Investigating MCE Chemical Library Drugs for Combinational Therapies for Clinical *Aspergillus fumigatus* isolates

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Abstract

Aspergillus fumigatus is a globally present pathogen capable of inflicting debilitating and life-threatening opportunistic infections in individuals, primarily those who are immunocompromised. Diagnosing *A. fumigatus* infections is often difficult, leading to a delay in treatment which can greatly impact patient outcomes. Furthermore, our lessening of antifungal development combined with increasing resistance generates a feasible scenario where only last resort options are viable. This has prompted the World Health Organization (WHO) to declare this pathogen a "critical priority" due to increased resistance and rising mortality rates. Azoles are utilized as primary treatment options for *Aspergillus fumigatus* infections such as voriconazole (VRC), itraconazole (ITC), and posaconazole (POS) with a reserve of Amphotericin B (AmB). In the past two decades, the emergence of resistance to azoles has contributed to a 90% mortality rate in resistant cases globally.

In this report, we investigated the MedChem Express (MCE) Drug Repurposing Compound Library (4,226 compounds) in conjunction with itraconazole at 0.06 µg/mL against *A. fumigatus* CDC #738. After the initial screening, we identified compounds known to be antifungals or antiseptics and deselected them. The remaining thirty selected compounds were evaluated through published literature and clinical trial data to determine those candidates with favorable characteristics/properties. Criteria for candidate selection consisted of evaluating the compounds; plasma concentration peak, the time to reach peak, protein binding, oral availability, and drug class. Six candidates were ranked the highest of the previous round –surprisingly 50% of those

compounds were HIV drugs, cobicistat, elvitegravir, lopinavir. The remaining three selected compounds are penfluridol, rilapladib, and rolapitant.

The combination of itraconazole (ITC), posaconazole (POS), and voriconazole (VRC), with the identified compounds demonstrated promising amounts of synergy, in resistant and susceptible isolates.

Further investigation revealed novel properties of ITC and POS when in combination with our compounds of interest. Rilapladib was able to reverse POS, ITC, and VRC resistant strain(s) to a sensitive profile. Growth kinetic assays demonstrate potent anti-germination properties not seen before in the sub-inhibitory doses of azoles. This work demonstrates that high-throughput screening as a viable technique to identify robust antifungal synergizers, allowing for tenable translation to a clinical setting.

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General Audience Abstract

Aspergillus fumigatus is a worldwide fungal organism capable of causing disease, particularly in immunocompromised individuals. Infections primarily occur when individuals inhale spores that can remain dormant until the person's immune system is weakened, via disease, cancer, or prescribed drug for surgery. When the immune system is weakened, the spores are more effective at lung colonization. *Aspergillus fumigatus* infections can be combatted with voriconazole (VRC), itraconazole (ITC), or posaconazole (POS). However, in the past two decades, some fungi have started to develop resistance to azoles, necessitating the use of amphotericin B (AmB), a highly intolerable and final treatment option.

In this report, we challenged *A. fumigatus* CDC isolate #738 with compounds from the MedChem Express (MCE) Drug Repurposing Compound Library (4,226 compounds) with itraconazole at 0.06 μg/mL, an ineffective concentration. We selected combinations and compounds that negated 90% of fungal growth those combinations that contained a known antifungal or antiseptic agent; once identified we deselected any known antifungal or antiseptic agents. The remaining selected compounds were evaluated for favorable drug properties, by reviewing published literature and clinical reports to determine those candidates with favorable characteristics/properties. The combination of ITC, POS, and VRC with the identified compounds demonstrated antifungal enhancement in resistant and susceptible isolates.

We observed the reversal of resistance to POS, ITC, and VRC in several isolates when the drug combinations were applied. This demonstrates the importance of evaluating approved and under current review drugs to identify novel properties to aid our dwindling number of effective

antifungals. This study provides promising combinational therapies for further evaluation in combating resistant *A. fumigatus* infections.

