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Differential Gene Expression in Auristatin PHE-Treated *Cryptococcus neoformans*

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The antifungal pentapeptide auristatin PHE was recently shown to interfere with microtubule dynamics and nuclear and cellular division in the opportunistic pathogen *Cryptococcus neoformans*. To gain a broader understanding of the cellular response of *C. neoformans* to auristatin PHE, mRNA differential display (DD) and reverse transcriptase PCR (RT-PCR) were applied. Examination of approximately 60% of the cell transcriptome from cells treated with 1.5 times the MIC (7.89 μM) of auristatin PHE for 90 min revealed 29 transcript expression differences between control and drug-treated populations. Differential expression of seven of the transcripts was confirmed by RT-PCR, as was drug-dependent modulation of an additional seven transcripts by RT-PCR only. Among genes found to be differentially expressed were those encoding proteins involved in transport, cell cycle regulation, signal transduction, cell stress, DNA repair, nucleotide metabolism, and capsule production. For example, *RHO1* and an open reading frame (ORF) encoding a protein with 91% similarity to the *Schizophyllum commune* 14-3-3 protein, both involved in cell cycle regulation, were down-regulated, as was the gene encoding the multidrug efflux pump Afr1p. An ORF encoding a protein with 57% identity to the heat shock protein HSP104 in *Pleurotus sajor-caju* was up-regulated. Also, three transcripts of unknown function were responsive to auristatin PHE, which may eventually contribute to the elucidation of the function of their gene products. Further study of these differentially expressed genes and expression of their corresponding proteins are warranted to evaluate how they may be involved in the mechanism of action of auristatin PHE. This information may also contribute to an explanation of the selectivity of auristatin PHE for *C. neoformans*. This is the first report of drug action using DD in *C. neoformans*.

The incidence of fungal infections has dramatically increased in recent years, due in part to improved medical techniques, including immunosuppressive therapy and organ transplantation, and to the onset of AIDS. The opportunistic fungal pathogen *Cryptococcus neoformans* was identified as a human pathogen more than 100 years ago (7). The incidence of invasive infections caused by this encapsulated yeast had risen dramatically by the late 1970s. By the 1990s and as a consequence of the AIDS epidemic, cryptococcosis was considered a widespread and common infection (8, 30). Cryptococcal meningitis is the most commonly encountered life-threatening manifestation of cryptococcosis and is fatal if untreated (8, 45). Existing therapies include polyene and azole antifungals (15, 57).

The unique linear pentapeptide auristatin PHE (dovaline-valine-dolaisoleuine-dolaproine-phenylalanine-methyl ester) is a synthetic structural modification of the marine natural product dolastatin 10 (41, 42). Dolastatin 10, isolated from the Indian Ocean sea hare *Dolabella auricularia* (40) and later from the associated marine cyanobacterium *Symploca* sp. strain VP642 (28), exhibits remarkable cytostatic and antineoplastic activities and is currently undergoing phase I and II cancer clinical trials. Its mammalian tubulin-binding activity has been investigated in detail (for a review, see reference 39). Briefly, dolastatin 10 inhibits mammalian tubulin polymerization and the associated GTP hydrolysis (3) and acts as a noncompetitive inhibitor of vincristine and vinblastine (4).

Broth microdilution assays (BMAs) with a large panel of fungi, with an emphasis on basidiomycetes, have shown that dolastatin 10 has selective antifungal activity against *C. neoformans*, and auristatin PHE has selective antifungal activity against *C. neoformans* and some species of *Trichosporon* (43, 61). Other species in the genus *Cryptococcus* are not inhibited by high concentrations of these peptides. The reason for the selectivity of the peptides is still unknown, but selectivity could be due to differences in compound access or efflux. Furthermore, the susceptible organisms may share a unique target that enhances binding and/or interaction with the drug(s). Compared to dolastatin 10, auristatin PHE had lower MICs (43, 61), exhibited concentration-dependent killing between the MIC and four times the MIC (43), and demonstrated increased activity as well as prolonged postantifungal effects in the presence of human serum (43, 61). Iterative deconvolution in conjunction with an optimized *C. neoformans* microtubule immunolabeling procedure enabled us to investigate the mechanism of action of auristatin PHE (62). Detailed visualization of the microtubule cytoskeleton in auristatin PHE-treated *C. neoformans* revealed complete disruption of first cytoplasmic and then spindle microtubules in a time- and concentration-dependent manner (62). Sub-MICs of auristatin PHE caused complete microtubule disruption within 4.5 h, accompanied by

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blockage of nuclear migration and nuclear and cellular division, resulting in cells arrested in a uninucleate, large-budded stage (62).

