Published in final edited form as:

Fungal Genet Biol. 2011 December ; 48(12): 1130–1138. doi:10.1016/j.fgb.2011.09.004.

MODULATION OF FUNGAL SENSITIVITY TO STAUROSPORINE BY TARGETING PROTEINS IDENTIFIED BY TRANSCRIPTIONAL PROFILING

Andreia S. Fernandes¹, A. Pedro Gonçalves¹, Ana Castro¹, Telma A. Lopes², Rui Gardner², N. Louise Glass³, and Arnaldo Videira^{1,4,*}

¹IBMC-Instituto de Biologia Molecular e Celular

²Instituto Gulbenkian de Ciência

³Department of Plant and Microbial Biology, UC-Berkeley, US

⁴ICBAS-Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Portugal

Abstract

An analysis of the time-dependent genetic response to the death-inducer staurosporine was performed in *Neurospora crassa* by transcriptional profiling. Staurosporine induced two major genes encoding an ABC transporter and a protein with similarity to regulatory subunits of potassium channels. The transcriptional response is dependent on the activity of a novel transcription factor. Deletion mutants in differentially expressed genes displayed altered sensitivity to staurosporine, underscoring significant proteins involved in the response to the drug. A null-mutant of the ABC transporter (*abc3*) is extremely sensitive to staurosporine, accumulates more staurosporine than the wild type strain and is defective in energy-dependent export of the drug, indicating that the ABC3 protein is the first described staurosporine transporter. It was located in the plasma membrane by immunofluorescence microscopy. The combination of inhibitors of ABC transporters or of potassium channels with staurosporine leads to an enhanced activity against *N. crassa* and pathogenic fungi paving the way to the development of more potent and specific antifungals. Our results highlight the general use of transcriptional profiling for the identification of novel proteins involved in cell death and their potential use as drug targets.

Keywords

staurosporine; cell death; transcriptional profiling; ABC transporter; *Neurospora crassa*

1. Introduction

Programmed cell death (PCD) is a process of cell suicide essential for the development and survival of the organism. The process was firstly described in higher eukaryotes, but more recently found to occur in fungi as well (Madeo et al., 1997). Interference with PCD has had

© 2011 Elsevier Inc. All rights reserved.

*Corresponding author. IBMC-Instituto de Biologia Molecular e Celular, Rua Campo Alegre 823, 4150-180 Porto, Portugal. Phone: +351-226074900; Fax: +351-226099157; avideira@ibmc.up.pt.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

increasing relevance in public health issues due to the fact that its stimulation may prove to be fundamental in cancer treatments and in the control of infectious diseases (Ramsdale, 2008; Reed, 2006; Sharon et al., 2009). Thus, an understanding of the complex networks that ultimately lead to cell death is necessary. It is anticipated that modulation of PCD can be achieved by the identification and targeting of key components regulating the process.

PCD can be induced with drugs like staurosporine (STS), an alkaloid from microbial origin firstly described as antibacterial and antifungal (Omura et al., 1977). STS has been widely used as a protein kinase inhibitor or as a PCD-inducing agent (Gescher, 2000). The STS concentration needed to induce PCD (100 nM to 10 μ M) is much higher than the K_i for inhibition of protein kinases ($\sim$ 5 nM), indicating that STS interferes with other cellular functions (Jarvis et al., 1994). Although STS effects on apoptosis have been largely studied, nothing is known so far about its trafficking and transport. Several STS analogues are in advanced clinical trials as anticancer agents (Osman et al., 2010).

The filamentous fungus *Neurospora crassa* has been a valuable research model organism (Davis & Perkins, 2002), particularly following its genome sequencing (Galagan et al., 2003) and targeted disruption (Colot et al., 2006). It undergoes PCD following the addition of external pro-apoptotic drugs (Castro et al., 2010; Castro et al., 2008; Videira et al., 2009) or as a consequence of heterokaryon incompatibility (Glass & Dementhon, 2006). Specific mutants of respiratory chain complex I are particularly sensitive to STS (Castro et al., 2010). In this work, we performed a transcriptomic analysis of *N. crassa* exposed to STS in order to identify novel mechanisms associated with PCD. We further showed that targeting specifically identified proteins by chemical means allows modulation of *N. crassa* sensitivity to STS and this effect could be extended to the pathogenic fungi *Aspergillus fumigatus* and *Candida albicans*. The higher activity of STS when combined with specific protein inhibitors against pathogenic fungi has potential medical implications.

2. Material and Methods

2.1. Strains, growth techniques and chemicals

Wild type *N. crassa* (FGSC 2489), the cell wall-less slime mutant (FGSC 4761), and several deletion strains generated by the Neurospora Genome Project (Dunlap et al., 2007), were obtained from the Fungal Genetics Stock Center (McCluskey, 2003). The mitochondrial complex I nuo mutants have been reviewed (Marques et al., 2005). Standard procedures were employed for growth and handling of *N. crassa*, *Aspergillus fumigatus* ATCC 46645 and *Candida albicans* SC5314 strains (Castro et al., 2010; Davis & de Serres, 1970). *N. crassa* conidial germination was evaluated by optical microscopy. For spot assays, serial 3-fold dilutions of cellular suspensions from all fungi were spotted on agar plates (GFS for *N. crassa* (Davis & de Serres, 1970), Sabouraud for *A. fumigatus* and *C. albicans*) containing drugs, so that the last spot contained $\sim$ 50 cells, and incubated at 26 °C (37°C for *A. fumigatus*). We prepared stock solutions of 10.7 mM STS/DMSO (LC-Laboratories), 50mM Verapamil/ethanol (Sigma-Aldrich), 1 M sodium orthovanadate (Sigma-Aldrich) and 100 mM 4-aminopyridine (Sigma-Aldrich).