Dedicated to my Mother, Jeff, Spenser, Paddington, Neil, Teal'C, Azul, Sabrina, Benny, Cooter, Roscoe, Woody and friends for their unconditional love and support

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List of Abbreviations

ABC	**ATP-Binding Cassette**
ADD	**Additive**
ADP	**Adenosine diphosphate**
ANOVA	**Analysis of variances**
ANT	**Antagonistic**
ATCC	**American Type Culture Collection**
A. fumigatus	***Aspergillus fumigatus***
BBB	**Blood Brain Barrier**
BCE	**Before Common Era**
BCRP	**Breast Cancer Resistance Protein**
BEI Resources	**Biodefence and Emerging Infections Research Resource Repository**
BP	**Base Pairs**
CDC	**Centers for Disease Control and Prevention**
CLSI	**The Clinical & Laboratory Standards Institute**
Cobi	**Cobicistat**
DMSO	**Dimethyl sulfoxide**
Elvit	**Elvitegravir**
FDA	**Food and Drug Administration**
FICI	**Fractional Inhibitory Concentration Index**
FLC	**Fluconazole**
FMGX	**Fosmanogepix**
GPCR(s)	**G protein-coupled receptors**
GPI	**Glycosylphosphatidylinositol**
H	**Histidine**
HR	**Hour**
HIV	**Human Immunodeficiency Virus**
Hmg-CoA	**Hydroxymethylglutaryl-CoA**
IBX	**Ibrexafungerp**
IND	**Indifferent**
I.N.D.	**Investigational New Drug (Application)**
ITC	**Itraconazole**
ISV	**Isavuconazole**
L	**Lysine**
LPV	**Lopinavir**
MCE	**MedChemExpress**
MDR1	**Multi-Drug Resistance-1 P-glycoprotein**
mL	**Milliliter**
MOA	**Mechanism of Action**
MRP3	**Multi-drug resistance protein 3**
MFS	**Major Facilitator Superfamily**
MIC	**Minimum Inhibitory Concentration**
NCC	**National Clinical Collection**
NIH	**National Institutes of Health**

OD	**Optical Density**
PBS	**Phosphate Buffer Saline**
PD	**Potato Dextrose**
Pen	**Penfluridol**
P-gp	**P-glycoprotein**
POS	**Posaconazole**
RZF	**Rezafungin**
SYN	**Synergy**
TR	**Tandem Repeat**
VRC	**Voriconazole**
WHO	**World Health Organization**

Chapter 1. Introduction

1.1 Azole Antifungals

The first fungal pathogen was described in 500 BCE; however novel pathogenic organisms have arisen within the past century [1]. Fungal infections represent a small and often overlooked group of diseases, with many states within the United States (US) rarely mandating reporting [2]. This contributes to lack of knowledge surrounding accurate infection and resistance rates.

Development of antifungals began in the early 1900s with the groundbreaking development of orally available drugs in the 1960s [3]. The initial class of antifungals, iodinated trichlorophenols marked a revolution in the ability to treat fungal diseases both topically and systemically[3, 4]. Arising from the increasing interest in antifungal therapies were imidazoles –a class of azoles, with benzimidazole, developed in 1944, and chlormidazole in 1958. The class azole represents a majority of frontline and secondary treatment options for a wide range of fungal infections. This class works by binding to an enzyme, lanosterol 14-α-demethylase, thereby inhibiting the formation of ergosterol, a crucial cell wall component [3, 5, 6]. These drugs target fungal cell wall synthase enzymes, and have an off-target side effect of inhibiting P540 mammalian enzymes, resulting in drug intolerance and toxicity [6]. Initially, miconazole became the first azole available for treatment for nearly two decades before being discontinued due to toxicity [6]. Ketoconazole arrived next and due to its oral formulation it became the standard treatment option. Following the advancement of these two antifungals, third generation azoles, fluconazole (FLC), and ITC were developed, each having increased efficacy against fungal infections and decreased side effects compared to their predecessors [6, 7]. Importantly, this third generation of azoles was rationally designed such that resistant strains could be effectively challenged [7]. FLC, while effective, safer, and purposefully designed, lacked the ability to effectively challenge molds, e.g.,

A. fumigatus; therefore, voriconazole (VRC) was approved to aid the armament of itraconazole (ITC) in 2002, as a triazole (3rd generation azoles) [7]. Researchers within Schering Plough Research Institute developed a new compound, SCH51048 that displayed antifungal properties well above expected values [8]. This led them to evaluate any active metabolites of the compound, thereby discovering posaconazole a broad-spectrum antifungal [7, 8]. Due to ever-increasing resistance, a second generation of triazoles (e.g., isavuconazole) were rationally designed to reduce off target interactions, generating tetrazoles (4th generation) with better selectivity [7].

