins spots of interest were excised from the gel, and in situ tryptic digestion was carried out as described earlier [28]. In the final step, the lyophilized tryptic digestion product was reconstituted in 4–5 μ l of 50% ACN/0.1% TFA to dissolve the extracted peptides for MS analysis.

Mass Spectrometric Analysis

Tryptic digests were analyzed with a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA) to acquire peptide mass fingerprint (PMF). Then, PMF data were interrogated for protein identification with NCBI database using Mascot search engine and analysis was done on global proteomic solutions (GPS) software version 3.6 (Applied Biosystems, Framingham, MA) automatically. For confirmation of protein identification by PMF, the peptides were further subjected to fragmentation by MS/MS in result-dependent analysis (RDA) mode. The resulting MS/MS ion spectra are again interrogated with NCBI

database (*A. fumigatus*) for confirmation of the protein IDs. The search parameters for MS and MS/MS analysis have been described previously [20]. Function and functional category of each differentially expressed protein on exposure to artemisinin were assigned from ExPASy database for biochemical pathways (<http://www.expasy.ch/tools/pathways/>).

Results

MIC₅₀ and MIC₉₀ of artemisinin against *A. fumigatus* strain Af293 in RPMI 1640, pH 7.4, after 24 h at 37°C, were determined to be 125 and 250 μ g/ml, respectively, whereas MIC₅₀ and MIC₉₅ of itraconazole were 0.154 and 0.5 μ g/ml respectively, (Fig. 1). When performed an in vitro study on antifungal action of artemisinin in combination with ITC, a standard antifungal drug, it showed a synergistic effect against *A. fumigatus* (Table 1).

In order to understand the molecular effects of artemisinin against *A. fumigatus*, the transcriptomic and proteomic profile of *A. fumigatus* was studied in 48 h grown fungal spores exposed to artemisinin for 3 h at MIC₅₀ concentration (see methods).

Microarray Analysis

From two independent dyes swap experiments (see “Materials and methods”), differentially expressed genes appearing in both the channels were considered for analysis and clustering. A total of 745 genes of *A. fumigatus* were differentially expressed on exposure to artemisinin. Of these, 352 genes were observed to be upregulated and 393 downregulated (Table S2 in supplemental material). Hypothetical proteins represented the largest group of genes ($n = 192$; 95 upregulated and 97 downregulated). Other differentially expressed genes were classified into groups such as those involving translation, transport proteins, transcription, cell wall and associated proteins, cell stress, lipid/fatty acid/sterol metabolism, carbohydrate metabolism, amino acid metabolism, oxidative phosphorylation, allergens, etc. (Fig. 2a). Some of the genes that may be specific or of special regulatory importance correspond to oxidative phosphorylation, cell wall and associated proteins, lipid/fatty acid/sterol metabolism, transport proteins, cell stress and oxidative

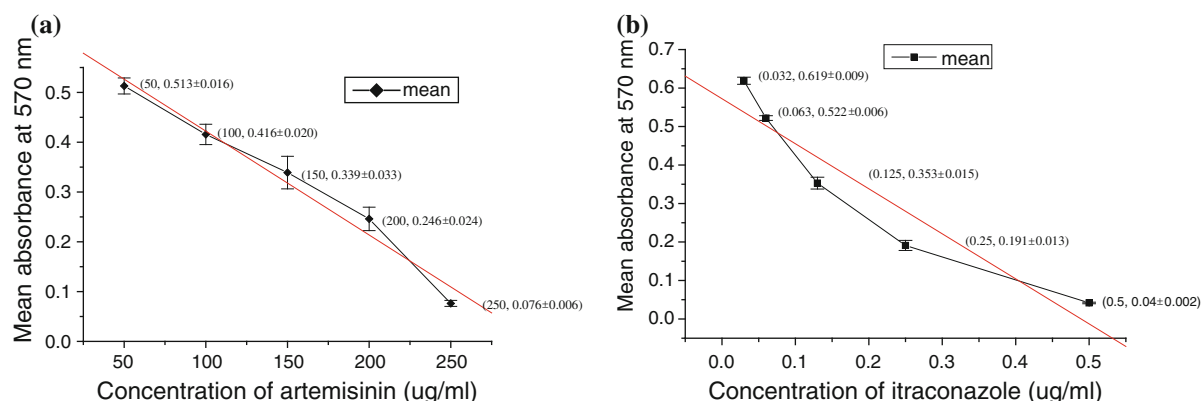


Fig. 1 Determination of MIC of artemisinin and itraconazole for *A. fumigatus*. **a** MIC₅₀ and MIC₉₀ of artemisinin for *A. fumigatus* were determined to be 125 and 250 µg/ml, respectively. The values in the graph represent concentrations of artemisinin and mean absorbance values with standard deviations. **b** The MIC₅₀ and MIC₉₅ of itraconazole (ITC) for *A. fumigatus* were determined to be 0.154 and 0.5 µg/ml,

respectively. Spores (1×10^6 per ml) of *A. fumigatus* were incubated with different concentrations of artemisinin or itraconazole or respective volume of solvent control for 24 h at 37°C in RPMI medium. An MTT assay was performed to study the inhibition of growth of *A. fumigatus* by artemisinin as described in “Materials and Methods”