2.2. Microarray experiments and data analysis

Closed-circuit designs were employed for microarray comparisons (Fig. 1A), because they are statistically robust and provide a higher resolution in identifying differentially expressed genes than designs that use a universal reference (Townsend, 2003). *N. crassa* conidia obtained from cultures grown for 7 days at 25°C under constant light (Kasuga et al., 2005) were germinated in Vogel's minimal medium (10^7 cells/ml) at 30°C with strong agitation.

STS (12.5 μ M) was added after 5 hours. At different times, mycelium samples were collected by quick filtration, frozen in liquid nitrogen and kept at -70°C.

The isolation of RNA with TRIzol (Invitrogen Life Technologies) and its purification with the RNeasy kit (Qiagen), cDNA synthesis and labeling with either Cy3 or Cy5 dyes (Amersham) and hybridization (Pronto kit, Corning) with gamma amino propyl silane slides printed with 70-mer oligonucleotides, which include the 10526 ORFs predicted in the *Neurospora* genome, have been detailed before (Kasuga et al., 2005; Videira et al., 2009). Each hybridization was duplicated, labeling one cDNA sample with Cy3 and the other with Cy5, and *vice-versa* (di-swap).

The hybridization images were obtained with a GenePix 4000B scanner and the signals were quantified with the GenePix Pro6 software, which automatically flagged low-quality spots. Then, slides were also inspected manually. Spots with a mean fluorescence intensity for at least one of the Cy3 or Cy5 dyes that was greater than the mean background intensity plus three standard deviations were selected for further analysis if less than 0.02% of the pixels were saturated.

Normalized ratio data were analyzed with the Bayesian Analysis of Gene Expression Levels (BAGEL) software in order to calculate a relative expression level and a credible interval for each gene in each sample (Townsend & Hartl, 2002). The genes were associated with functional categories using the FunCat catalog created by MIPS (Ruepp et al., 2004). Microarray data have been deposited at the NCBI gene expression and hybridization array data repository (GEO, <http://www.ncbi.nlm.nih.gov/geo/>). Table S1 in the supplemental material lists mRNA profiling results and functional annotations.

2.3. Quantification of gene expression by real time RT-PCR

RNA was extracted with Illustra RNAspin Mini kit (GE Healthcare) from hyphae grown in the same conditions used for microarray experiments, quantified with the ND 1000 spectrophotometer (Nanodrop) and used to produce cDNA with SuperScript First-Strand Synthesis System kit (Invitrogen). Specific oligonucleotide primers (Table S2), an iCycler iQ5 and SYBR Green Supermix kit (Bio-Rad) were used for qRT-PCR. Triplicate data was obtained in each qRT-PCR experiment and threshold cycle values within an interval of ± 0.5 cycle in the same experiment were accepted. Gene concentration was determined by the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). Relative quantifications employed the NCU04173 gene (actin) as control and the wild type *N. crassa* not exposed to STS as calibrator. Two independent experiments were performed.

2.4. Polyclonal antisera and western blotting

A 905 bp fragment of the 3'-end of the *NCU09975 (abc3)* cDNA (corresponding to the C-terminal 301 aminoacid residues) was amplified by PCR with specific primers (Table S2) from a first strand cDNA obtained as described in the previous section, ligated in the pCRII-TOPO vector (Invitrogen) and cloned in *E. coli* DH5 α . DNA sequencing confirmed the correct sequence. The fragment was then excised with *Bam*HI and *Nde*I, ligated in the pET19b expression vector and cloned in *E. coli* BL21(DE3) (Novagen). The protein was expressed following induction with IPTG, solubilized from cellular inclusion bodies with 8M urea, purified by Ni²⁺-affinity chromatography HisTrap HP (GE Healthcare), precipitated with TCA, quantified with the Bio-Rad Protein Assay kit and used for rat immunization by established procedures at IBMC animal house by following the European Directive 86/609/EEC for animal experimentation and the guidelines on antibody production of the Canadian Council on Animal Care. The collected rat antisera was diluted 1/1000 for

use in western blots of total protein extracts of *N. crassa* resolved by SDS-PAGE in 12 % acrylamide gels (Zauner et al., 1985).

2.5. Immunofluorescence microscopy

The cell wall-less slime mutant was grown for 3 h in SeM medium (Schulte & Scarborough, 1975) from an initial concentration of 5×10^6 cell/ml in the presence or absence of 5 μ M STS. Cells were suspended in PBS, fixed in 2 % paraformaldehyde for 1 h, washed, and permeabilized with 0.1 % Triton X-100, 0.1 % sodium citrate for 2 min. They were sequentially incubated with 0.1 % BSA for 1 h, 1/250 diluted rat antisera (see above) for 16 h, and 1/1000 diluted secondary antibody conjugated with Alexa Fluor 594 (Invitrogen) for 1 h. The slides were mounted in Vecta Shield (Vector Laboratories) and observed under a Laser Scanning Confocal Microscope Leica SP2 AOBS SE.

2.6. Staurosporine determination by flow cytometry and UV-vis absorption spectroscopy

STS absorbs radiation on the UV region of the spectrum and, when excited in this region, it fluoresces on the range 370-410 nm (Iyer et al., 2008). These spectroscopic properties allowed its intra- and extracellular quantification. For the determination of STS concentration in the extracellular medium, and in order to observe variations, the experiments employed 50-fold more concentrated cellular suspensions (2.5×10^8 cells/ml). Conidial cells, previously incubated in liquid media containing STS, were collected by centrifugation. STS concentration in supernatants (extracellular medium) was measured by visible absorption spectroscopy at $A_{372} \text{ minus } A_{395}$ in a Shimadzu UV-160A spectrophotometer and normalized with the absorption of a standard STS solution. The cell pellet was washed with PBS and intracellular STS concentration was measured by flow cytometry as detailed in Fig. S1.