1.2 Antifungal Resistance in *Aspergillus fumigatus*

A. fumigatus infections are often associated with 40-90% mortality, due to treatment failure and delayed diagnosis [9]. Treatment failure can result from a delay in proper treatment as well as antifungal resistance. Antifungal resistance is described as the ability of the pathogen to overcome antifungal drug therapy via a variety of mechanisms, e.g., efflux, and target modification [10]. Novel mechanisms of resistance are being debated and proposed as we gain further insight into this pathogen [11, 12]. We are aware of know mechanisms of resistance that readily adapt to novel drugs [15, 18].

It is important to understand the resistance mechanisms employed by the fungal pathogen against previous generations of azoles because they can contribute to resistance against newer generations. A primary driver for increased resistance to azoles can be attributed to off-target agricultural fungicide application. While *Aspergillus spp.*, are capable of colonizing humans, they do not need a human host and can persist in the environment. This means that *Aspergillus spp.*, encounters antifungal azoles—mefentrifluconazole, paclobutrazole, and prothioconazole, utilized in agriculture resulting in environmental isolates gaining resistance to clinical azoles without pre-exposure to them[13]. In one case it was determined that >90% of isolates retained genetic

resistance hallmarks while being described as clinically azole naïve [14]. This demonstrates that agricultural resistance mechanisms can readily translate into clinical resistance mechanisms. Agricultural azoles are preferred as common pesticide applications due to their relative broad-spectrum activity, efficacy on plant fungal infections, and low cost [13, 15]. The over-application of these azoles allows for increased interaction with azole naïve *A. fumigatus*. The WHO has observed high azole resistance "rates of 15-20% … in parts of Europe and over 80% … in Asia." among *A. fumigatus* isolates as a result of these interactions [16]. These increasing resistance rates carry with them an even more concerning statistic, a 90% mortality rate observed in clincs [14, 17]. Furthermore, as *A. fumigatus* infections are not commonly reported, the numbers for resistance are possibly inaccurate and at worse underestimates [16].

Mechanisms of resistance in *A. fumigatus* primarily rely on modification of the azole target (Table 1.1). A single point mutation within the gene *cyp51A,* contributes to a variety of treatment failures. Such mutations result in single amino acid substitutions e.g., G54, M220, L98H, and Y121F [14]. Alongside the mutations within the protein structure, tandem repeats (TR) are frequently observed with the L98H mutation. These tandem repeats occur within the 34, 46, or 53 bp (base pair). The most observed mutation pattern generating azole resistance is TR_{34}/L98H [14, 17]. Others, such as TR_{46}/Y121F/T289A, form VRC resistance [15, 18]. A TR_{46}/Y121F/T289A mutation is less common but carries with it the potential to pass on resistance to recently developed azoles [15, 18].

In the past decade, interest has grown in the involvement of efflux pumps, ATP-binding cassette (ABC), and major facilitator superfamily (MFS) transporters in antifungal resistance [19]. They have been implicated in resistance due to increased activity observed for azoles, a substrate of select efflux pumps[19]. Comprising the ABC superfamily are three subsets, *ABCB*, *ABCC*, and

ABCG, which are then placed into the category of multi-drug resistance family due to their capabilities to export antifungals, virulence factors and secondary metabolites [19]. Alongside these subsets are *CDR1* and *CDR2*, which are well-defined in *C. albicans* [19]. Research has shown that in response to azole exposure, e.g., itraconazole *A. fumigatus*, *abcA-D*, *atrF*, *Mdr1*, *mfsA, -C,* and *CDR1* are all elevated, 4–14 fold in response [20]. Furthermore, in voriconazole and posaconazole resistant strains, it was observed that the basal expression of *CDR1* increased 5–75 fold compared to a wild-type strain *A. fumigatus* #293 [20]. Novel cryptic mutations are coming to light that produce similar effects of *cyp51A* point mutations while being a wholly separate mechanism. Hmg-CoA Reductase mutations have demonstrated an ability to enhance ergosterol levels in a similar fashion to *cyp51A* mutations [11, 12]. In summation, rising levels of ergosterol are capable of producing azole resistance isolates, through a variety of *cyp51A* point mutations and TRs, and HMG-CoA mutations. When these mutations work in tandem with elevated efflux pump levels, treatment options for these isolates rapidly diminish. Therefore, it is of urgent importance that novel treatment options are available to reduce azole resistance generation, through agricultural and clinical treatment modification.