Table 1 An in vitro study on antifungal action of artemisinin in combination with ITC

Itraconazole (µg/ml) ^a	Artemisinin (µg/ml)				
	0	31.25	62.5	125	250
0.5	5%	5%	0%	0%	0%
0.25	27%	20%	8%	2%	0%
0.125	55%	40%	10%	7%	0%
0.0625	74%	60%	30%	25%	6%
0.03125	88%	85%	65%	44%	10%
0	100%	85%	73%	50%	10%
Combination [MIC alone (µg/ml)]*			MIC (µg/ml) of artemisinin/ITC **		
Artemisinin/ITC ^b			62.5/0.125 [S, 0.5]; 62.5/0.25 [S, 0.75]		
[250/0.5]			125/0.125 [S, 0.75]; 125/0.25 [A, 1]		

* MICs of artemisinin and ITC, respectively, are shown in brackets

** Effective drug combination that caused total growth inhibition together; First number indicates the concentration of artemisinin, and the second is the concentration of ITC. In brackets, the type of interactions (A additive; S synergy) is indicated as inferred from FIC index

^a Schematic representation of results obtained from interaction of artemisinin (31.25–250 µg/ml) in combination with ITC (0.032–0.5 µg/ml) against *A. fumigatus*. The numbers in checkerboard represent growth percentages calculated by dividing average OD of each well divided by average OD of solvent control well. *A. fumigatus* spores (1×10^6 spores/ml) were incubated in RPMI 1640 medium with DMSO or various concentrations of ITC along with artemisinin, as mentioned above, at 37°C for 24 h. An MTT assay was performed to study the inhibition of growth of *A. fumigatus*

^b Artemisinin showed synergistic or additive effect in its interaction with ITC against *A. fumigates* as determined by FIC index

stress-related genes and are listed in Table 2. On the basis of significance of genes from above-mentioned functional classes, eight target genes and a control gene, 18s rRNA gene, were selected for confirmation with real-time PCR. Seven of them showed

expression profiles similar to those observed in the microarray data, reflecting the consistency in microarray and real-time PCR data (Table 3). However, for one of these eight target genes, namely extra-cellular fruiting body protein, we did not observe

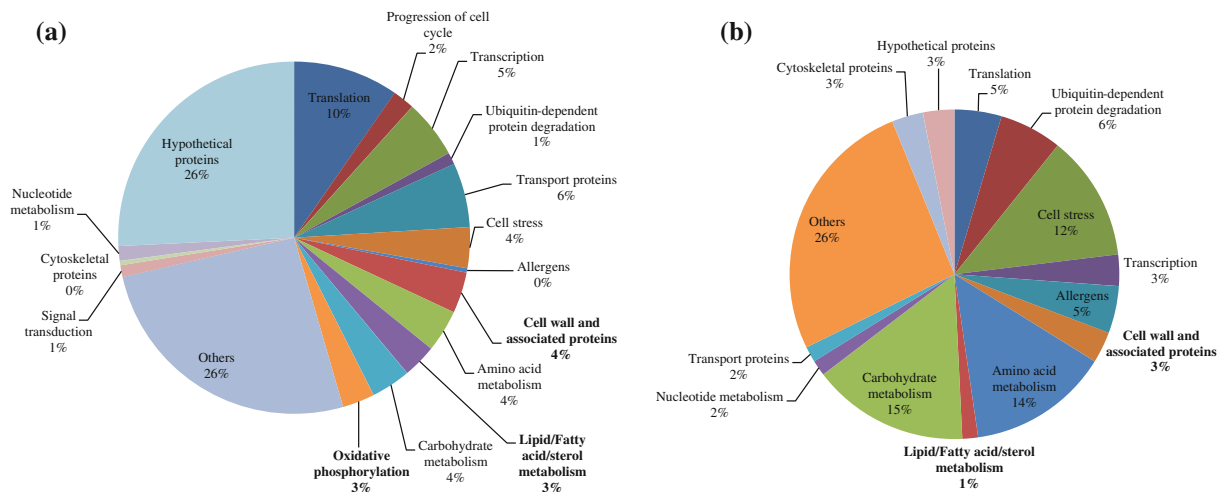


Fig. 2 Pie chart grouping responsive genes ($n = 745$) (a) and unique proteins ($n = 65$) (b) of *A. fumigatus* with 2.0-fold changes or more on exposure to artemisinin. According to the microarray study, 26% of the responsive genes encode hypothetical proteins, 10% of the identified proteins are associated with translation, 6% with transport proteins, 5% with transcription, 4% with cell stress, 4% with cell wall and associated proteins, 4% amino acid metabolism, 4% carbohydrate metabolism, 3% with lipid/fatty acid/sterol metabolism, 3% with oxidative