2.7. TUNEL assay

DNA strand breaks were analyzed by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) using the *in situ* cell death detection kit fluorescein (Roche Applied Science), as described previously (Castro et al., 2008). *N. crassa* conidia were incubated in minimal medium in the presence or absence of 12.5 μ M STS for 3 h and washed in PBS. Spheroplasts were prepared (Duarte et al., 1995), and fixation was performed 4.5 h after treatment with the drug. Flow cytometry analysis was performed in a FACSCalibur (BD Biosciences). Twenty-thousand cells per sample were analyzed. Data were acquired with CELLQuest PRO 3.3 (BD Biosciences).

3. Results

3.1. Analysis of gene expression patterns following staurosporine treatment

Staurosporine induces programmed cell death in many organisms, including *N. crassa* (Castro et al., 2010). In order to identify molecular pathways associated with the response to STS, we analyzed gene expression patterns after treatment with the drug during a time-course and utilizing full genome microarrays available for *N. crassa* (Fig. 1A). Conidial cells were germinated in liquid medium during 5 hours and further incubated in the presence of STS for a period of 2 hours. Samples collected at different time points were used to prepare fungal RNA for evaluation of relative expression levels using full genome *N. crassa* microarrays.

In these experiments, we obtained statistical support for expression levels for 4596 genes, representing about half of the fungal genome (Table S1). Genes that displayed no expression under these conditions or that only displayed expression only in some samples were eliminated during BAGEL analyses (Townsend & Hartl, 2002). Individual scrutiny of the

time-course profiles of gene expression patterns revealed 135 genes (~3%) that, under our conditions, display differential expression in the presence or absence of STS. Most genes show increased expression levels in the presence of the drug indicating an active transcriptional response. Assignment of functional categories, as defined by the FunCat catalogue (Ruepp et al., 2004), revealed that this set of affected genes is highly enriched in the “Metabolism” category (~50%). About 1/3 of the genes affected by STS treatment have no attributable function (Table S1). The list of genes differentially expressed point at putative novel mechanisms involved in the drug response.

In this work, we focused on two genes, *NCU09141* and *NCU09975* (*abc3*), which show a higher-fold induction 30-60 min upon STS treatment as compared to basal levels of expression (Fig. 1B). Quantification of gene expression by qRT-PCR confirmed that the expression of both genes is highly induced when *N. crassa* cells are exposed to STS (Fig. 1C). The expression of *NCU07546*, a paralog of *NCU09975* (Fig. S2A) was used as control, and showed similar expression levels in the presence or absence of STS. Because specific mutants of respiratory chain complex I are particularly sensitive to STS (Castro et al., 2010), we tested the expression of *NCU09141* and *NCU09975* in complex I mutants. However, the expression response of *NCU09141* and *NCU09975* in the complex I mutants was similar to that of the wild type strain following STS treatment (Fig. 1C). Western blots of total fungal protein extracts with a rat antiserum against *NCU09975* (ABC3) show undetectable basal levels of the protein, but which is readily induced by treatment of *N. crassa* with STS (Fig. 1D), confirming the results of gene expression experiments. The use of this antiserum with immunofluorescence microscopy showed that the protein distributes at the cell surface in a pattern compatible with a plasma membrane localization (Fig. 1F), thus supporting the assigned function *NCU09975* as a member of the ABC transporter superfamily (see below).

In order to further investigate the involvement of the *NCU09141* and *NCU09975* proteins in mediating the effects of STS, we checked the drug sensitivity of mutant strains carrying deletions in the respective genes. The Δ *NCU09975*, hereafter designated *abc3* mutant, is extremely sensitive to STS in comparison to wild type, as observed by its growth in GFS solid medium, which induces colonial growth (Fig. 2A), and a marked impairment of germination in liquid medium (Fig. S3A), which is also reflected by a higher impairment of cell size increase over time as measured by the forward scatter parameter in flow cytometry (Fig. S3B). This sensitivity is rather specific since the strain is not particularly sensitive to another cell-death inducer such as phytosphingosine nor to reactive oxygen species, like hydrogen peroxide or those generated by treatment with paraquat. Moreover, STS induces much higher chromatin fragmentation in the *abc3* mutant than in the wild type strain, a PCD hallmark detected by the TUNEL assay (Fig. 2D). The Δ *NCU09141* mutant is only slightly more sensitive to STS than wild type (Fig. 2B), possibly due to the presence of redundant cellular functions.

We also investigated the reaction to STS of other mutants carrying deletions in genes whose expression is affected by exposure of fungal cells to the drug (Table S3). As controls, we used in these assays the complex I deletion mutants *nuo51* and *nuo21.3c*, which are more sensitive and more resistant to STS than wild type, respectively (Castro et al., 2010). Fig. 2C shows additional strains that are clearly more sensitive (Δ *NCU21652*, previously called Δ *NCU09974*) or more resistant (Δ *NCU03229*, Δ *NCU06419*, Δ *NCU04512*) to STS than the wild type strain, indicating that the respective proteins play a significant role in the *N. crassa* response to the drug and pointing directions for future work. Taken together, our results also underline the general use of transcriptional profiling for the identification of novel proteins involved in the cellular reaction to death inducers.

Interestingly, the product of the *NCU21652* gene, which is located adjacent to *abc3* in the *N. crassa* genome, contains a domain present in transcription factors. Despite that it was not included in a previous characterization of transcription factor mutants (Colot et al., 2006), we hypothesized that *NCU21652* encodes a transcription factor involved in the genetic response to STS. Indeed, RT-PCR experiments demonstrate that the increased expression levels of *NCU09141* and *abc3* genes is impaired in the presence of STS in a *NCU21652* deletion-mutant background (Fig. 1E). These data represent strong evidence that *NCU21652* is a novel transcription factor required to set up a genetic response to STS and explain the high STS-sensitivity of the Δ *NCU21652* strain.