Table 1.1 Resistance mechanisms in *Aspergillus fumigatus*

Aspergillus fumigatus	*Resistance Mechanisms*	*Clinical Outcome*	*Reference*
CDC # 731, 733, 734, Netherlands 2007, Germany 2012 (2), India 2012, Iran 2013	L98H/TR34	N/A, N/A, N/A, N/A, Alive (2), N/A, N/A	[21], [14]
CDC # 732, 735	F495I/L98H/S297T/TR34	N/A, N/A	[21]
Netherlands 2008	L98H/TR34/M220V	Alive (7), Died (2)	[14]
Netherlands 2009	TR53	Cured	[14]
Netherlands 2011, Belgium 2011, 2014, US 2016, France, 2016, Tanzania 2016	TR46/Y121F/T289A	Died, Died, Died, Died, Died, Died	[14]
Germany 2015	L98H/TR34/Y121F/T289A	Died (7)	[14]
Netherlands 2013	TR46/Y121F/T289A, L98H/T34	Alive (8), Died (5), Persisting (2)	[14]
US 2016	G448S	N/A	[12]

1.3 New Treatments in Development

As discussed in 1.1, newer generation azoles are currently in development with one passing through phase two trials, isavuconazole[22]. However, there is a growing concern that while these novel azoles are being developed the resistance of the fungal pathogens to the previous azole will contribute to a greater ease in gaining resistance to newer azoles. Isavuconazole is one such case, approved in 2014, with a mode of action akin to voriconazole. Already known and present mutations within *cyp51A* are capable of generating resistance to isavuconazole [23]. Known resistance mechanisms, such as those in Table 1.1, G448S, TR46/Y121F/T289A already contribute to voriconazole and now isavuconazole resistance [23].

Ibrexafungerp is one of the latest non-azole antifungals to be utilized and approved [24, 25]. This compound works by inhibiting glucan synthase [24, 26]. This mechanism is similar to echinocandins but the binding sites differ, contributing to a surprisingly minimal amount of side effects with better therapeutic potential [24, 26]. In 2021, ibrexafungerp—a triterpenoid antifungal, was approved for *Candida spp*., infections, making it the most recent antifungal to receive approval, and more importantly, is a as a non-azole drug [25]. Thus allowing for a slight increase in treatment diversity. Rezafungin is another glucan synthesis inhibitor that holds promise as well; yet both are only approved for *Candida spp*., infections. Both these drugs carry with them better fungal penetration, and better tolerability; with ibrexafungerp have the added effect of activity against echinocandin resistant strains, [27].

Novel non-azole drugs such as fosmanogepix (APX001) are being investigated in phase-two trials [28, 29]. APX001 works by targeting the glycosylphosphatidylinositol (GPI) synthesis pathway [28, 29]. Exposure to this drug inhibits inositol acylation during the generation of GPI anchoring proteins. Inhibition of inositol acylation compromises cell wall stability, reduces virulence traits, and negates germ tube formation [28, 29].

Olorofim is gaining interest as well due to its fungal over mammalian cell specificity and activity against multi-drug resistant fungi[27]. Olorofim works by inhibiting a fungal-specific dehydrogenase (dihydroorotate dehydrogenase) that is crucial for pyrimidine biosynthesis. Importantly, this drug is in clinical trials for the specific treatment of invasive aspergillosis, one of the deadliest disease states [30].

G protein-coupled receptors (GPCRs) are also gaining attention. GPCRs are utilized for extracellular sensing in response to environmental cues. Fungi rely heavily on the usage of GPCRs in regulating growth/metabolism, virulence, and fungal toxin production [31, 32]. Most importantly, these fungal receptors so far do not share much similarity with human GCPRs; meaning there would be a reduction in off-target effects if one were to attempt targeting these receptors [31]. Research has shown that *gprC* and *gprD* are crucial for hyphal development and virulence traits in *A. fumigatus* [32]. Removal of these receptors resulted in growth defects, increased sensitivity to oxidative stress, and attenuated virulence *in vivo* [31, 32]. In turn deletion of *gprK* results in an increased germination rate demonstrating their dual role [31, 33]. Researchers in the process of characterizing certain GPCRs, *gprM*, and *grpJ* discovered novel features relating to their control over melanin production and cell wall integrity [34]. They determined that overexpression of *gprM* and *grpJ* results in decreased growth fitness and reduced radial expansion [34]. As well, the researchers observed the increased sensitivity to congo red and calcofluor white, suggesting compromised cell wall stability [34]. These initial investigations demonstrate the crucial need for a better understanding of GCPRs and their potential as druggable targets for their use as novel antifungal therapies.