phosphorylation, 2% with progression of cell cycle, etc. (a). According to the proteomic study, 15% with carbohydrate metabolism, 14% of the identified proteins are associated with amino acid metabolism, 12% with cell stress, 6% with ubiquitin-dependent protein degradation, 5% allergens, 5% with translation, 3% with cell wall and associated proteins and 3% cytoskeletal proteins, etc. (b). Differentially expressed proteins and genes belonging to various functional categories were annotated from the ExPASy database (<http://expasy.org/uniprot>)

significant modulation in expression by real-time PCR.

Proteomic Analysis

We used 2-DE-MS-based proteomics approach for the analysis of proteins from *A. fumigatus* treated with solvent control or artemisinin. The experimental conditions used in protein profiling to study the modulation of proteins induced by artemisinin (125 $\mu\text{g/ml}$, MIC_{50}) allowed access to approximately 500 proteins of the fungus (according to the number of protein spots seen on the gel), of which 175 were differentially expressed (66 were upregulated and 109 were downregulated). Peptide mass fingerprinting led to the identification of 85 proteins—29 upregulated and 56 downregulated proteins, corresponding to 65 unique proteins (Fig. 3). The differentially expressed proteins belong to carbohydrate metabolism, cell stress, amino acid metabolism, translation, ubiquitin-dependent protein degradation, transcription, cytoskeletal proteins, cell wall and associated proteins and others including hypothetical proteins (Fig. 2b; Tables S3 and S4 in supplemental material). The

proteins that may be specific or of special regulatory importance in fungus include cell wall and associated proteins, lipid/fatty acid/sterol metabolism, transport proteins and cell stress-related proteins (Table 2).

Discussion

In the present study, we show the antifungal activity of artemisinin against *A. fumigatus*, a human pathogenic filamentous fungus. MIC_{50} of artemisinin against *A. fumigatus* strain Af293 was determined to be 125 $\mu\text{g/ml}$ (442 nM) (Fig. 1) whereas MIC_{50} of artemisinin against *Candida albicans* has previously been reported to be 8 $\mu\text{g/ml}$ [6]. Artemisinin, being less effective on *A. fumigatus*, may not be a potential antifungal drug alone, but, in view of possibility of a distinct mechanism of action, it may be useful in combination with other antifungal drugs. An in vitro experiment performed to study the antifungal action of artemisinin in combination with ITC showed a synergistic effect (see Table 1) suggesting its potential use in combination with ITC. To identify the pathways targeted by artemisinin, transcriptomic and

Table 2 Integrated microarray and proteomic data showing differential expression levels of genes and proteins of *A. fumigatus* belonging to some of the functional classes on exposure to artemisinin