3.2. The ABC3 protein has a role in staurosporine export

The predicted amino acid sequence of *NCU09975* (ABC3) displays high homology with members of the superfamily of ABC transporters (Fig. S2A) and this type of proteins have been implicated in ATP-dependent drug efflux (Cannon et al., 2009). The high sensitivity of the *abc3* mutant strain to STS led us to hypothesize that it is a STS exporter. In order to assess its function, we compared the accumulation of STS in wild type and the *abc3* mutant strain. Taking advantage of STS fluorescence (Iyer et al., 2008), we devised a flow cytometry methodology to measure its intracellular concentration. Conidial suspensions (5×10^6 cells/ml) were incubated for 1 hour at room temperature in minimal medium containing 25 μ M STS, after which cells were washed and their STS intracellular content measured. Fig. 3A shows that STS accumulation is ca 3.5 times higher in the mutant than in wild type cells. Since STS partially fluoresces in the visible region of the spectrum when excited with UV radiation, spots of cells loaded with STS should fluoresce in a trans-UV illuminator. Indeed, when spotted in GFS solid medium with a high STS concentration, in which growth is barely observed (middle panel in Fig. 3B), mutant cells exhibit much higher fluorescence over UV-transillumination than wild type cells (Fig. 3B, lower panel), confirming a higher STS accumulation in the *abc3* mutant cells.

We sought thus to test if the higher accumulation of STS in the mutant was due to a limited drug efflux capacity. Since drug transport by ABC proteins requires ATP (Cannon et al., 2009) and we did not observe higher intracellular accumulation of STS in the mutant *versus* wild type when cells are incubated in water instead of growth medium, we assessed the rate of STS extrusion between wild type and *abc3* mutant strains. This was done by incubating conidia from both strains for 15 min at room temperature in minimal medium containing 25 μ M STS and devoid of sugars to avoid active STS export. Afterwards, sucrose was added, aliquots were collected at different time points and the STS internal content was assessed. It was observed that, over a period of 100 min, the internal STS content of wild type cells decreases to ca 1/5 of the initial value, whereas in the *abc3* mutant cells the STS content oscillates around the initial value (Fig. 3C). In accordance with higher intracellular STS concentration, the extracellular concentration of STS in the mutant liquid culture was smaller than in the wild type liquid culture (Fig. 3D). The higher STS accumulation in the mutant cells, together with lower extracellular concentrations and limited STS export capacity observed in the mutant strain, points to an essential role of ABC3 protein in the export of STS.

3.3. Targeted drug inhibitors modulate fungal sensitivity to staurosporine

Since the *abc3* strain is highly sensitive to STS and the ABC3 protein plays a role in STS export, we reasoned that inhibiting the ABC3 protein would also make wild type cells much more sensitive to STS. Therefore, we searched the literature and tested the effects on *N. crassa* of a series of compounds targeting ABC-type proteins when used in combination with STS (Table S4). While some compounds revert the sensitivity of wild type to STS, others stimulate the effects of STS, indicating capability to modulate fungal sensitivity to

STS. Figure 4A shows that STS has a much stronger antifungal effect against wild type *N. crassa* cells when combined with either Verapamil or sodium orthovanadate. Orthovanadate is a universal inhibitor of ABC transporters, binding at the phosphate binding site and impairing ATP hydrolysis (Sarkadi et al., 2006). Verapamil has been described as a specific competitive inhibitor of human P-glycoprotein (Tsuruo et al., 1982). While these compounds have little if any effect on *N. crassa* when used alone, their combination with STS leads to a dramatic effect on fungal growth. This effect could be also observed in other *N. crassa* strains, such as the nuo78 complex I mutant that show a similar transcription of *abc3* as the wild type strain (not shown), corroborating the ability to modulate staurosporine sensitivity with inhibitors.

In order to confirm that Verapamil and orthovanadate were indeed inhibiting STS export, the intracellular accumulation of STS in the presence of each of these compounds was assessed in both wild type and *abc3* mutant cells (Fig. 5C and D). After one hour of incubation with STS together with either Verapamil or orthovanadate, the STS intracellular content of the wild type cells increases significantly, whereas in the *abc3* mutant the differences are not significant. Accordingly, extracellular levels of STS in wild type cells treated with Verapamil are lower than in untreated cells (Fig. 5A and B). These results provide an explanation for the enhanced antifungal effects of the combination of STS with either Verapamil or orthovanadate. By preventing STS elimination from fungal cells, these two inhibitors potentiate the cellular effects of STS treatment.

To determine whether the interaction of drug combinations on cell growth was specific for *Neurospora* or could be generalized to other fungi, we tested the effect of combining STS with an inhibitor of ABC transporters in two common human pathogens, *Candida albicans* (Fig. 4B) and *Aspergillus fumigatus* (Fig. 4C). Like in *N. crassa*, the combination of STS and sodium orthovanadate results in an impressive effect on growth of both of the pathogenic species. These results unravel the potential of this type of drug combinations to be used as antifungal agents.

Despite the fact that the Δ NCU09141 mutant is not particularly sensitive to STS, the drug-induced expression of the respective gene observed in microarray hybridizations advocates that the encoded protein may play a role in the fungal response to STS. NCU09141 is classified as a “probable pyridoxine dehydrogenase”, suggesting an interesting link between cell death and vitamin biosynthesis. However, we found that it also displays similarity to β -subunits (regulators) of the shaker-related family of voltage-gated potassium channels (Fig. S2B). These proteins that have been implicated in programmed cell death including STS-induced apoptosis in mammalian cells (Szabo et al., 2008; Szabo et al., 2010) and NCU02887 (annotated as a voltage-gated potassium channel subunit) is also induced upon *N. crassa* exposure to STS (Table S1). Therefore, as rationalized for ABC transporters above, we tested the effects of combining compounds targeting voltage-gated potassium channels with STS on fungal growth (Table S4). Figure 4(A-C) illustrates the strong enhancement effect of the combination between STS and 4-aminopyridine on growth of *N. crassa*, *C. albicans* and *A. fumigatus*. These results suggest that NCU09141 may indeed be associated with potassium channels and support their involvement in the response to STS. Altogether, our results point out the power of transcriptional profiling for identifying novel proteins with potential to become drug targets.