In the present report, mRNA differential display and reverse transcriptase PCR (RT-PCR) were used to gain a broader understanding of the response of *C. neoformans* to auristatin PHE. Genes differentially expressed in the presence of auristatin PHE, including genes encoding proteins involved in transport, cell cycle regulation, signal transduction, cell stress, DNA repair, purine biosynthesis, and capsule production, as well as proteins of unknown function, are described.

MATERIALS AND METHODS

Yeast strain and growth conditions. The clinical isolate *C. neoformans* ATCC 90112 was obtained from the American Type Culture Collection (Manassas, Va.), maintained on yeast morphology (YM) agar (Difco Laboratories, Detroit, Mich.), and grown in YM broth (Difco Laboratories) at 35°C in a rotary shaker at 280 rpm.

Antifungal agent. Auristatin PHE (M_r = 760) was synthesized and purified by standard purification procedures as previously described (41, 42). Aliquots were stored desiccated in the dark at room temperature. Prior to each experiment, the peptide was reconstituted in dimethyl sulfoxide (DMSO) and diluted in YM broth to the appropriate concentrations, while maintaining the DMSO concentration at 0.8%.

Susceptibility testing of *C. neoformans* isolates. Antifungal susceptibility testing of *C. neoformans* was performed by the reference BMA as previously described (61). The MIC was defined as the lowest concentration of auristatin PHE that inhibited all visible growth of the test organism (optically clear).

Drug treatments and isolation of total RNA. Early-logarithmic-phase cultures of *C. neoformans* ATCC 90112 in YM broth were treated with 1.5 times the MIC of auristatin PHE (7.89 μM; as determined by BMA in YM), while maintaining the DMSO concentration at 0.8%. Control cells were treated with DMSO only. Drug-treated and control cultures were incubated in a rotary shaker at 35°C and 280 rpm for 90 min.

For RNA isolation, cells were harvested by centrifugation, washed in ice-cold water, and quickly frozen in dry ice. Frozen cells were lyophilized overnight, and cell walls were mechanically disrupted by vortexing for several minutes with 0.425- to 0.6-mm glass beads. RNA was extracted first with Trizol (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions and then again with phenol-chloroform (Pierce Biotechnology, Inc., Rockford, Ill.) and precipitated with isopropanol. To remove chromosomal DNA from the RNA preparation, 50 μg of total RNA was incubated with 6 U of RNase-free DNase I (Qiagen, Valencia, Calif.) in the presence of 10 U of RNase inhibitor (Superase-In; Ambion, Inc., Austin, Tex.). After 30 min at 37°C, the RNA was extracted with phenol-chloroform, precipitated with isopropanol, and dissolved in diethyl pyrocarbonate (DEPC)-treated water, and the RNA was purified with the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically, and RNA integrity was visualized by electrophoresis through a denaturing gel.

mRNA differential display. Differential display was performed with the RNAimage kit (GenHunter, Nashville, Tenn.) according to the manufacturer's instructions, with minor modifications. Briefly, 500 ng of total yeast RNA was reverse transcribed with one of three different one-base anchored oligo(dT) primers (0.2 μM H-T_{11}A, H-T_{11}C, and H-T_{11}G) and 100 U of Moloney murine leukemia virus RT in the presence of 20 μM deoxynucleoside triphosphates (dNTPs) at 37°C for 1 h. After the enzyme was inactivated at 75°C for 5 min, 10% of the RT mixture was used as the template in the differential display-PCR mixture, which contained 0.2 μM T_{11}N primer (from previous reaction) in combination with a 0.2 μM 13-mer arbitrary primer (H-AP) in the presence of 0.2 μl of [α-^{33}P]dATP (2,000 Ci/mmol; New England Nuclear, Boston, Mass.), 1 U of *Taq* DNA polymerase (Qiagen), and 2 μM dNTPs. The cycling parameters were as follows: 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s (40 cycles, followed by a 5-min extension at 72°C). A total of 70 different combinations of anchor primers and arbitrary primers were used, and all reactions were run in duplicate. The radiolabeled PCR products were separated on a 6% denaturing polyacrylamide gel and visualized by autoradiography. The cDNA bands representing differentially expressed mRNAs were excised, and the DNA was recovered from the dried gel by boiling followed by ethanol precipitation. The recovered cDNAs were reamplified with the same primer sets and amplification conditions, except that the isotope was omitted and dNTPs were used at a 20 μM final concentration. Reamplified PCR products were verified on a 2% agarose gel.