As we continue to develop novel antifungals, we must take into consideration that while improving upon readily available drugs allows for ease in design and increased safety, they also

run the risk of being susceptible to already known resistance mechanisms by *Aspergillus spp.* Therefore, novel target discovery is crucially important for this clearly stated reason process that one must evaluate to aid our armament. While searching for novel drugs, novel applications of old drugs with identified synergizers holds promise for extending currently available azoles. We should consider drug repurposing, because while these antifungals may not be suitable for treatment due to resistance, novel combinations can reveal clinical applications that were not present. One should not overlook the lengthy and costly process to evaluate novel compounds when compared to drug repurposing. The process of Investigational New Drug (I.N.D.) applications through the Food and Drug Administration (FDA) is often arduous and expensive [35, 36]. The average span for an I.N.D. application is 10 to 15 years with a cost of–$2-3 billion USD [36, 37]. Through drug repurposing, one can expect 3-9 years of further evaluation depending on the stage of clinical trial, at a much lower price tag [37]. As well with previously approved drugs one has a plethora of clinical trial data alongside research articles published pre and post clinical trials. This allows the repurposing process to aid a researcher in understanding the properties of their identified drug/compound in a shorter time with less labor involved. In conclusion, drug repurposing for discovering novel antifungals or antifungal enhancers is a viable alternative to the cumbersome process of developing novel and derivative drugs.

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Chapter 2. Screening of MCE Drug Library against *Aspergillus fumigatus*

Identification of azole synergizers through high throughput library screening against clinical *Aspergillus fumigatus* isolates.

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Abstract:

Aspergillus fumigatus is a globally distributed opportunistic organism capable of debilitating and life-threatening infections in immunocompromised individuals. Azoles, comprised of voriconazole (VRC), posaconazole (POS), and itraconazole (ITC) are widely used to combat this pathogen. In the past two decades, we have witnessed the emergence of resistance to azoles on a global scale, vastly increasing the already alarming mortality rate. We seek to increase our therapeutic options through novel drug combinations identified through high-throughput screening and drug repurposing. In this report, we investigated the MedChem Express (MCE) Drug Repurposing Compound Library—comprised of 4,226 compounds, in conjunction with itraconazole at 0.06 μg/mL against *A. fumigatus* CDC #738. After the initial screening, we determined six compounds were of paramount interest: cobicistat, elvitegravir, lopinavir, penfluridol, rilapladib, and rolapitant. The combination of ITC, POS, and VRC with the identified compounds demonstrated promising amounts of synergy, in resistant and susceptible isolates. Further investigation revealed novel fungicidal properties of ITC and POS when in combination with our compounds of interest. One hit was observed to reverse ITC, POS, and VRC resistant strain(s) to a sensitive profile. Growth kinetic assays demonstrate potent anti-germination properties and curious morphological features such as swelling and branching abnormalities. Utilization of high throughput screening techniques to determine robust antifungal synergizers offers promising possibilities for extending the usability and tolerability of azoles.

Keywords: *Aspergillus fumigatus*, drug repurposing, azole resistance, re-sensitization, fungicidal

Introduction:

Drug repurposing has attracted an ever-growing crowd of researchers due to reduced costs, time, and inclusion of de-risked compounds[1-3]. De-risked compounds can be described as drugs currently approved by the FDA or undergoing phase two clinical trials as the safety parameters and adverse effects are well described[1, 2, 4]. Furthermore, it should not be understated the measurable decrease in cost and time to develop for drug repurposing. Currently, a new drug discovery process takes 10–15 years with a price tag of $2–3 billion dollars from private and public funds[1-3]. Drug repurposing in turn allows for a much faster turnaround of 6–9 years with a marked decrease in costs due to known drug parameters and previously completed clinical trials[3]. These associated benefits are not going unnoticed by researchers in human health[1, 5]. Our research in turn seeks to contribute to this growing knowledge base through the examination of the MedChemExpress Drug Repurposing Library to identify candidates for combinational therapies to often underutilized azoles, POS, and ITC. We also seek to determine if our identified compounds can enhance frontline azoles such as VRC. Of the over 4,000 compounds within the library our research identified six with the promise to enhance and re-sensitize various azoles. Another 24 were identified as candidates, by demonstrating efficacy (>90% growth inhibition with a sub-effective azole dose) and were unidentified as antifungals or antiseptics. They did not pass our criteria yet demonstrate potential through rigorous evaluation as azole enhancers through further review.