4. Discussion

The ability to modulate programmed cell death has broad implications in the medical field, from cancer treatment to fighting infectious diseases (Ramsdale, 2008; Reed, 2006; Sharon et al., 2009). Knowledge about its molecular basis is expectedly valuable for designing

drugs targeting specific proteins that can be used to modulate programmed cell death. Our data support this assumption and prove the underlying principle. We analyzed time-course alterations in *N. crassa* gene expression resulting from exposure to the death-inducer STS. Novel proteins involved in the response to the drug were found as judged by alterations in gene expression profiles and/or altered sensitivity to the drug of specific null-mutant strains. Among these, an ABC transporter protein (NCU09975) and a protein with similarity to voltage-gated potassium channels subunits (NCU09141) were identified. Then, we showed that treating *N. crassa* with inhibitors of either of this type of proteins allows modulation of the fungal sensitivity to STS. In particular, some inhibitors extremely enhance the antifungal effect of STS, namely the general ABC transporter inhibitor orthovanadate (Sarkadi et al., 2006), the competitive inhibitor of mammalian p-glycoprotein Verapamil (Aller et al., 2009) and the 4-aminopyridine inhibitor of voltage-gated potassium channels (Chin et al., 1997). This effect is observed not only against *N. crassa*, but also against common fungal pathogens, such as *A. fumigatus* and *C. albicans*. Thus, these drug combinations have potential applications as antifungals and, possibly, anticancer agents.

While NCU09141 is assigned as a probable pyridoxine dehydrogenase, we noticed its similarities to regulatory subunits of voltage-gated potassium channels of the shaker-related subfamily Kv β 2 (Xu & Li, 1997). While this remains to be proved, our data clearly associate voltage-gated potassium channels with the response to STS. *NCU09141* and another gene (*NCU02887*, annotated as a voltage-gated potassium channel subunit) are induced upon *N. crassa* exposure to STS. In addition, the inhibitor of voltage-gated potassium channels 4-aminopyridine (Chin et al., 1997) reinforces the antifungal properties of STS. These channels are involved in apoptosis, although it remains controversial whether their activation leads to or protects from apoptosis (Szabo et al., 2010). The action of β 2 subunits on the regulation of voltage-gated K⁺ channels is not clear, but its proposed role in channel activation (Xu & Li, 1997) lead us to speculate that STS-driven overexpression of NCU09141 may protect against cell death by activating K⁺ efflux.

The product of *NCU09975* (ABC3) displays high homology with members of the superfamily of ABC transporters. These proteins have been often implicated in conferring multidrug resistance to chemotherapy both to tumor cells and to infectious organisms (Cannon et al., 2009; Nascimento et al., 2003). The best human homologue of ABC3 is p-glycoprotein (33 % identity and 54 % similarity), a multiple drug exporter involved in apoptosis protection (Sarkadi et al., 2006). In a phylogenetic analysis of fungal ABC proteins, *N. crassa* ABC3 was placed in Group III of the ABC-B subfamily (Kovalchuk & Driessen, 2010), but no clear functions have been attributed so far to members of this group. Expression of *A. fumigatus* AtrC, another member of the group, increases in the presence of various toxicants but the respective knockout mutant is not differentially sensitive to them (Andrade et al., 2000). The *Magnaporthe grisea* ABC3 homolog is required for host cell penetration and for protection from oxidative stress (Sun et al., 2006). By functional characterization, we show that an ABC3 deletion-mutant accumulates more STS than wild type *N. crassa* when exposed to the drug. In sharp contrast to the mutant, wild type is able to extrude STS upon energization of the cells indicating that ABC3 is involved in STS transport. This conclusion is further supported by the fact that STS accumulation in the wild type strain is enhanced by two inhibitors of p-glycoprotein, orthovanadate and Verapamil, while the same inhibitors did not affect significantly the STS content of the mutant strain. Despite the high accumulation of STS in the mutant, its sensitivity to STS observed from spot assays results from STS-induced PCD and not just through STS toxicity resulting from deficient efflux, as demonstrated by the TUNEL assay. ABC3 appears to have narrower substrate specificity than p-glycoprotein, which might reflect the fact that, facing changing environments, fungal genomes possess a higher density of ABC transporters than the human genome (Ren et al., 2007). To our knowledge, ABC3 is the first described transporter of the

widely used death-inducer STS. The fact that ABC3 is highly induced in the presence of STS and the *abc3* mutant is extremely sensitive to the drug and that inhibitors of ABC transporters enhance the effects of STS against *N. crassa* and pathogenic fungi illustrates common mechanisms of drug resistance among fungi and pinpoints a major role of ABC3 in these processes.

Multidrug resistance often arises from mutations in regulators of multidrug resistance genes like the ABC transporters (Akache et al., 2004). In this work, we also identified a novel transcription factor NCU21652 that can be elicited as a drug target. We showed that NCU21652 is required for the STS-dependent overexpression of the *NCU09141* and *NCU09975* genes, indicating that it has a pivotal role in the control of the genetic response of *N. crassa* to STS. It should be noticed that all these proteins seem to be also at least partially involved in the response to other death-inducers, since the respective genes are induced in *N. crassa* cells exposed to phytosphingosine (Videira et al, 2009). In summary, the identification of proteins responsible for drug response by transcriptional profiling, with subsequent protein targeted inhibition combined with drug insult revealed to be a powerful strategy to overcome drug resistance in fungi. These data stand as a basis for designing novel drugs/strategies for attacking tumor or pathogenic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge Paula Magalhães for help with Real-Time PCR, Dr Isabel Carvalho and Tânia Ribeiro for rat immunization and antiserum preparation, and Dr Paula Sampaio for help with immunofluorescence microscopy. This work was supported by a “Programa Ciência” fellowship financed by POPH-QREN (typology 4.2) with co-funding from ESF and MCTES to ASF, a Fundação Calouste Gulbenkian PhD fellowship to APG, NIH grant GM60468 to NLG, research grants from FCT Portugal and the European POCI program of QCAIII (co-participated by FEDER) and a sabbatical fellowship from Fundação Luso-Americana to AV.