cDNA subcloning and sequence analysis. PCR products were cloned with the TOPO TA cloning system (Invitrogen) according to the manufacturer's instructions. Briefly, cDNAs were ligated into pCR 2.1-TOPO and transformed into One Shot chemically competent *Escherichia coli* cells. The plasmid DNA was isolated with a Mini Prep 24 (MacConnell Research Corp., San Diego, Calif.), ethanol precipitated, and sequenced with the CEQ dye terminator cycle sequencing Quick Start kit and the CEQ L (Beckman Coulter, Inc., Fullerton, Calif.). Sequence data were analyzed with CEQ L software. Homology searches with the Basic Local Alignment Search Tool (BLAST) were performed by using the GenBank database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the *C. neoformans* Genome Project, Stanford Genome Technology Center (SGTC) database (http://www-sequence.stanford.edu/group/C.neoformans/index.html). Open reading frames (ORFs) were determined according to the preliminary annotations of the SGTC *C. neoformans* Genome Project database and the tentative annotations of The Institute for Genome Research (TIGR) *C. neoformans* database (http://www.tigr.org/tdb/e2k1/cna1/).

cDNA synthesis and real-time semiquantitative RT-PCR. For cDNA synthesis, 2 μg of total RNA was reverse transcribed by poly(A) priming with SuperScript II RNase H^- RT (Invitrogen) according to the manufacturer's instructions. Semiquantitative RT-PCR was performed with a LightCycler (Roche Applied Science, Indianapolis, Ind.). Amplification was carried out as recommended by the manufacturer with the LightCycler FastStart DNA Master SYBR Green I kit (Roche). Briefly, 2 μl of a 1:20 dilution of cDNA was added to the LightCycler FastStart Mastermix (0.5 μM specific primer, 2 to 5 mM $MgCl_2$, 1 μl of FastStart DNA Master SYBR Green I), and amplification was carried out for 20 to 45 cycles (95°C for 10 s, 55 to 64°C for 6 s, 72°C for 4 to 12 s). Real-time accumulation of double-stranded DNA was detected by fluorimetry after each elongation step at a temperature 2°C below the determined melting point for the PCR product being analyzed. Specificity of amplification was verified by melting curve analysis and conventional agarose gel electrophoresis. The threshold cycles for the genes of interest were normalized to that for 18S rRNA. The normalized threshold cycle differences between control and auristatin PHE-treated samples were calculated, and the final *n*-fold changes in gene expression levels were defined as 2^x, where x is the difference in threshold cycle. Standard deviations (SD) were calculated from expression levels in two independent experiments. Differential expression was defined as at least a twofold change. Oligonucleotide primers (Table 1) used for amplification of specific sequences were designed with Oligo, version 5.0, and synthesized by Qiagen.

RESULTS AND DISCUSSION

Identification of genes differentially expressed in *C. neoformans* cells exposed to auristatin PHE in vitro. Genome sequencing projects have resulted in complete or partial sequencing of many genomes, facilitating the assembly of cDNA microarrays representing the complete or almost complete transcriptome of a given organism (52). Gene expression patterns obtained with cDNA microarrays for *Candida albicans* in response to a 24-h exposure to 10 μM itraconazole (13), as well as for *Saccharomyces cerevisiae* after a 90-min exposure to 0.5 times the MIC of the polyenes amphotericin B and nystatin (63), have been described. Furthermore, gene expression profiling has been used to predict drug targets. For example, Bammert and Fostel (5) evaluated differential gene expression in antifungal-treated (0.5 times the MIC of clotrimazole, fluconazole, itraconazole, ketoconazole, PNU-144248E, voriconazole, amorolfine, and terbinafine for 90 min) *S. cerevisiae* strains with genetic alterations (ERG2, ERG5, and ERG6 deletions) in the same targeted pathway, ergosterol biosynthesis. Moreover, gene expression profiles obtained by cDNA microarray have been reported for drug-resistant versus drug-susceptible fungal pathogens, such as fluconazole-resistant and -susceptible *C. albicans* (47), and for the stepwise acquisition of fluconazole resistance by *C. albicans* (48).

