Targeting *Aspergillus fumigatus* hypoxia response pathways to potentiate contemporary antifungal therapies

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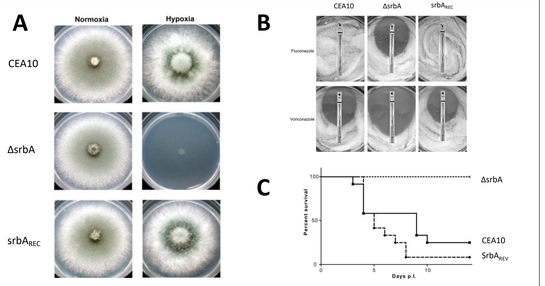
SrbA Is Required For Hypoxia Adaptation, Azole Drug Resistance, And Virulence In *Aspergillus fumigatus*

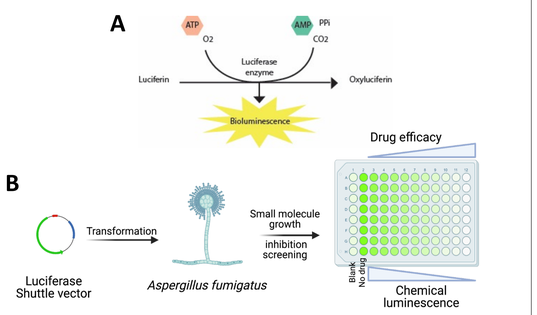


A. SrbA is required for hyphal growth under hypoxic conditions. 1×10^6 conidia of CEA10, *ΔsrbA*, $srbA_{REC}$ *(ΔsrbA+srbA)* were plated on GMM plates and incubated at 37°C under normoxic and hypoxic conditions for 96 hours.

B. SrbA mediates resistance to Fluconazole (FL) and Voriconazole (VO) in Aspergillus fumigatus. E-test strips (AB Biodisk, N.J.) plastic strips impregnated with a gradient of Fluconazole and Voriconazole were used per manufacturers' instructions. Each strip was placed onto a RPMI-1640 (Sigma Aldrich) agar plate containing a lawn of conidia and growth inhibition was measured after 24 and 48 hours by direct observation of the plates at 37°C.

C. SrbA is necessary for virulence in murine model. Outbred CD-1 mice (n = 12) were immunosuppressed by i.p. injection of cyclophosphamide (150 mg/kg) 2 days prior to infection and s.c. injection of Kenalog (40 mg/kg) 1 day prior to infection and injection of 150 mg/kg cyclophosphamide 3 days post-inoculation and 40 mg/kg Kenalog 6 days post-inoculation. Mice were inoculated intranasally with 10^6 conidia in a volume of 40 µl of wild type CEA10, *ΔsrbA* mutant strain and the *srbA* reconstituted strain. P value for comparison between *ΔsrbA* and wild type CEA10, P = 0.0002.

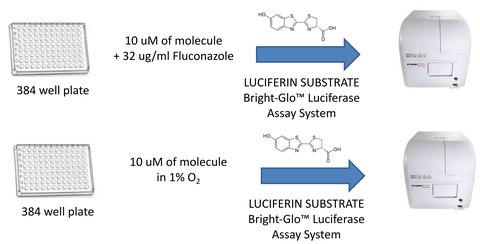
Adapting a gpdA-luciferase Reporter for High Throughput Screening of *Aspergillus fumigatus*



Overview of bioluminescence based high-throughput phenotypic screen for small molecules with anti *Aspergillus fumigatus* activity.

A. Mono-oxygenation of luciferin substrate into oxyluciferin is mediated by the luciferase enzyme to output measurable bioluminescence. (Promega protocols). B. A construct containing a luciferase encoding gene driven by a strong constitutive promoter is introduced into an *A. fumigatus* strain of choice and used in a phenotypic screen to identify small molecules with antifungal activity as indicated by a reduction in bioluminescence compared to controls. Figure Created with Biorender.com

Adapting a gpdA-luciferase Reporter for High Throughput Screening of *Aspergillus fumigatus*



C. Summary of high throughput azole potentiator and hypoxia inhibition screens

An *A. fumigatus* strain containing a luciferase encoding gene driven by a strong constitutive promoter is used in a phenotypic screen to identify small molecules with azole potentiator properties or increased hypoxia efficacy as indicated by a 70% reduction in bioluminescence compared to controls.