As a result of the selection process, three HIV drugs were identified from the MCE library, cobicistat, elvitegravir and lopinavir. These were selected for due to impressive cellular penetration, known use in *A. fumigatus* risk group and high safety[6-11]. Cobicistat is an FDA-approved drug for the treatment of HIV as a pharmacokinetic enhancer[8]. Cobicistat (Cobi) works

through the selective inhibition of cytochrome P-450 3A allowing for decreased metabolism of antiviral medications thereby prolonging the co-drugs effect. Tolerance of cobicistat was evaluated in phase II and III clinical trials and was determined to be acceptable[8]. Furthermore, cobicistat was found to inhibit intestinal P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP)[7]. Elvitegravir (Elvit) is also used to combat HIV infections. However, this drug is utilized to combat HIV in patients through inhibition of integrase[9, 11]. HIV inserts its genome into the host cell through the usage of integrase so inhibition of this resulted in a stoppage of viral replication[11]. Elvitegravir is often given in combination with cobicistat to achieve a synergistic effect, known to be safe for co-administration[10]. Lopinavir (LPV) is utilized as a protease inhibitor for HIV therapy[12]. Lopinavir and cobicistat have been evaluated as efflux pump inhibitors. One such study demonstrated the impressive retention of cytotoxic substances in mammalian cells. Researchers determined that Multi Drug Resistance-1 P-glycoprotein (MDR1) -P-gp BCRP, and Mutli Drug Resistance Protein 3 (MRP3) are inhibited and that ABC transporters are blocked due to LPV being a poor substrate for efflux[13].

Amongst the other candidates' compounds identified, there are two that bind to receptors located within the central nervous system. Penfluridol (Pen) is a long-acting and orally deliverable neuroleptic given for the treatment of seizures[14]. Penfluridol works by selectively binding to dopamine receptors[15]. Further interest in this drug has grown within the cancer community. Researchers have begun showing promising signs of repurposing this drug for renal cell carcinoma due to its ability to induce apoptosis[15]. Rolapitant on the other hand is utilized to prevent nausea in patients undergoing chemotherapy. This drug works by selectively inhibiting NK1 receptors, fortunately, as with penfluridol, it can easily traverse the blood-brain barrier (BBB)[16]. These compounds together are of great interest for their affinity for subfamilies of G protein-coupled

receptors (GPCRs) within humans demonstrating potential for interaction with fungal GPCRs[17, 18]. Rilapladib is our final identified compound with the potential to cross the BBB and work as a therapy for dementia patients[19, 20]. This drug was originally defined as a lipoprotein-associated phospholipase A2 inhibitor for the treatment of atherosclerosis plaque formation[19, 20]. It completed phase-two clinical trials and has recently been announced to be utilized for further proof of concept clinical studies[21].

We have effectively utilized a high throughput screening assay in this study to evaluate the MCE Drug Repurposing Library against an *A. fumigatus* clinical isolate. Resulting in six identified compounds of interest due to well-described drug parameters and *in vitro* efficacy studies. We observe much promise and variability in these compounds to synergize effectively with the azoles most often used to combat *A. fumigatus* infections. In turn, we hope to contribute to further advancing the goal of drug repurposing in fungal diseases with limited and ever-decreasing antifungal therapies.

Results:

Screening of MCE Drug Repurposing Library

The MCE Drug Repurposing Compound Library (consisting of >4000 compounds) was screened at a concentration of 16μM in conjunction with ITC at 0.06μg/mL against *A. fumigatus* CDC #738. In the initial screening assay 91 compounds were identified with 30 being removed due to their role as known antifungals/antiseptic agents. Following the secondary selection process six compounds were identified due to their acceptable half-life, drug purpose (i.e., anticancer or antipsychotic), oral availability, absorption, and toxicity, Table 2.1 and 2.2.

Minimum inhibitory concentration (MIC) and microdilution checkerboard

To further examine the six potential drug candidates we challenged an *A. fumigatus* CDC isolate panel via an MIC assay, per CLSI M38[22]. The antifungal activity of our azoles, ITC, POS, and VRC ranged from 1-64, 1, and 0.25-2 μg/mL respectively. Cobicistat, elvitegravir, rolapitant, and lopinavir all displayed MICs above 256 μg/mL with rilapladib displaying an MIC above 64 μg/mL. Penfluridol however displayed a surprisingly low MIC range of 4-16 μg/mL across a variety of azole-susceptible and resistant isolates, Table 2.2.