Gene ID	Gene name	Function	Fold change ^a	
			Microarray data	Proteomic data
<i>Oxidative phosphorylation</i>				
Afu2g05450	64 kDa mitochondrial NADH dehydrogenase	Complex I of oxidative phosphorylation	−3.3	
Afu3g08770	Mitochondrial NADH:ubiquinone oxidoreductase B16.6 subunit	Complex I of oxidative phosphorylation	2.4	
Afu1g06610	Ferredoxin-like iron-sulfur subunit of mitochondrial complex I	Complex I of oxidative phosphorylation	3.7	
Afu2g09130	NADH-ubiquinone dehydrogenase 24 kDa subunit, putative	Complex I of oxidative phosphorylation	2.8	
Afu5g02080	NADH-ubiquinone oxidoreductase	Complex I of oxidative phosphorylation	3	
Afu4g04750	NADH-ubiquinone oxidoreductase 21 kda subunit	Complex I of oxidative phosphorylation	−2.3	
Afu4g11050	NADH-ubiquinone oxidoreductase 51 kDa subunit precursor	Complex I of oxidative phosphorylation	2.1	
Afu5g07380	NADH-ubiquinone oxidoreductase b12 subunit	Complex I of oxidative phosphorylation	3.5	
Afu6g04620	NADH-ubiquinone oxidoreductase B14 subunit, putative	Complex I of oxidative phosphorylation	2.3	
Afu1g12290	Possible NADH-ubiquinone oxidoreductase	Complex I of oxidative phosphorylation	3.6	
Afu5g10370	Succinate dehydrogenase iron-sulfur protein	Complex II of oxidative phosphorylation	6.6	
Afu1g15590	Succinate dehydrogenase membrane anchor subunit, putative	Complex II of oxidative phosphorylation	11	
Afu3g07810	Succinate dehydrogenase, flavoprotein subunit	Complex II of oxidative phosphorylation	2	
Afu1g04540	NADH-cytochrome b5 reductase precursor	Complex III of oxidative phosphorylation	2.3	
Afu4g06790	Ubiquinol-cytochrome c reductase complex 14 kDa protein	Complex III of oxidative phosphorylation	2.6	
Afu4g11390	Ubiquinol-cytochrome c reductase complex 17 kd protein	Complex III of oxidative phosphorylation	3.5	
Afu5g10610	Ubiquinol-cytochrome c reductase iron-sulfur subunit precursor	Complex III of oxidative phosphorylation	2.7	
Afu4g01260	Mitochondrial chaperone ATPase (Bcs1), putative	Chaperone necessary for the assembly of mitochondrial respiratory chain complex III	2.1	
Afu5g02750	Cytochrome c oxidase subunit Va, putative	Complex IV of oxidative phosphorylation	2.5	
Afu6g07670	Cytochrome c oxidase assembly protein cox15	Complex IV of oxidative phosphorylation	3.1	
Afu4g09360	ATP synthase proteolipid P2, putative	Complex V of oxidative phosphorylation	2.8	
Afu2g05060	Alternative oxidase	Alternative oxidase in oxidative phosphorylation	3.4	
<i>Cell wall and associated proteins</i>				
Afu3g01150	GPI anchored cell wall protein, putative	Cell wall protein	5.6	
Afu8g04370	GPI anchored protein, putative	Cell wall protein	2.2	

Table 2 continued

Gene ID	Gene name	Function	Fold change ^a	
			Microarray data	Proteomic data
Afu7g00970	GPI anchored protein, putative	Cell wall protein	−2.3	
Afu3g13360	Glycosylphosphatidylinositol anchor protein homolog	Cell wall protein	−2	
Afu4g08200	GPI transamidase component PIG-U, putative	Essential for transfer of GPI to proteins	2.3	
Afu5g07600	SH2-containing inositol 5-phosphatase 2	Binds filamin and regulates submembraneous actin	2.1	
Afu2g07790	Glycine rich cell wall structural protein, putative	Cell wall protein	−2.4	
Afu1g12350	Extracellular fruiting body protein, putative	G-protein coupled receptor protein signaling, play an important role in the terminal differentiation pathway	−2.4	
Afu3g09690	Extracellular thaumatin domain protein, putative	Cell wall protein	−2.3	
Afu1g17250	Conidial hydrophobin RodB	Cell wall protein	−64.4	2.4, 3.4 (2 isoforms)
Afu2g14420	Cutinase, putative	Degrades cutin	−27.3	
Afu6g02510	Chitin biosynthesis protein (Chs5), putative	Chitin biosynthesis	−3.1	
Afu1g12040	Chitin biosynthesis protein (Chs7), putative	Chitin biosynthesis	−3.3	
Afu4g01290	Endo-chitosanase, pseudogene	Hydrolyzes chitosan	−4.5	
Afu4g08410	Mannose-6-phosphate isomerase, class I	Synthesis of the GDP-mannose and dolichol-phosphate-mannose	−6.1	
Afu6g07620	GDP-mannose pyrophosphorylase A	Involved in GDP-alpha D-mannose; Involved in cell cycle progression	−2.5	
Afu2g05150	Antigenic cell wall galactomannoprotein, putative	Cell wall protein	2.6	
Afu5g03080	Septin	GTP-binding proteins and key players in cytokinesis, surface growth	−9.3	
Afu1g08850	Septin	GTP-binding proteins and key players in cytokinesis, surface growth	−4.7	
Afu5g08540	Septin	GTP-binding proteins and key players in cytokinesis, surface growth	−2.5	
Afu2g14490	Endoglucanase, putative	Cell separation, involved in extending and rearranging 1,3- β -glucan chains, and cross-linking these polymers to other wall components	−4.5	
Afu1g12560	Endo-1,4-beta-glucanase, putative	Glycoside hydrolase	−3.1	
Afu1g16190	Cell wall glucanase Crf1	Cell separation, involved in extending and rearranging 1,3- β -glucan chains, and cross-linking these polymers to other wall components	2.4	
Afu5g08780	Cell wall glucanase, putative	Cell separation, involved in extending and rearranging 1,3- β -glucan chains, and cross-linking these polymers to other wall components	2.1	