TABLE 1. DNA sequences for primers used in RT-PCR

Sequence amplified	Primer sequences[a] (5′–3′)
ADE2	F: GCT GGT ATC TTT GGC GTT GAG R: GGC AGC GGA TGG AAC ACG A
AFR1	F: CGT CTT ATC AGC GGT CTT GT R: CCG GTA GAG TCA GGG TTA T
Amino acid permease	F: GCG TTT ACG AGT TTG GTC AG R: AGT CAA GAG CCA CGA TAA GAA
CAC1	F: GCC GCT GAT GGA GTA TCT GA R: CGC CCA TGT CTC TAA CCT CT
CAP60	F: ACT TCC TGC GTC TGC GTT CC R: GGC CAC CAA ACA AGT CGA AAG
DIP5	F: CGC TAT AAT AAA CGC TTG GTA R: CGG CTA GTA CAC TTG GTA AA
HSP104	F: ATG ATC CTT CCT GCG AGT GG R: TGT GGG CAA CTT CAC CGT C
RAD51	F: CGA ATC CAC ACT CCG CTC ATT R: GGC TGA ACC AAA CGC AAA GG
Related to ITRI	F: ACG CCA TTG TTT ATT ACA CC R: AGA TGC CCA AAG CGA TAC AT
RHO1	F: CGA CGA TGG CAA GAC TAT CC R: CCT AAA GCA CCA CAC AAC CCT
14-3-3	F: AGC TTC CTG GCG AAT CGT CT R: TCG GCA AGG TAT CGG TGG TAA
CN11014294	F: TTC CAC CGC CAC CCA AAG R: TTC TTC AAA CGC CTT CGC CA
CN11018463	F: GCT TGT ATG GGA GGG CTT GAT R: CGA GGG CAT TGG AAA CGT ATT
CN11028834	F: TTC GAC TAC CAA CGC CCA AC R: AGG ACC GGC AGC AGC TTT C
18S rRNA	F: TAG AGG GAC TGT CGG CGT CT R: CTC TCG GCA AGG CGG TAA GA

[a] F, forward; R, reverse.

As there are no cDNA microarrays available for *C. neoformans*, mRNA differential display was performed in an attempt to identify genes differentially expressed with auristatin PHE treatment. Differential mRNA display is a powerful method for detecting transcriptional modulation of known and novel genes in different cell populations or among cells under altered conditions (27). When drug exposure is investigated, as we did with auristatin PHE, the generated expression patterns can point to the involvement of previously uncharacterized genes and can provide information about drug mechanism of action and possible resistance mechanisms.

Total RNA was isolated from *C. neoformans* ATCC 90112, a cerebrospinal fluid isolate, the same strain used to examine the mechanism of action of auristatin PHE via immunofluorescence (62). Cells were treated with 1.5 times the MIC (7.89 μM or 6 μg/ml, as determined by broth microdilution in YM) of auristatin PHE or an equivalent volume of DMSO (control) for 90 min. We previously determined that, at this exposure time and concentration, cytoplasmic microtubules are partially disrupted and spindle microtubules are present (62) but cells are still viable (43, 61). Large-budded, uninucleate cells (approximately 25% of the population) become apparent after a 90-min exposure to 1.5 times the auristatin PHE MIC (data not shown). The viability of *C. neoformans* under these conditions is also supported by time-kill (43) and postantifungal effect studies (61). Seventy different combinations of anchor primers and arbitrary primers were used, covering approximately 60% of the cryptococcal transcriptome. This approximation is based on a theoretical calculation described by Liang (26) and assumes 10,000 mRNA species expressed per cell. Twenty-nine differentially displayed genes, the majority of them down-regulated, were detected. The seven gene fragments exhibiting the highest levels of differential expression, three up-regulated and four down-regulated, were subcloned and sequenced. Sequences were subjected to a BLAST search using the GenBank database and the SGTC *C. neoformans* Genome Project database. ORFs were determined according to the preliminary annotations of the SGTC *C. neoformans* Genome Project database and the tentative annotations of TIGR *C. neoformans* database. The *C. neoformans* Genome Project was launched in 1999 (21) and is a research collaboration between scientists at the SGTC and TIGR. The sequencing goal of a 12- to 13-fold genome coverage of *C. neoformans* was reached in October 2001, and the assembled contigs were posted publically. Errors are still being identified and removed. Sequence comparison of the differentially displayed cDNAs identified the *C. neoformans* adenylate cyclase gene and three ORFs with similarity to known genes in other fungi, as well as three ORFs which have not yet been associated with any function (Table 2). An inherent difficulty with *C. neoformans* differential display is that many ORFs code for transcripts of still unknown function. Differential expression for these transcripts was confirmed by RT-PCR normalized to 18S rRNA (Table 2; Fig. 1). Expression of seven additional differentially expressed genes was examined by RT-PCR only (Table 2; Fig. 1).