After determining the MICs for our compounds, the next step was to determine at what levels they best synergize with our azole antifungals, Table 2.3–.8. This is performed through a microbroth dilution checkerboard assay, as previously described [23, 24]. Resulting from this assay is a Fractional Inhibitory Concentration Index (ΣFICI), which is utilized to determine the range and therefore qualifications for synergy (SYN) ($\leq$ 0.5), additive effect ($>$ 0.5 to $\leq$ 1), and indifference (IND) ($>$ 1 to $\leq$ 4) of the drug combinations When evaluated with cobicistat we observed 60% synergy with ITC, 80% synergy and 20% additive with POS, and 20% synergy and 60% additive with VRC, Table 2.3. ΣFICI values for cobicistat ranged from 0.125–1.063, 0.064–0.531, and 0.375–1.125, respectively. Elvitegravir displayed 50% synergy with ITC, 70% synergy, and 30% additive with POS and 20% synergy and 50% additive with VRC, Table 2.4. ΣFICI values for elvitegravir ranged from 0.188–1.125, 0.064–0.563, and 0.375–1.125. Lopinavir demonstrated a 50% synergy and 10% additive with ITC, 80% synergy and 20% additive with POS, and 40% additive with VRC, Table 2.5. ΣFICI values for LPV ranged from 0.266–1.063, 0.281–0.563, and 0.563–1.002, for ITC, POS, and VRC. Penfluridol displayed 60% synergy and 10% additive with ITC, 80% synergy and 20% additive with POS, and 100% indifference with VRC, Table 2.6. ΣFICI values for penfluridol ranged from 0.313–1.5, 0.064–0.531, and 1.5–2.12, for ITC, POS, and VRC. Rilapladib when utilized demonstrated 80% synergy and 20% additive with ITC, 100% synergy

with POS, and 50% synergy and 30% additive with VRC, Table 2.7. ΣFICI values for rilapladib ranged from 0.252–0.563, 0.132–0.375 and 0.375–1.125. Rolapitant displayed 50% synergy with ITC, 70% synergy and 30% additive with POS, and 10% synergy with VRC, Table 2.8. ΣFICI values for rolapitant ranged from 0.188–1.125, 0.064–0.563, and 0.375–1.125 for ITC, POS and VRC, Table 2. Elvitegravir and rolapitant demonstrated a reversion of isolate #731 from a resistant POS strain to one within the area of technical uncertainty, Tables 2.4 and 2.7. Rilpladib demonstrated the most potential in reverting resistant strains to ITC, POS, and VRC. Isolates #731, 733, and 734 demonstrated a reversion from an MIC of 64 to 0.5–0.125 μg/mL and isolate #732 demonstrated a reversion to a susceptible VRC of 0.5 μg/mL. All isolates demonstrated a reversion to a susceptible POS MIC of 0.25 μg/mL when rilapladib was applied. All isolate's resistance and susceptibility values were determined from EUCAST as currently no CLSI values are determined[25].

Growth kinetics of *A. fumigatus* CDC #738

To understand the impact of the drugs in combination or alone on fungal growth we employed a growth kinetics assay. *A. fumigatus* #738 when challenged with azoles at subinhibitory-MIC concentrations and MCE selected compounds at concentrations derived from the checkerboard (Table 2.3-2.8) displayed no diminished growth over 72 hrs, not shown. When combined VRC and Elvit, LPV, Pen, and Rola displayed no significant differences while only slightly inhibiting growth, Figure 2.2A. VRC with Cobi ($p<0.001$) and Rila ($p<0.0001$) at 72 hr demonstrated significant growth reduction, Figure 2.2A. ITC with all selected compounds demonstrated high statistical significance at 48 and 72 hr ($p<0.0001$) compared to ITC alone. Of note, Elvit, Rola, and Cobi maintained constant OD values throughout the course of the experiment, displaying minimal to no fungal development, Figure 2.2B. POS with Pen

demonstrated statistical significance at 48 and 72 hr ($p<0.0001$) when compared to POS alone but did not inhibit growth. Alternatively, all other compounds demonstrated growth inhibition at 48 and 72 hr ($p<0.0001$). These combinations as well demonstrated very little change in their OD value over the course of 72 hrs reflecting remarkable inhibitory properties of the drug combinations, Figure 2.2C.