References

- Akache B, MacPherson S, Sylvain MA, Turcotte B. Complex interplay among regulators of drug resistance genes in *Saccharomyces cerevisiae*. *J Biol Chem*. 2004; 279:27855–27860. [PubMed: 15123673]
- Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science*. 2009; 323:1718–1722. [PubMed: 19325113]
- Andrade AC, Van Nistelrooy JG, Peery RB, Skatrud PL, De Waard MA. The role of ABC transporters from *Aspergillus nidulans* in protection against cytotoxic agents and in antibiotic production. *Mol Gen Genet*. 2000; 263:966–977. [PubMed: 10954082]
- Cannon RD, Lamping E, Holmes AR, Niimi K, Baret PV, Keniya MV, Tanabe K, Niimi M, Goffeau A, Monk BC. Efflux-mediated antifungal drug resistance. *Clin Microbiol Rev*. 2009; 22:291–321. [PubMed: 19366916]
- Castro A, Lemos C, Falcao A, Fernandes AS, Glass NL, Videira A. Rotenone enhances the antifungal properties of staurosporine. *Eukaryot Cell*. 2010; 9:906–914. [PubMed: 20435699]
- Castro A, Lemos C, Falcao A, Glass NL, Videira A. Increased Resistance of Complex I Mutants to Phytosphingosine-induced Programmed Cell Death. *J Biol Chem*. 2008; 283:19314–19321. [PubMed: 18474589]
- Chin LS, Park CC, Zitnay KM, Sinha M, DiPatri AJ Jr, Perillan P, Simard JM. 4-Aminopyridine causes apoptosis and blocks an outward rectifier K⁺ channel in malignant astrocytoma cell lines. *J Neurosci Res*. 1997; 48:122–127. [PubMed: 9130140]

- Colot HV, Park G, Turner GE, Ringelberg C, Crew CM, Litvinkova L, Weiss RL, Borkovich KA, Dunlap JC. A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. *Proc Natl Acad Sci USA*. 2006; 103:10352–10357. [PubMed: 16801547]
- Davis RH, de Serres FJ. Genetic and microbiological research techniques for *Neurospora crassa*. *Methods Enzymol*. 1970; 17A:79–143.
- Davis RH, Perkins DD. Timeline: *Neurospora*: a model of model microbes. *Nat Rev Genet*. 2002; 3:397–403. [PubMed: 11988765]
- Duarte M, Sousa R, Videira A. Inactivation of genes encoding subunits of the peripheral and membrane arms of *Neurospora* mitochondrial complex I and effects on enzyme assembly. *Genetics*. 1995; 139:1211–1221. [PubMed: 7768434]
- Dunlap JC, Borkovich KA, Henn MR, Turner GE, Sachs MS, Glass NL, McCluskey K, Plamann M, Galagan JE, Birren BW, Weiss RL, Townsend JP, Loros JJ, Nelson MA, Lambreghts R, Colot HV, Park G, Collopy P, Ringelberg C, Crew C, Litvinkova L, DeCaprio D, Hood HM, Curilla S, Shi M, Crawford M, Koerhsen M, Montgomery P, Larson L, Pearson M, Kasuga T, Tian C, Basturkmen M, Altamirano L, Xu J. Enabling a Community to Dissect an Organism: Overview of the *Neurospora* Functional Genomics Project. *Adv Genetics*. 2007; 57:49–96.
- Galagan JE, Calvo SE, Borkovich KA, Selker EU, Read ND, Jaffe D, FitzHugh W, Ma L-J, Smirnov S, Purcell S, Rehman B, Elkins T, Engels R, Wang S, Nielsen CB, Butler J, Endrizzi M, Qui D, Ianakiev P, Bell-Pedersen D, Nelson MA, Werner-Washburne M, Selitrennikoff CP, Kinsey JA, Braun EL, Zelter A, Schulte U, Kothe GO, Jedd G, Mewes W, Staben C, Marcotte E, Greenberg D, Roy A, Foley K, Naylor J, Stange-Thomann N, Barrett R, Gnerre S, Kamal M, Kamvysselis M, Mauceli E, Bielke C, Rudd S, Frishman D, Krystofova S, Rasmussen C, Metzenberg RL, Perkins DD, Kroken S, Cogoni C, Macino G, Catcheside D, Li W, Pratt RJ, Osmani SA, DeSouza CPC, Glass L, Orbach MJ, Berglund JA, Voelker R, Yarden O, Plamann M, Seiler S, Dunlap J, Radford A, Aramayo R, Natvig DO, Alex LA, Mannhaupt G, Ebbole DJ, Freitag M, Paulsen I, Sachs MS, Lander ES, Nusbaum C, Birren B. The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature*. 2003; 422:859–868. [PubMed: 12712197]
- Gescher A. Staurosporine analogues - pharmacological toys or useful antitumour agents? *Crit Rev Oncol Hematol*. 2000; 34:127–135. [PubMed: 10799837]
- Glass NL, Dementhon K. Non-self recognition and programmed cell death in filamentous fungi. *Curr Opin Microbiol*. 2006; 9:553–558. [PubMed: 17035076]
- Iyer GH, Taslimi P, Pazhanisamy S. Staurosporine-based binding assay for testing the affinity of compounds to protein kinases. *Anal Biochem*. 2008; 373:197–206. [PubMed: 18047825]
- Jarvis WD, Turner AJ, Povirk LF, Traylor RS, Grant S. Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. *Cancer Res*. 1994; 54:1707–1714. [PubMed: 7511048]
- Kasuga T, Townsend JP, Tian C, Gilbert LB, Mannhaupt G, Taylor JW, Glass NL. Long-oligomer microarray profiling in *Neurospora crassa* reveals the transcriptional program underlying biochemical and physiological events of conidial germination. *Nucleic Acids Res*. 2005; 33:6469–6485. [PubMed: 16287898]
- Kovalchuk A, Driessen AJ. Phylogenetic analysis of fungal ABC transporters. *BMC Genomics*. 2010; 11:177. [PubMed: 20233411]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Madeo F, Frohlich E, Frohlich KU. A yeast mutant showing diagnostic markers of early and late apoptosis. *J Cell Biol*. 1997; 139:729–734. [PubMed: 9348289]
- Marques I, Duarte M, Assunção J, Ushakova AV, Videira A. Composition of complex I from *Neurospora crassa* and disruption of two “accessory” subunits. *BBA-Bioenergetics*. 2005; 1707:211–220. [PubMed: 15863099]
- McCluskey K. The Fungal Genetics Stock Center: from molds to molecules. *Adv Appl Microbiol*. 2003; 52:245–262. [PubMed: 12964247]
- Nascimento AM, Goldman GH, Park S, Marras SA, Delmas G, Oza U, Lolans K, Dudley MN, Mann PA, Perlin DS. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-