Effect on genes involved in transport. Three ORFs with similarity to transport proteins and the ABC transporter AFR1 were differentially regulated in auristatin PHE-treated *C. neoformans* (Table 2). Two of the ORFs, one down-regulated 3.9-fold and the other down-regulated 2.8-fold, showed 20% sequence identity to one of the *Schizosaccharomyces pombe* amino acid permeases and 41% sequence identity to *S. cerevisiae* Dip5p. The *S. pombe* amino acid permease (accession number T41612) has recently been identified (60), and no substrate has been defined. Dip5p, encoded by the *DIP5* gene, is a dicarboxylic amino acid permease, which mediates high-affinity transport of L-glutamate and L-aspartate in *S. cerevisiae* (46). Homologues of two amino acid permeases were down-regulated 2.7- and 3.7-fold in *C. albicans* after treatment with 10 μM itraconazole (13). Down-regulation of amino acid permeases may reflect slowing of metabolism in auristatin PHE-treated *C. neoformans*. The third ORF, encoding a protein which showed 47% similarity to the related to *myo*-inositol transport protein ITR1 in *Neurospora crassa*, was up-regulated 2.2-fold. No function has yet been assigned to the products of *related to ITR1* or *ITR1* in *N. crassa*. In *S. cerevisiae*, the *ITR1* gene encodes the *myo*-inositol transport protein ITR1, which is the major permease for inositol uptake (34). ITR1 belongs to the sugar transporter superfamily and is regulated by inositol and choline (33). Inositol is an essential component of the phospholipids in eukaryotic cells. Furthermore, phosphoinositides act as second messengers in a range of eukaryotic

TABLE 2. Changes in *C. neoformans* ATCC 90112 gene expression as obtained by RT-PCR in response to a 90-min exposure to 1.5 times the MIC of auristatin PHE

Role and gene name[a]	Accession no.	*C. neoformans* ORF[b] or TC[c] (% homology)[d]	Gene product or function	Mean fold change in gene expression (±SD)[e]
Transport				
Amino acid, inositol transport				
Weakly similar to amino acid permease (*S. pombe*)	T41612[g]	*TC8721* (partial, 20)	Amino acid permease	−3.94 (±0.14)
Similar to *DIP5* (*S. cerevisiae*)[f]	NP_015058[g]	*CN11001895* (41)	Dicarboxylic amino acid permease	−2.75 (±0.25)
Similar to related to *ITR1* (*N. crassa*)[f]	T51198[g]	*CN11011509* (47)	Related to *myo*-inositol transport protein	2.19 (±0.18)
Multidrug resistance				
AFR1	AJ318062		ABC transporter in drug resistance	−4.76 (±0.86)
Signal transduction pathways				
CAC1[f]	AF290191		Adenylate cyclase	−2.37 (±0.25)
RHO1	AB017639		G protein	−3.50 (±0.56)
Cell stress and DNA repair				
Similar to *HSP104* (*P. sajor-caju*)[f]	AF188207[g]	*CN11024231* (57)	Heat shock protein	3.08 (±0.09)
Similar to *RAD51* (*U. maydis*)	Q99133[g]	*TC10153* (partial, 43)	DNA repair protein	−4.98 (±0.68)
Purine biosynthesis				
ADE2	U70673		Phosphoribosylaminoimidazole carboxylase	3.64 (±0.25)
Capsule				
CAP60	AF030696		Capsule protein	−3.00 (±0.18)
Other				
Homolog to 14-3-3 (*S. commune*)	AAK33011[g]	*TC8511* (partial, 91)	14-3-3 protein	−2.21 (±0.28)
Unknown				
CN11014294[f]	NA[h]	*CN11014294*	Unknown	−3.12 (±0.32)
CN11018463[f]	NA	*CN11018463*	Unknown	3.62 (±0.43)
CN11028834[f]	NA	*CN11028834*	Unknown	−3.99 (±1.58)

