Effects of Organoantimony Compounds on Fungal Pathogens *Cryptococcus neoformans* and *Candida albicans*

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Abstract

Cryptococcus neoformans is an opportunistic pathogen that causes pulmonary cryptococcosis and cryptococcal meningitis in immune-compromised individuals. *Candida albicans*, also opportunistic, can cause pulmonary candidiasis, genitourinary tract infections, candidemia, and oral candidiasis. Fungal infections are responsible for approximately 1.7 million annual deaths. With few antifungal drugs, high toxicity, and increased resistance to antifungals, the importance of finding new antifungal therapies is crucial. We hypothesized that novel organoantimony compounds would effectively restrict fungal growth. We tested approximately 20 compounds against *C. neoformans* and *C. albicans* in minimum inhibitory concentration (MIC) assays. Compounds A, B, E, I, F, and G were effective against *C. neoformans* with MIC concentrations of 10.94 µg/ml, 19.79 µg/ml, 18.75 µg/ml, 12.5 µg/ml, 20.83 µg/ml, and 2.60 µg/ml, respectively. Compounds E and G were effective against *C. albicans* at 15.625 µg/ml and 25 µg/ml, respectively. Compounds I and G were fungicidal against *C. neoformans* at concentrations 50 µg/ml and 25 µg/ml, respectively, and compound G was fungicidal against *C. albicans* at 50 µg/ml. Cytotoxicity assays showed that antifungal compounds A, B, E, I, F, and G were non-toxic. RNA sequencing studies have identified several *C. neoformans* genes involved with the compounds' inhibitory effects.

Introduction

Cryptococcus neoformans is a fungal pathogen found around the world in soil and bird droppings. While most individuals who encounter the fungi do not suffer any negative effects, those who are immune-compromised, such as patients with AIDS, transplants, or chemotherapy, can become infected. This opportunistic pathogen is known to cause pulmonary cryptococcosis, or an acute or chronic infection in the lungs, and cryptococcal meningitis, an infection of the brain and spinal column. It is estimated that *C. neoformans* infects 275,000 and kills approximately 180,000 people each year. Another fungal pathogen, *Candida albicans*, is commonly found in the vaginal and digestive mucosa. *C. albicans* has been known to cause vaginal infections (predicted to affect 75% of women in their lifetime), oral candidiasis in 90% of HIV-infected individuals with AIDS, and nosocomial infections (with an estimated mortality rate of 60%). In contrast to antibiotics, the quantity of available antifungal drugs remains low. There are only four main classes of antifungal drugs: polyenes, azoles, allylamines, and echinocandins. With the increased resistance to antifungals in recent years, the importance of finding new options for antifungal therapy is crucial. In this study we sought out to determine if a new class of organoantimony compounds had fungistatic or fungicidal effects on *C. neoformans* and *C. albicans*.

Methods

Strains and Media: *C. neoformans* strain H99 and *C. albicans* strain SC5314 were grown in a YPD (Yeast Peptone Dextrose) broth at 30ºC in a shaking incubator for 18hrs. The cells were then washed with sterile phosphate-buffered saline (PBS) three times with the use of a centrifuge. The concentration of cells was counted using a hemacytometer, with trypan blue being used to exclude dead cells. The concentrations for the assay inoculums were determined using the hemacytometer counts. Macrophage cell line J774A.1 was grown in J774 media and passaged every 3-4 days. The macrophages were washed with sterile J774 media three times with the use of a centrifuge and counted similarly to *C. neoformans* and *C. albicans*.

Compounds: Novel organoantimony compounds produced by Dr. Nikolay Gerasimchuck's lab at Missouri State University were tested to determine their fungistatic and fungicidal properties

Minimum Inhibitory Concentration (MIC) Assay: *C. neoformans* and *C. albicans* were incubated with different organoantimony compounds to test the compounds' inhibitory effects. A 96 well plate was filled with differing concentrations of the tested compound, diluted 1:2 with RPMI-MOPS, from 100 µg/ml down to 0.098 µg/ml. Each row on the plate served as a replicate, and the first column contained only RPMI-MOPS, serving as the control. A concentration of $0.5x10^3$ cell/ml of the tested fungi (also diluted in RPMI-MOPS) was mixed into each well of the plate. The plate was then incubated in a humidified incubator at 35ºC for 48 hours. Following incubation, the wells were resuspended, and visual inspection and an optical density at 490 was measured on a plate reader (BioTek) to determine MIC. The concentration that resulted in reduced fungal growth compared to the control was determined to be the MIC.

Minimum Fungicidal Concentration (MFC) Determination: If a MIC was found, two replicates from the the MIC column and two concentrations above were plated on a YPD plate. The plates were then incubated in an inverted position for 48 hours at 30ºC. Following incubation, the number of colony forming units (CFUs) on each plate were counted. The MFC is the concentration of the column corresponding to the plate with no fungal growth. If each of the plates have growth, then either the compound does not have an MFC, or it is greater than 100 µg/ml.