- level resistance to itraconazole. *Antimicrob Agents Chemother.* 2003; 47:1719–1726. [PubMed: 12709346]
- Omura S, Iwai Y, Firano A, Nakagawa A, Awaya F, Tsuchiya T, Masuma R. A new alkaloid AM-2282 of *Streptomyces* origin taxonomy, fermentation isolation and preliminary characterization. *J Antibiot.* 1977; 30:275–282. [PubMed: 863788]
- Osman AB, Gani SM, Engh RA. Protein kinase inhibition of clinically important staurosporine analogues. *Nat Prod Rep.* 2010; 27:489–498. [PubMed: 20336234]
- Ramsdale M. Programmed cell death in pathogenic fungi. *Biochim Biophys Acta.* 2008; 1783:1369–1380. [PubMed: 18294459]
- Reed JC. Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. *Nat Clin Pract Oncol.* 2006; 3:388–398. [PubMed: 16826219]
- Ren Q, Chen K, Paulsen IT. TransportDB: a comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. *Nucleic Acids Res.* 2007; 35:D274–279. [PubMed: 17135193]
- Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, Tetko I, Guldener U, Mannhaupt G, Munsterkotter M, Mewes HW. The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Res.* 2004; 32:5539–5545. [PubMed: 15486203]
- Sarkadi B, Homolya L, Szakacs G, Varadi A. Human multidrug resistance ABCB and ABCG transporters: participation in a chemoinnity defense system. *Physiol Rev.* 2006; 86:1179–1236. [PubMed: 17015488]
- Schulte TH, Scarborough GA. Characterization of the glucose transport systems in *Neurospora crassa* sl. *J Bacteriol.* 1975; 122:1076–1080. [PubMed: 125261]
- Sharon A, Finkelstein A, Shlezinger N, Hatam I. Fungal apoptosis: function, genes and gene function. *FEMS Microbiol Rev.* 2009; 33:833–854. [PubMed: 19416362]
- Sun CB, Suresh A, Deng YZ, Naqvi NI. A multidrug resistance transporter in *Magnaporthe* is required for host penetration and for survival during oxidative stress. *Plant Cell.* 2006; 18:3686–3705. [PubMed: 17189344]
- Szabo I, Bock J, Grassme H, Soddemann M, Wilker B, Lang F, Zoratti M, Gulbins E. Mitochondrial potassium channel Kv1.3 mediates Bax-induced apoptosis in lymphocytes. *Proc Natl Acad Sci USA.* 2008; 105:14861–14866. [PubMed: 18818304]
- Szabo I, Zoratti M, Gulbins E. Contribution of voltage-gated potassium channels to the regulation of apoptosis. *FEBS Lett.* 2010; 584:2049–2056. [PubMed: 20102714]
- Townsend JP. Multifactorial experimental design and the transitivity of ratios with spotted DNA microarrays. *BMC Genomics.* 2003; 4:41. [PubMed: 14525623]
- Townsend JP, Hartl DL. Bayesian analysis of gene expression levels: statistical quantification of relative mRNA level across multiple strains or treatments. *Genome Biol.* 2002; 3:RESEARCH0071.
- Tsuruo T, Iida H, Yamashiro M, Tsukagoshi S, Sakurai Y. Enhancement of vincristine- and adriamycin-induced cytotoxicity by verapamil in P388 leukemia and its sublines resistant to vincristine and adriamycin. *Biochem Pharmacol.* 1982; 31:3138–3140. [PubMed: 7150340]
- Videira A, Kasuga T, Tian C, Lemos C, Castro A, Glass NL. Transcriptional analysis of the response of *Neurospora crassa* to phytosphingosine reveals links to mitochondrial function. *Microbiology.* 2009; 155:3134–3141. [PubMed: 19520721]
- Xu J, Li M. Kvbeta2 inhibits the Kvbeta1-mediated inactivation of K⁺ channels in transfected mammalian cells. *J Biol Chem.* 1997; 272:11728–11735. [PubMed: 9115226]
- Zauner R, Christner J, Jung G, Borchart U, Machleidt W, Videira A, Werner S. Identification of the polypeptide encoded by the URF-1 gene of *Neurospora crassa* mtDNA. *Eur J Biochem.* 1985; 150:447–454. [PubMed: 3160590]

Abbreviations used

PCD Programmed cell death

STS Staurosporine

Highlights

- Transcriptional profiling identifies proteins responding to staurosporine treatment
- The *Neurospora* ABC3 protein is a staurosporine transporter
- Drug combinations lead to synergistic activity against *N. crassa* and pathogenic fungi

Fig. 1.