[a] Listed are *C. neoformans* gene names or gene names for other species (in parentheses), to which the *C. neoformans* ORFs or tentative consensus (TC) sequences of interest exhibited highest sequence identity. ORF names are shown if no homology exists.
[b] *C. neoformans* (CN) ORFs are from the SGTC database. Nucleotide sequence identities between differential display cDNA clones and SGTC ORFs were at least 90%.
[c] TC sequences were created by the assembly of expressed sequence tags into virtual transcripts and are derived from TIGR database.
[d] Homology seaches and tentative annotations derived from the SGTC and TIGR databases.
[e] Differential gene expression is shown as the change during drug treatment relative to expression in DMSO-treated control strains, normalized to 18S rRNA. SD represent expression data of two independent experiments.
[f] Gene candidates and ORFs derived from the differential display.
[g] Accession numbers are for protein sequences.
[h] NA, not applicable.

signaling cascades and are regulators of membrane traffic (for reviews see references 6 and 14). Nine genes encoding products that facilitate membrane transport were up-regulated in *S. cerevisiae* with thiuram treatment, including the sugar transporter-encoding *JEN1* (23). If auristatin PHE influx occurs passively, induction of the *related to ITR1* gene could be involved in altering phospholipid content and thus membrane fluidity in an attempt to reduce drug uptake.

AFR1, down-regulated 4.8-fold, encodes the ABC transporter AntiFungal Resistance 1 (Afr1p) (44). Afr1p belongs to the multidrug efflux pump proteins of the ABC transporter family. Several fungal ABC transporters have been implicated in resistance to antifungal compounds, e.g., CDR1, CDR2 (29, 50), and pmd1$^+$ (35) and possibly play a role in virulence (56). Recently, Afr1p was shown to be involved in fluconazole resistance in *C. neoformans* (44). The apparent paradoxical down-regulation of a multidrug efflux pump in auristatin PHE-treated *C. neoformans* suggests that *C. neoformans* would have one less strategy for overcoming drug treatment. Whether or not this change at the transcriptional level has any clinical relevance remains to be seen.

Effect on genes involved in signal transduction pathways. *CAC1*, a gene involved in a conserved signal transduction pathway controlling cyclic AMP (cAMP) production, was down-regulated 2.4-fold (Table 2). The *CAC1* gene encodes the cryptococcal adenylate cyclase (1). The cAMP generated by adenylate cyclase is a ubiquitous second messenger molecule that enables a cell to respond to extracellular signals. It is activated or inhibited by the Gα subunits of heterotrimeric G proteins in response to ligand-activated G-protein-coupled receptors (1, 55). Although many signaling elements have yet to be described, the cryptococcal cAMP signal transduction pathway plays a central role in cellular pro-

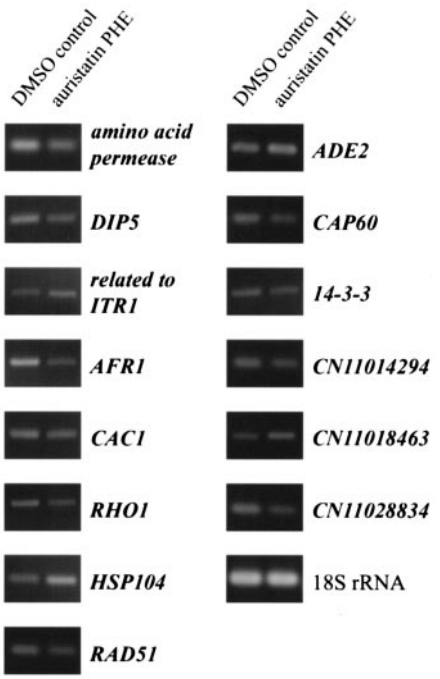

FIG. 1. RT-PCR of differentially expressed genes with auristatin PHE treatment in *C. neoformans* ATCC 90112. 18S rRNA served as a control.

cesses including encapsulation, melanin production, filamentation, mating, and virulence (1, 17, 59).