High Throughput Screen - Results Summary

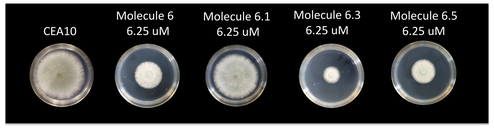
Table 1. Summary of *A. fumigatus* Fluconazole Potentiator High Throughput Screen

Screening Stage	Number of Compounds	% Total
Total Screened	206,240	100
Primary Hits	1266	0.614
Med. Chem. Pass	1203	0.583
Confirmed Hits	236	0.114
Re-Ordered from Vendor	53	0.026
MIC ≤ 10 µM (+ 32 µg/ml Fluc.)	39	0.019
$MIC_{(-Fluc)}$ / $MIC_{(+Fluc)}$ ≥ 4	**29**	**0.014**

Table 2. Summary of *A. fumigatus* Hypoxia High Throughput Screen

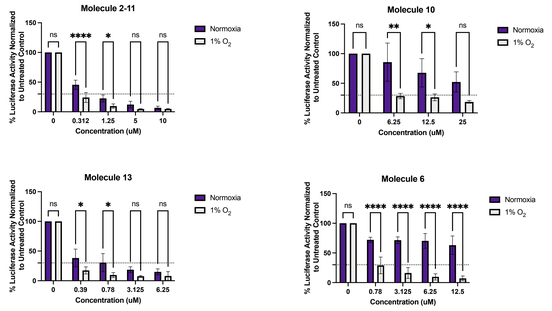
Screening Stage	Number of Compounds	% Total
Total Screened (to date)	84,273	100
Primary Hits	474	0.563
Med. Chem. Pass	171	0.613
Confirmed Hits	47	0.168
Hypoxia Specific	1	0.004
Re-Ordered from Vendor	1	0.004
MIC ≤ 10 µM Hypoxic Growth	1	0.004
$MIC_{Normoxia}$ / $MIC_{Hypoxia}$ ≥ 4	**1**	**0.004**

We Have Identified Analogs of Drug-Like Compounds With Increased Efficacy Against *A. fumigatus*



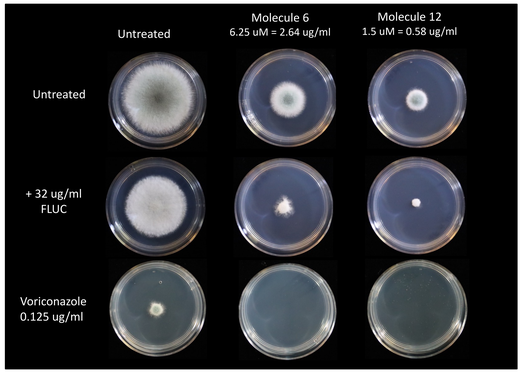
1x10^3 conidia of laboratory strain CEA10 were inoculated onto glucose minimal media plates treated with molecule 6 or analogs 6.1, 6.3 and 6.5 and incubated for 72 hours (37°C, 5% CO_2).