Table 2 continued

Gene ID	Gene name	Function	Fold change ^a	
			Microarray data	Proteomic data
Afu6g00550	Glycosyl transferase family 8 family	Biosynthesis of disaccharides, oligosaccharides and polysaccharides	2.4	
Afu1g11460	1,3-beta-glucanosyltransferase Bgt1	Cell wall morphogenesis	3.4	
Afu6g11390	1,3-beta-glucanosyltransferase Gel2	Cell wall morphogenesis	−2.6	
Afu1g14300	Fasciclin domain family	Cell adhesion molecule	4	
gil133920236	Cell wall protein PhiA [<i>Aspergillus fumigatus</i>]	Cell wall protein		−3.4
<i>Lipid/Fatty acid/sterol metabolism</i>				
Afu4g03630	Sterol 24-c-methyltransferase, putative, ERG6	Ergosterol biosynthesis	6.4	
Afu1g03150	c-14 sterol reductase, ERG24	Ergosterol biosynthesis	2	
Afu1g05720	c-14 sterol reductase, ERG24	Ergosterol biosynthesis	−2.3	
Afu8g02440	c-4 methyl sterol oxidase, ERG25	Ergosterol biosynthesis	−3.4	
Afu2g00320	Sterol delta 5,6-desaturase ERG3	Ergosterol biosynthesis	−3.1	
Afu1g07480	Coproporphyrinogen III oxidase, putative, HEM13	Heme biosynthesis	−3	
Afu1g02580	Lipase, putative	Lipid degradation	50.4	
Afu5g02040	Extracellular lipase, putative	Lipid degradation	−2.5	
Afu3g14680	Lysophospholipase Plb3	Lipid degradation	7.7	
Afu4g08720	Lysophospholipase	Lipid degradation	2.4	
Afu2g16520	Phospholipase D (PLD), putative	Lipid degradation	−2	
Afu5g06500	Acyl-CoA dehydrogenase family protein	Fatty acid beta oxidation	2.2	
Afu1g12650	3-ketoacyl-CoA ketothiolase (Kat1), putative	Fatty acid beta oxidation	3.9	
Afu4g10950	3-ketoacyl-coA thiolase peroxisomal A precursor	Fatty acid beta oxidation	3.4	
gil70986952	Electron transfer flavoprotein alpha subunit	Important in beta oxidation of fatty acids and catabolism of amino acids and choline		4.8
Afu6g11210	3-oxoacyl-(acyl-carrier-protein) reductase	Lipid synthesis, fatty acid synthesis	4.2	
Afu1g05870	scs3 protein	Phospholipid biosynthesis	2.2	
Afu6g04240	PAP2 domain protein	Biosynthesis of phospholipids and triacylglycerol	−2.6	
Afu6g07270	fadD6	Fatty acid CoA synthetase	2.4	
Afu5g02760	Fatty acid elongase (Gns1), putative	Fatty acid biosynthesis	3.1	
Afu3g04220	Fatty acid synthase beta subunit, putative	Fatty acid biosynthesis	−2.4	
Afu7g05350	Delta-9 fatty acid desaturase	Fatty acid biosynthesis	−2.3	
Afu2g11990	Pten 3-phosphoinositide phosphatase alpha, putative	Lipid metabolism	2.5	

Table 2 continued

Gene ID	Gene name	Function	Fold change ^a	
			Microarray data	Proteomic data
Afu2g02310	sur7 protein, putative	Involved in sporulation and affects the sphingolipid composition of the plasma membrane	3.5	

The list shows those altered gene and protein expression of *A. fumigatus* which are related to the molecular mechanisms of artemisinin and that of other antifungal drugs. The complete list of genes and proteins with altered expression levels belonging to various functional classes is given in Table S2 (microarray analysis) and S4 (proteomic analysis) in the supplemental material

^a Values shown are ratios for *A. fumigatus* treated with artemisinin for 3 h versus *A. fumigatus* cultured with solvent control for 3 h

Table 3 Comparison of ratios of gene expression for *A. fumigatus* treated with artemisinin and with solvent control obtained after microarray hybridization and real-time RT-PCR

Locus tag	Gene description	Fold increase ^a	
		Microarray hybridization	Real-time PCR
Afu2g05450	64 kDa mitochondrial NADH dehydrogenase	−3.3	−3.9
Afu2g00320	Sterol delta 5,6-desaturase, ERG3	−3.1	−3.8
Afu1g17250	Conidial hydrophobin RodB	−64.0	−12.10
Afu3g09690	Extracellular thaumatin domain protein	−2.3	−3.1
Afu1g12350	Extracellular fruiting body protein, putative	−2.3	−1.5
Afu4g09110	Cytochrome C peroxidase	4.8	7.8
Afu2g17300	Glutathione S transferase	3.3	5.2
Afu2g04060	NADH:flavin oxidoreductase/NADH oxidase family protein	−2.8	−2.6