The cryptococcal *RHO1* gene, down-regulated 3.5-fold in the present study (Table 2), encodes the Rho1 protein, which has a high degree of identity with Rho1p in other fungal species (10, 54). The Rho GTPases belong to the Ras-related superfamily of small G proteins. These proteins act as molecular switches between the GTP-bound form (active) and the GDP-bound form (inactive) and interact with many different genes in different pathways. In yeast cells, such pathways include cell wall biosynthesis (2, 16), actin organization (18, 24), and bud emergence (22). Rho1p activates a (1-3)β-D-glucan synthase (2, 16) involved in polarized cell growth (16), is required for bud formation (22), and is involved in the regulation of cytoskeletal reorganization (24). Rho1p is localized at growth sites including the bud tip and the cytokinesis site (22, 24). Our microscopic documentation of arrested nuclear and cellular division in auristatin PHE-treated *C. neoformans* suggested that cell cycle checkpoints were disturbed, and thus down-regulation of *RHO1* is not surprising. Examination of Rho1p localization in auristatin PHE-treated *C. neoformans* cells would be of particular interest. Down-regulation of *CAC1* and *RHO1*, whose products are involved in many major cellular processes, may also indicate that auristatin PHE has a multifaceted mechanism.

Effect on genes linked to stress. An ORF encoding a protein with 57% identity to the heat shock protein HSP104 in *Pleurotus sajor-caju* was up-regulated 3.1-fold in auristatin PHE-treated *C. neoformans* (Table 2). In yeasts, this molecular chaperone plays an essential role in the induction of thermotolerance, as strains lacking HSP104 lose this ability (49). *HSP104* expression is drastically up-regulated in response to high temperature and during stationary-phase growth. Treatment with various agents, such as H_2O_2, cadmium, pesticides, and amphotericin B, has been shown to induce heat shock proteins (11, 20, 23, 31, 51, 63). HSP104 expression is induced after a 30-min exposure to the pesticide and fungicide thiuram in yeasts (23). Thus, it is not surprising that HSP104 was up-regulated in response to auristatin PHE.

Effect on genes linked to DNA repair. Another ORF, encoding a protein which shows 43% identity to the RAD51 protein in *Ustilago maydis*, was down-regulated fivefold (Table 2). RAD51, encoded by *RAD51*, is a DNA damage repair protein involved in the recombination repair pathway. As a structural and functional homolog of the bacterial RecA recombinase, the yeast RAD51 stimulates pairing and strand exchange between homologous single-stranded and double-stranded DNA (36). *RAD51* deletion strains of *S. cerevisiae* are viable but highly sensitive to ionizing radiation and are defective in meiosis (53). The major role for recombination during mitosis is most likely the eradication of DNA damage, including repair of DNA double-strand breaks and cross-links (37). In the presence of a DNA-damaging chromoprotein, *RAD50*, *RAD51*, and *RAD52* were up-regulated (51). However, in human PC-14 cells treated with TZT-1027, another synthetic derivative of dolastatin 10, the DNA repair protein XRCC1 was down-regulated 10-fold (32). Furthermore, a homolog of RAD26 involved in transcription-coupled DNA repair was down-regulated 2.8-fold in *C. albicans* after treatment with 10 μM itraconazole (13). Down-regulation of *RAD51* with auristatin PHE exposure suggests that auristatin PHE is not a DNA-damaging agent. Additionally, down-regulation of *RAD51* could be due to the loss of a cell cycle checkpoint.

Effect on genes linked to purine biosynthesis. *ADE2*, up-regulated 3.6-fold (Table 2), encodes phosphoribosylaminoimidazole carboxylase, which is one of the key enzymes in purine biosynthesis (19, 38). This enzyme is necessary for virulence of *C. neoformans*, as shown in the rabbit meningitis model (38). The relationship of *ADE2* up-regulation to the target or mechanism of action of auristatin PHE is unknown. As with any of the genes differentially expressed with auristatin PHE treatment, *ADE2* may simply be coregulated with genes that are directly or indirectly involved.

Effect on a capsule gene. There was a threefold decrease in *CAP60* expression during auristatin PHE treatment (Table 2). The cryptococcal polysaccharide capsule is one of the most important virulence factors of *C. neoformans* (9, 25). *CAP60* is one of the capsule-associated genes coding for Cap60p, which is primarily localized to the nuclear membrane (9). The exact function of *CAP60* is still unclear; however, it is required for capsule biosynthesis, a pathway yet to be defined. In addition, Cap60p may have as yet undescribed functions that are not related to capsule biosynthesis. Since Cap60p is located in the nuclear membrane, the decreased expression with auristatin PHE treatment may be related to the arrest of nuclear migration and division. It is also possible that down-regulation of capsule biosynthesis is part of an overall attempt to conserve energy for the repair of cellular damage.