Cytotoxicity Assay: J774A.1 cells at a concentration of $1.0x10^6$ µg/ml were incubated with different organoantimony compounds at their MIC concentration for 24 hours with J774 media alone and untreated J774A.1 cells serving as negative controls and J774A.1 cells treated with 100X cell-lysis buffer for the positive control. Vybrant Cytotoxicity Assay Protocol was followed to determine percent cytotoxicity. Percent cytotoxicity below 30% was considered non-toxic.

RNA Extraction and Sequencing: *C. neoformans* and *C. albicans* at concentrations of $10x10^6$ µg/ml were incubated with different organoantimony compounds at the MIC concentrations for 24 hours. RNA was then extracted and purified according to the Qiagen AllPrep Fungal DNA/RNA/Protein Quick-Start Protocol. RNA purity was verified via 260/280 optical density measurements on a plate reader (BioTek). RNA was sent to Novogene for sequencing.

Data Analysis: GraphPad Prism version 5.00 for Windows was used to create graphs and conduct statistical analyses. One-way ANOVA with the Tukey's multiple comparison test (to compare pairs of columns) was used to compare data between 3 groups, and an unpaired t-test was used to compare data between 2 groups.

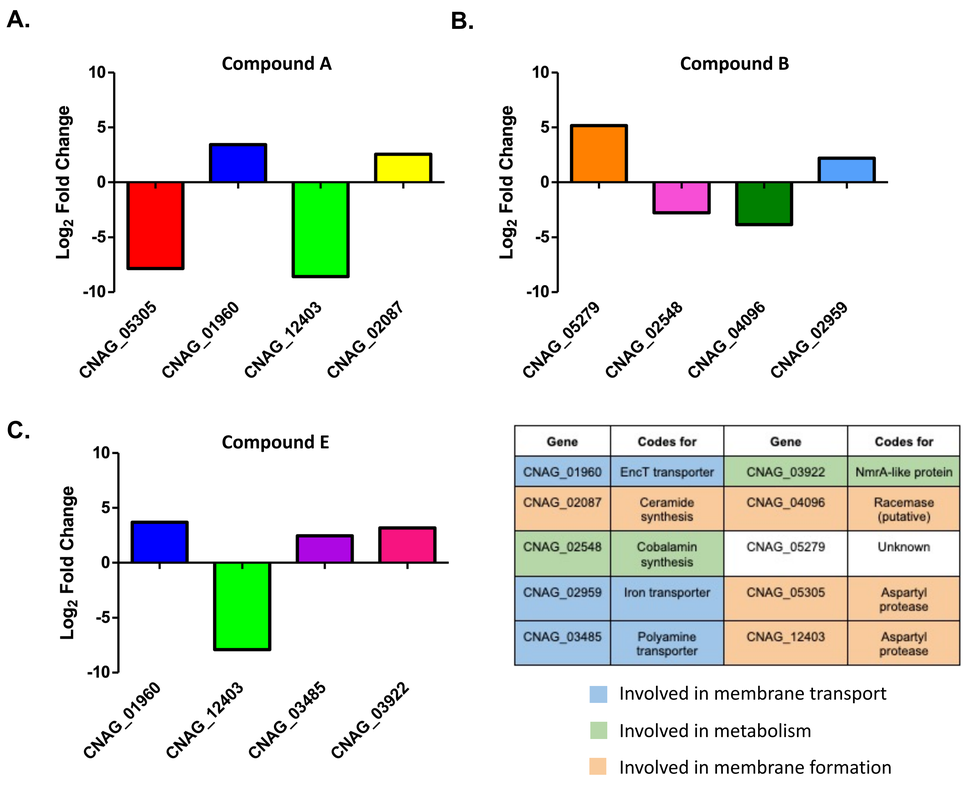
Results

Figure 1. MIC Concentrations of Compounds with *C. neoformans* and *C. albicans*

Backbone Controls MIC, µg/ml	*C. n.*	*C. a.*	Side Group Controls MIC, µg/ml	*C. n.*	*C. a.*	Compounds MIC, µg/ml	*C. n.*	*C. a.*
Control 1	41.67	50	Control 3A	>100	>100	Compound A	10.93	31.25
			Control 3B	>100	>100	Compound B	19.79	32.14
						Compound C	33.33	100
						Compound D	25	100
			Control 3E	>100	>100	Compound E	18.75	15.625
Control 2	>100	>100	Control 3F	>100	>100	Compound F	20.83	100
			Control 3G	>100	>100	Compound G	2.604	25
						Compound H	>100	>100
						Compound I	12.5	50
			Control 3J	25	100	Compound J	>100	>100
			Control 3K	25	100	Compound K	>100	>100
						Compound L	100	>100