Expression levels and protein localization after STS treatment. (A) Scheme of the microarray experiment. *N. crassa* conidia were germinated in minimal medium for 5 hours (time 0) and then incubated in the absence (C) or in the presence of 12.5 μ M STS (S). Samples were withdrawn at the indicated time points (minutes) and used for di-swap microarray hybridizations (arrows, with the arrowhead pointing the sample labelled with Cy5) and quantification of gene expression levels. (B) Time-course relative expression levels of the genes encoding NCU09141 (upper panel) and ABC3 (lower panel). (C) Quantification by RT-PCR of relative expression levels of the genes encoding ABC3, NCU07546 and NCU09141 performed in the wild type strain (wt) and the complex I

mutants (nuo51 and nuo78) following 5-hour germination and 30 minutes incubation in the absence (black bars) or presence of STS (grey bars). (D) Western blots of total protein extracts from 16 h-grown fungal cells exposed to 12.5 μ M STS for 3 h or untreated-cells using antiserum against ABC3 and antiserum against the constitutive 30.4 kDa subunit of complex I, as control. Standard molecular weights (kDa) are indicated on the left. (E) Quantification by RT-PCR of relative expression levels of the genes encoding NCU09975 (*abc3*) and NCU09141 (shown above) performed in the wild type strain and the Δ NCU21652 mutant (shown below) following 5-hour germination and 30 minutes incubation in the absence (black bars) or presence of STS (grey bars). (F) Localization of the ABC3 protein by immunofluorescence using confocal microscopy in the *N. crassa* slime mutant after 16 h of growth followed by 3 h in the presence (left) or absence (right) of 12.5 μ M STS.

Fig. 2.

Drug-sensitivity of different strains. (A-C) Serial dilutions of conidial suspensions of the designated strains were spotted and incubated in control medium (left panels of A, B and C) or medium containing 5 μ M STS or 10 μ g/ml phytosphingosine (PHS) or 1.5 mM hydrogen peroxide or 200 μ M paraquat, as indicated. (D) TUNEL staining. Quantification by fluorescence-activated cell sorter analysis of DNA fragmented cells from wild type (wt) or *abc3* mutant cells that were incubated for 3 h in the absence (grey shadow histogram) or presence (unshadow histogram) of 12.5 μ M STS. FITC, fluorescein isothiocyanate. The median of the fluorescein isothiocyanate fluorescence in the absence (grey) or presence (black) of STS is indicated on the right graph.

Fig. 3.

Mutant *abc3* cells accumulate high levels of STS as a result of defective drug extrusion. (A) Quantification of intracellular STS by flow cytometry in wild type (wt) and *abc3* mutant strain after 1 h incubation with 25 μ M STS. Bar graphs show average $\pm$ standard deviation of the median of STS fluorescence from three independent experiments. A Mann-Whitney test was performed (* $p < 0.05$). (B) Nine spots of serial 3-fold dilutions of conidial suspensions of wild type (wt) and *abc3* strains were incubated in GFS medium in the absence (top) or presence of 7.5 μ M STS. The plates were scanned in Gel Doc instrument (Bio Rad) in epi-white mode or trans-UV mode. (C) Wild type (grey) and *abc3* mutant cells (black) were incubated in medium containing 25 μ M STS, but devoid of sugars. After sucrose addition, samples were collected at the indicated time point and intracellular STS concentration was determined by flow cytometry. A representative experiment performed in triplicate is shown. PI-positive or dead cells monitored throughout the time-course were always $< 1.5\%$. (D) Visible absorption spectra of culture media from wild type (solid black lines) and *abc3* cells (solid grey lines) after the indicated 2 or 60 minutes of incubation in 12.5 μ M STS is shown in the left panel, with the dashed line representing the spectrum of control (media with 12.5 μ M STS and no cells). The time-course concentration of staurosporine measured from visible absorption spectra of culture media of wild type

(circles) and *abc3* (squares) is shown in the right panel. The data represent the average $\pm$ standard deviation from three independent experiments.

Fig. 4.

Antifungal effects of drug combinations. Serial dilutions of cellular suspensions of *N. crassa* (A), *C. albicans* (B) and *A. fumigatus* (C) were spotted and incubated in control medium (first panels of A, B and C) or medium containing STS or sodium orthovanadate (OV) or Verapamil or 4-aminopyridine (4-AP), or combinations of drugs. Drug concentrations used were 5, 15 and 10 μ M STS and 5, 1 and 1 mM OV (A, B and C, respectively), 5 mM 4-AP and 0.5 mM Verapamil.

Fig. 5.

Verapamil and orthovanadate increase STS intracellular accumulation and a concomitant decrease of extracellular STS concentration. (A) Visible absorption spectra of concentrated external media of 5 h-grown *N. crassa* (initial concentration 10^7 conidia/ml) in minimal medium at 30 °C followed by 30 min in the presence of 12.5 μM STS and in the absence (a) or presence of 0.5 mM Verapamil (b). (B) STS extracellular concentration in the absence (grey) or presence of Verapamil (black) calculated from the spectra in A. (C) and (D) Quantification by flow cytometry of relative intracellular STS concentration in the indicated strains in the absence (grey) or presence (black) of 5 mM Verapamil (C) or 5 mM orthovanadate (D). The data in B, C and D represent the average $\pm$ standard deviation of three independent experiments. Statistical significance was determined with the Mann-Whitney test (* $p \leq 0.05$).