Effect on other genes. There was a 2.2-fold reduction in the expression of an ORF encoding a protein exhibiting 91% similarity to the *Schizophyllum commune* 14-3-3 protein (Table 2).

14-3-3 proteins form a group of highly conserved, abundant, acidic, dimeric proteins, which are involved in a vast number of cellular processes, such as apoptosis, signal transduction, and cell cycle regulation (for a review, see reference 58). Their exact role in these processes remains unclear. If they are involved in cell cycle regulation in *C. neoformans*, their decreased expression in the presence of auristatin PHE would not be unexpected.

Effect on transcripts of unknown function. Transcripts *CN11014294* and *CN11028834*, of unknown function, were down-regulated 3.1- and 4-fold, respectively, and transcript *CN11018463* was up-regulated 3.6-fold (Table 2). Sequences of these transcripts did not show similarities to that of any other gene. Investigation of their protein products could yield clues to the mechanism of action and the mechanism of selective fungicidal activity of auristatin PHE.

Conclusion. In the last decade, differential display has become an extensively used technique for the sensitive detection of altered gene expression. However, there are associated disadvantages and limitations, including the high incidence of false positives and, most importantly, the technical difficulty in achieving complete genome coverage unless an inordinate number of reactions are performed. Differential display is thus by no means comprehensive, but it can point to novel and unforeseen correlations between biological pathways.

This is the first report of drug action using differential display in *C. neoformans*. This technique, as well as RT-PCR, was used due to the unavailability of *C. neoformans* cDNA microarrays. A portion of the cryptococcal transcriptome was analyzed in response to a single dose of auristatin PHE at a single time point, a dose and duration of exposure in which cells are viable but a fraction of the population begins to exhibit microtubule and cell division aberrations (43, 61, 62). A complete *C. neoformans* genome annotation and the availability of cDNA microarrays would make a multi-time point study much more feasible. We demonstrated microscopically that auristatin PHE affects microtubule dynamics in *C. neoformans*, resulting in abnormal nuclear migration and arrested cell division (62). In *C. neoformans*, there are two β-tubulin genes, *TUB1* and *TUB2* (12). *TUB1* is more conserved and more highly expressed and encodes a β-tubulin with approximately 81, 82, and 84% homology to β-tubulins from *Aspergillus nidulans*, humans, and *Schizophyllum commune*, respectively (12). In the present study, the cryptococcal β-tubulin gene *TUB1* (12) was not differentially expressed after a 90-min exposure to 1.5 times the auristatin PHE MIC (data not shown). Similarly, cDNA macroarray analysis of human cell lines treated for 6 h with the dolastatin 10 derivative TZT-1027 (another tubulin-damaging agent) revealed no alteration in expression of tubulin genes (32). In both cases, the expression changes noted may precede microtubule damage. Alternatively, microtubule disruption and the accompanying alterations to the cell cycle may be unrelated to tubulin gene expression. Auristatin PHE and related peptides may affect the function of nontubulin targets with roles in the cell cycle. Two transcripts down-regulated in the present study are involved in cell cycle regulation, *RHO1* and the ORF encoding a protein with 91% similarity to the *S. commune* 14-3-3 protein. The fact that there are other auristatin PHE-responsive genes, involved in transport, signal transduction pathways, cell stress, DNA repair, purine biosynthesis, and capsule production, suggests that additional cellular processes are affected by auristatin PHE. The effects of auristatin PHE on gene expression may be direct or indirect; for example, the cascade of gene expression changes that result from the global stress response is a common indirect effect of altered cellular conditions. Further investigation of the differentially expressed genes and their products, in combination with phenotypic studies, such as studies of capsule or melanin production and the localization of Rho1p, is warranted. Identification of the auristatin PHE target(s) will aid in the design of other *C. neoformans*-specific drugs.

As sequences in the cryptococcal genome gradually become available in public databases and a consensus on the sequence annotation is reached, genome-wide transcript profiling using *C. neoformans* cDNA microarrays will be possible. This will allow greatly expanded functional genomic analyses to help elucidate modes of action of auristatin PHE and other antifungal agents in *C. neoformans*. Knowing more about the mechanism of action of auristatin PHE will facilitate the design and synthesis of related compounds with enhanced pharmacological profiles.

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