Conclusion. MICs for each of the compounds and their controls tested with *C. neoformans* (*C. n.*) and *C.* albicans (*C. a.*). Detected MICS are shown in green and those with resistance up to 100 µg/ml are shown in red. Results indicate that Compounds A, B, E, I, F, and G were most effective at inhibiting *C. neoformans* growth with MIC concentrations of 10.94 µg/ml, 19.79 µg/ml, 18.75 µg/ml, 12.5 µg/ml, 20.83 µg/ml, and 2.60 µg/ml, respectively. Compounds E and G were most effective at inhibiting *C. albicans* growth with MICs of 15.625 µg/ml and 25 µg/ml, respectively. Compounds I and G were fungicidal against *C. neoformans* with MFCs 50 µg/ml and 25 µg/ml, respectively, and compound G was fungicidal against *C. albicans* with an MFC of 50 µg/ml (not shown). All MIC experiments were done in triplicates to quadruplicates.

Figure 2. Genes Up- and Down-regulated in *C. neoformans* Treated with Antifungal Compounds

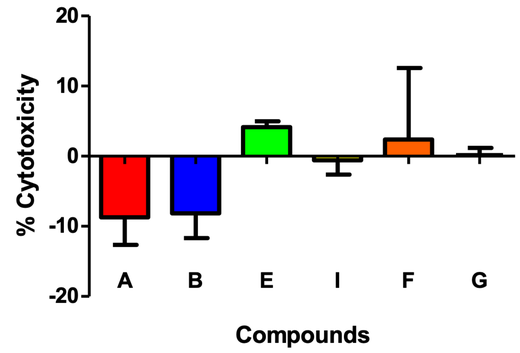


Gene	Codes for	Gene	Codes for
CNAG_01960	EncT transporter	CNAG_03922	NmrA-like protein
CNAG_02087	Ceramide synthesis	CNAG_04096	Racemase (putative)
CNAG_02548	Cobalamin synthesis	CNAG_05279	Unknown
CNAG_02959	Iron transporter	CNAG_05305	Aspartyl protease
CNAG_03485	Polyamine transporter	CNAG_12403	Aspartyl protease

Involved in membrane transport
Involved in metabolism
Involved in membrane formation

Conclusion. RNA sequencing of untreated *C. neoformans* is compared with *C. neoformans* treated with compounds A (A), B (B), and E (C). Treatment with compound A resulted in the up-regulation of genes CNAG_01960 and CNAG_02087 and the down-regulation of genes CNAG_05305 and CNAG_12403 (A). Treatment with compound B resulted in the up-regulation of genes CNAG_05279 and CNAG_02959 and down-regulation of genes CNAG_02548 and CNAG_04096 (B). Treatment with compound E resulted in the up-regulation of genes CNAG_01960, CNAG_03485, and CNAG_03922, and the down-regulation of gene CNAG_12403 (C). RNA sequencing was performed in triplicates and all $\log_2$ fold changes had a p-value of $1x10^{-38}$ or lower.

Figure 3. Percent Cytotoxicity of Antifungal Compounds



Conclusion. Cytotoxicity assay results with J774A.1 cells. Compounds A, B, E, I, F, and G had cytotoxicity percentages of -8.73%, -8.13%, 4.15%, -0.59%, 2.37%, and 0.15%, respectively. All the cytotoxicity percentages are below 30% and the compounds are therefore deemed non-toxic.

Conclusions & Future Directions

MIC/MFC Assays:
- Compounds A, B, E, I, F, and G were most effective at inhibiting *C. neoformans* growth
  - Compounds I and G exhibited fungicidal activity against *C. neoformans* at 50 µg/ml and 25 µg/ml
- Compounds E and G were most effective at inhibiting *C. albicans* growth
  - Compound G exhibited fungicidal activity against *C. albicans* at 50 µg/ml

RNA Sequencing:
- Identified genes associated with membrane transport, membrane formation or metabolism that have been up- or down-regulated in *C. neoformans* cells treated with Compounds A, B, or E

Cytotoxicity Assays:
- Compounds A, B, E, I, F, and G are non-toxic

Future Studies:
- Continue RNA sequencing studies using compounds A, B, E, I, F, and G with *C. albicans* and compounds I, F, and G with *C. neoformans*
- Synergistic studies with existing drugs such as Amphotericin B and caspofungin
- Test compounds against drug resistant strains of *C. neoformans* and *C. albicans*
- Test the effectiveness of the compounds in a *C. neoformans* and *C. albicans* larval moth infection model using *Galleria mellonella*

Mechanistic studies
- Scanning and transmission electron microscopy (SEM and TEM) will be performed to identify the compounds' effects on the fungal cell wall and membrane
- Test compounds against mutant strains of *C. neoformans* that are knock-outs for the genes identified to be up- or down-regulated in RNA sequencing in order to identify genes involved in antifungal resistance and point to antifungal mechanism

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