^a Values shown are ratios for *A. fumigatus* treated with artemisinin for 3 h versus *A. fumigatus* cultured with solvent control for 3 h. Changes in expression levels of target genes are averages for replicates, normalized to 18s rRNA mRNA expression

proteomic profiles of *A. fumigatus* spores cultured for 48 h (late exponential phase) and then treated with artemisinin for 3 h (at 125 µg/ml concentration) were analyzed. Initial studies were performed to study the molecular profile at the proteomic level after 1, 2 or 3 h of incubation with artemisinin (data not shown). Incubation for less than 3 h was not sufficient to note the drug effects, and incubation for more than 3 h might have resulted in more of secondary effects. Also, we did not observe significant inhibition (approximately 6%) of *A. fumigatus* growth (data not shown) at this culture condition, which suggests that the molecular effects observed are due to artemisinin.

In the present study, differential gene expression information was extracted from microarray data with 10,003 genes. Hypergeometric probability performed with the microarray data showed significant over-representation ($P < 0.05$) of the functional classes:

ubiquitin-dependent protein degradation, transport proteins, cell stress, carbohydrate metabolism, translation, progression of cell cycle, amino acid metabolism, lipid/fatty acid/sterol metabolism and signal transduction (Figure F1 in supplemental material). On the other hand, the proteomic data were from a set of about 500 protein spots separated in 2-D gel in the pI range of 5–8, with 175 of them differentially expressed and identified. The difference in the number of genes and proteins did not permit a direct comparison of differentially expressed transcripts with proteins. Nevertheless, some of the genes and proteins affected on artemisinin treatment were found to be common and showed positive correlation in both microarray and proteomic analysis (Table S4 in supplemental material). The profiles observed in the present study possibly include the specific targets of the drug, as some of the effects were similar to the reported effects of antifungal drugs [29–32] or