Low Oxygen Conditions Potentiate The Effect of Drug-Like Compounds Against *Aspergillus fumigatus*



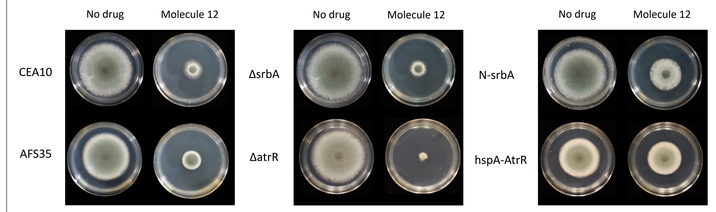
Molecules with significant antifungal activity in hypoxia were further validated using the wild-type *A. fumigatus* luciferase reporter strain. 5x10^4 *A. fumigatus* spores were inoculated into 96-well microplates in liquid glucose minimal medium and incubated for 24 hours (normoxia) or 27 hours (1% oxygen, hypoxia) in the presence or absence of the respective small molecules. Luciferase activity was measured by incubating each plate with 10 µL of BrightGlo Luciferase reagent for 20 minutes and plates were read using a Synergy Neo2 multi-mode plate reader. Relative light units (RLU) were normalized to the untreated controls. Data represent the mean and SEM from 3 independent biological replications. Statistical significance was measured with two-way ANOVA with Tukey's multiple comparisons test. A – molecule 6 ****P = < 0.0001; B – molecule 10 **P = 0.0015, *P= 0.0206; C – molecule 13 *P = 0.0199; D – molecule 2-11 ****P = < 0.0001, *P = 0.0150.

Treatment With Drug-Like Compounds Potentiates Azoles Against *Aspergillus fumigatus*



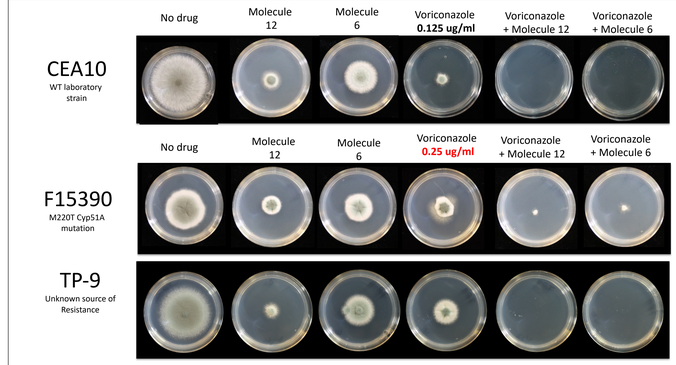
Molecules with significant synergy with fluconazole were further validated using the wild-type *A. fumigatus* CEA10 laboratory strain. 1x10^3 *A. fumigatus* spores were inoculated into glucose minimal medium plates supplemented with fluconazole (32 ug/ml), voriconazole (0.125 ug/ml), molecule 6 (6.25 uM), molecule 12 (1.5 uM) or a combination between the azoles and the molecules. Plates were incubated for 72 hours. Data shown is representative images of 3 independent biological replications.

Over-expression Of Hypoxic Regulators SrbA And AtrR Increases Resistance To Drug-like Compounds Suspected To Target The Hypoxia Response Pathway



A. 1x10^3 conidia of laboratory strains CEA10 and AFS35, SrbA loss of function mutant (ΔsrbA, CEA10 background), SrbA overexpression strain (N-srbA, CEA10 background), AtrR loss of function mutant (ΔatrR, AFS35 background) and AtrR overexpression strain (hspA-AtrR, AFS35 background) were inoculated onto glucose minimal media plates treated with Molecule 12 (1.5 uM = 0.58 ug/ml). Plates were incubated for 72 hours (37°C, 5% CO_2).

Co-Treatment With Drug-like Compounds Increases Efficacy Of Voriconazole Against Voriconazole Resistant Clinical Isolates



1x10^3 conidia of laboratory strain CEA10 **(A)**, and voriconazole resistant clinical isolates F15390 **(B)** and TP-9 **(C)** were inoculated onto glucose minimal media plates treated with voriconazole (0.125 ug/ml or 0.25 ug/ml), Molecule 6 (6.25 uM = 2.64 ug/ml), Molecule 12 (1.5 uM = 0.58 ug/ml) or combination treatment of voriconazole with one of the indicated molecules. Plates were incubated for 72 hours (37°C, 5% CO_2).

Funding and Acknowledgements

The Moye-Rowley Lab for A. fumigatus strains (AFS35, ΔatrR and hspA-AtrR)