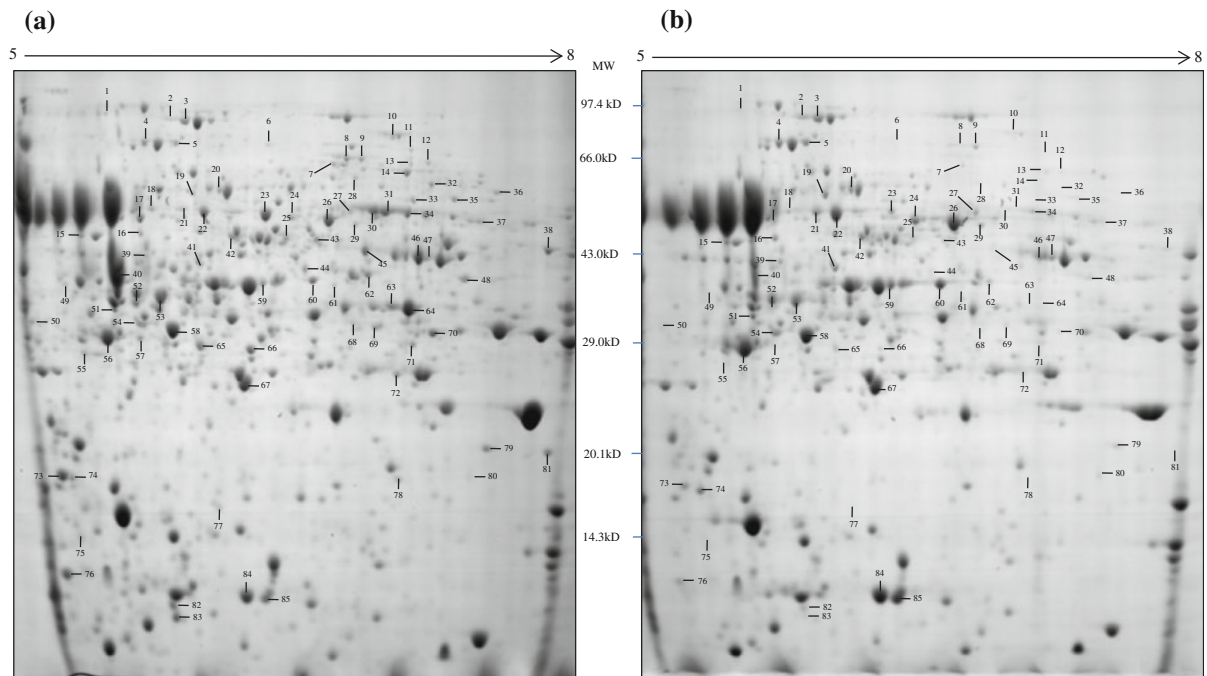


Fig. 3 2-D PAGE of proteins extracted from *A. fumigatus* treated with solvent control **(a)** and treated with artemisinin at 125 µg/ml **(b)**. Proteins (600 µg) were separated by IEF on an IPG strip (17 cm; pI range, 5–8) and SDS–PAGE on 12.0% Laemmli gels and stained with Coomassie blue R 250. 2-D gel images were compared to identify differential expression levels of *A. fumigatus* proteins on exposure to artemisinin, using PDQuest software. Differentially expressed proteins were then

subjected to MALDI-TOF and MALDI-TOF–TOF analysis, which resulted in identification of a total of 85 proteins (marked with arrows). A total of 29 proteins were observed to be upregulated, and 56 proteins were downregulated **(b)**. The functions and functional categories of the differentially expressed proteins identified are given in Table 1 and Table S4 in the supplemental material. MW, molecular mass

artemisinin as an antimalarial [18, 19]. We performed stress response element (STRE) sequence CCCCT analysis in the putative promoter region (1,000 bp region upstream of the transcription start codon) of 32 genes (upregulated fivefold and above on artemisinin treatment). Twenty-four out of them showed presence of STRE sequence, while 8 of these upregulated genes did not have STRE sequence in the putative promoter region (Table S5 in supplemental material). This suggests that genes upregulated in response to artemisinin are due to both a generalized stress response and a specific response to artemisinin. We highlight differential gene and protein expression belonging to those functional classes that are reported in mechanisms of action of artemisinin on *Plasmodium* and yeast (oxidative phosphorylation, cell stress) [8, 18, 19] or that are reported to be altered on exposure to standard antifungal drugs (cell wall and associated proteins, sterol biosynthesis pathway, transport proteins, cell stress) [29–32].

Molecular Effects

Oxidative Phosphorylation Pathway

A previous study showed that NADH dehydrogenase is the direct target of artemisinin, as NADH deletion in yeast provided more resistance to the fungus, whereas overexpression of the same led to more susceptibility to the yeast [18]. It was demonstrated that the inhibitory effect of artemisinin is mediated by disrupting the normal function of mitochondria through depolarizing their membrane potential. Recently, even in malarial parasite, mitochondria have been implicated as specific target of artemisinin [19]. Another study reported the downregulation of a closely related enzyme vacuolar ATP synthase subunit alpha and beta in *Plasmodium* on exposure to artemisinin using proteomic approach [33], which was also observed to be altered similarly in our study. In addition, two of the genes of complex I (64 kDa

mitochondrial NADH dehydrogenase, NADH-ubiquinone oxidoreductase 21 kDa subunit) were observed to be downregulated in microarray data (3.3 and 2.3-fold, respectively) and downregulation of 64 kDa mitochondrial NADH dehydrogenase was further confirmed by real-time PCR (3.9-fold), which is in agreement with the previous reports. However, many of the differentially expressed genes belonging to protein subunits or enzymes of complex I–V of oxidative phosphorylation pathway were observed to be upregulated on exposure to artemisinin. Therefore, we speculate that the artemisinin is targeting primarily 64 kDa mitochondrial NADH dehydrogenase in *A. fumigatus*, which is a specific response of artemisinin against *A. fumigatus*, and in response the fungus overexpresses the other genes belonging to this pathway to re-establish the membrane potential disrupted by artemisinin. Importantly, genes related to oxidative phosphorylation pathway are not observed to be significantly altered in *A. fumigatus*, *S. cerevisiae* and *C. albicans* on exposure to the standard antifungal drugs [20, 29–32, 34].

Cell Wall and Associated Proteins

We have observed the downregulation of most of the differentially expressed structural cell wall proteins (conidial hydrophobin RodB, extracellular thaumatin domain protein, extracellular fruiting body protein, galactomannoprotein, cell wall protein PhiA), the enzymes associated to the biosynthesis of structural cell wall carbohydrates such as 1,3-beta-glucan (1,3-beta-glucanotransferase Gel2) and chitin (chitin biosynthesis protein Chs5 and Chs7), and enzymes associated to hyphal growth such as glucanases (endoglucanase, endo-1,4-beta-glucanase). Cell wall maintenance-related genes are previously reported to be downregulated in *Trichophyton rubrum* on exposure to AMB [34] and *A. fumigatus* on exposure to AMB for 24 h [20].

Conidial hydrophobin RodB, a filamentous fungus-specific cell wall protein presumably present as highly insoluble complexes in the outermost layer of the fungal wall and mainly expressed in spores, was observed to be downregulated in microarray data (approximately 64-fold) and real-time PCR analysis (12-fold). Conidial hydrophobin protein B was earlier observed to be downregulated in response to voriconazole in *A. fumigatus* in microarray analysis [32]

and in response to 24 h exposure of *A. fumigatus* to AMB [20]. The role of RodB is not understood [35, 36]. Our data and the previous report of downregulation of RodB while no significant modulation in expression of RodA in *A. fumigatus* on exposure to voriconazole suggest that RodB, which is expressed in mycelia, might be one of the targets for antifungal action of artemisinin against *A. fumigatus*. The two isoforms of this protein were observed to be upregulated in proteomic data (2.4 and 3.4-fold) in our study; however, the fold change was not as high as in microarray data, suggesting the differential regulation of this gene at mRNA and protein level.

Extracellular thaumatin domain protein, reported to be a cell wall protein and a minor allergen [37], was observed to be downregulated in microarray analysis. Cell wall protein PhiA, a minor allergen [38], was observed to be downregulated in proteomic data. We also observed the downregulation of the enzyme associated to the elongation of the structural cell wall carbohydrate 1,3-beta-glucan such as 1,3-beta-glucanotransferase Gel2, reported to have a role in morphogenesis and virulence in *A. fumigatus* [39, 40]. Endoglucanase and endo-1,4-beta-glucanase enzymes, having a role in hydrolyzing existing cell wall structures, allowing hyphal branching as well as germ tube emergence or the formation of the numerous free reducing and non-reducing ends necessary for the activity of 1,3-beta-glucanotransferase [40], were observed to be downregulated in the present study. The above information suggests that artemisinin is targeting cell wall structural proteins, carbohydrates and associated enzymes.

Ergosterol Biosynthesis Pathway

Modulation of genes of ergosterol biosynthesis pathway observed in the present study is similar to earlier observations in response to various classes of antifungals [20, 32, 34, 41, 42]. Significant upregulation of ERG6 (6.4-fold) and downregulation of ERG3 (3.1-fold) (downstream to ERG6 in the pathway) in microarray analysis suggests that the fungus modulates the ergosterol pathway toward production of secondary sterols. Co-factor heme is required for enzymatic activities of Erg3p (C-5 sterol desaturase), Erg5p (C-22, 23 desaturase) and regulation of expression of Erg11p [43]. One of the enzymes of heme biosynthesis pathway, coproporphyrinogen III

oxidase (HEM13), with a role in sterol synthesis and regulation, has been observed to be downregulated. Since, heme is required for artemisinin action, the lowered production of heme could be a survival strategy of the fungus. Interestingly, upregulation of HEM13 was reported in *S. cerevisiae* in response to azoles [29] and *A. fumigatus* in response to AMB [20].

Other Genes/Proteins

Other genes/proteins with altered expression under the effect of the drug include some of the major allergens of *A. fumigatus* such as Asp f 2 (a fibrinogen binding protein) [44, 45], Asp f 3 (peroxisomal protein) [44] and Asp f 4 (with unknown function) [44], which were observed to be significantly downregulated on exposure to artemisinin in proteomic or microarray study. Translationally controlled tumor protein (TCTP), reported to react with artemisinin in situ and in vitro in the presence of hemin and as well as heme itself in the malarial parasite, *Plasmodium falciparum* [46], was observed to be downregulated (2.4-fold) on exposure to artemisinin in microarray analysis. Domain of unknown function (DUF) domain proteins seem to play important role in fungal response to artemisinin, as a number of DUF domain proteins were found to be downregulated (36.2, 2.8, 2.6, 2.4-fold) (see Table S2) in response to this drug in microarray analysis. We also observed significant upregulation of transport and cell stress proteins suggesting the adaptation of the fungus toward the stress due to artemisinin similar to the earlier observations with other antifungal drugs [47–50]. In addition, a large number of hypothetical proteins were observed to be significantly altered (>four fold) (24 upregulated and 18 downregulated). We do not clearly understand the implications of these alterations but believe that they may suggest yet newer mechanistic effects of the drug against *A. fumigatus*.

In summary, artemisinin targets oxidative phosphorylation pathway in *A. fumigatus* that has not been a target for the antifungal drugs in use, besides targets that are in common with other antifungal drugs such as cell wall and associated proteins/enzymes, ergosterol biosynthesis pathway, transport proteins and cell stress proteins. Interestingly, in vitro assay suggests a synergistic effect of artemisinin in combination with ITC against *A. fumigatus* that needs to be confirmed in vivo.

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