Germination Assay

To further interrogate the growth kinetics assay, another plate was imaged over the selected reading periods, from the growth kinetics assay, via a Nikon ECLIPSE Ti2 at 20X objective in brightfield with a scale bar of 100uM. At zero hours all spores within the test and control groups remained ungerminated and static. Slight precipitates of Pen, Rila, and Rola can be observed and were observed in the controls for the drug hits with and without the organism present. Over 72 hrs VRC alone and with the selected compounds did not inhibit germination or result in vast morphological abnormalities, e.g., altered branching structure, Figure 2.3 A-AB. When ITC is utilized to challenge spores over the course of 72hrs we observed vast hyphal growth, fig 2.4 V. At 24hr with ITC and LPV or Pen we observe hyphal development and spore swelling, fig. 2.4 K and L. In turn, Cobi, Elvit, Rila and Rola with ITC prevented germination, fig 2.4. I, J, M, N. At 48 hr Rola and ITC can be observed to fail in preventing growth, fig. 2.4 U. Of note branching in the Pen+ITC group is increased when compared to ITC alone, fig. 2.4 S. At 72hr Rila and Elvit with ITC are observed to have no hyphal development; while Cobi+ITC resulted in swelling and initialization of hyphae growth, fig 2.4 X, AA, and W respectively. For POS at 24 hr only Pen+POS resulted in hyphal growth, fig. 2.5 L. At 48hr all other combinations, except Pen+POS prevented germination, yet again increased branching can be observed in this combination group, fig. 2.5 L. Increased branching is a morphological feature observable in *A.* fumigatus only under stressed

condition or through selective mutations. POS and LPV at 72 hrs did not prevent germination of spores, while POS and Rola is observed to have preventing hyphal development yet spore swelling can be observed, fig. 2.5 Y, AB. Cobi, Elvit, and Rila preventing fungal germination at 72hrs, fig. 2 W, X, AA.

Materials and Methods:

Isolates and Compounds. Fungal strains employed within this report, Table 2, were obtained from the CDC (Atlanta, GA, USA). Media components were purchased from Thermo Fisher Scientific (Waltham, MA), RPMI 1640, Sigma-Aldrich (St. Louis, MO) 3-(N-Morpholino) propane sulfonic acid (MOPS), Becton, Dickinson and Company (Franklin Lakes, NJ), Potato Dextrose (PD) broth and agar. Itraconazole, posaconazole, and voriconazole were purchased from Biosynth Carbosynth (San Diego, CA USA). The MCE drug repurposing library and elvitegravir were purchased from MedChem Express (New Jersey, USA). Lopinavir was purchased from Acros Organics (New Jersey, USA), cobicistat and rilapladib from Asta Tech (Bristol, PA, USA), while rolapitant and penfluridol were purchased from Cayman Chemical (Ann Arbor, MI, USA). Reagents and dyes for fungal cell imaging are as follows: formaldehyde (Sigma Aldrich, St. Louis MO.), triton X-100 (Acros Organics, New Jersey, USA), and calcofluor-white (Sigma-Aldrich, St. Louis, MO).

Library Screening, microdilution checkerboard, and minimum inhibitory concentration (MIC) assays.

The MCE drug repurposing library was screening at 16μM against CDC clinical isolate *A. fumigatus* #738; in the presence or absence of itraconazole, 0.06μg/mL. In brief spore suspensions of *A.* fumigatus #738 were utilized in RPMI, at $5x10^4$ spores/mL. Aliquots of 100μL of the

aforementioned solution were transferred to 96-well plates containing the 16uM concentration of the drug and 0.06 µg/mL ITC. Positive hits were those that inhibited 95% or more of fungal growth after 48hr at 37ºC, plates were read at 530nm. To assess the activity of the identified hits with either itraconazole (ITC), or posaconazole (POS) against a variety of *Aspergillus* species, we conducted a standard broth microdilution checkerboard assay, as previously described [26-30]. Fractional inhibitory concentration indexes (ΣFICI) were calculated to determine drug interactions between the drugs [31]. Synergistic (SYN) drug interactions were determined when ΣFICI ≤ 0.5, indifferent (IND) at >0.5 to ≤4, and antagonistic (ANT) when values were > 4 [28, 32, 33].

Growth kinetics of *A. fumigatus* CDC #738.

A growth kinetics assay was performed to determine growth inhibition resulting from combinational therapies on *A. fumigatus* strain CDC #738 [34, 35]. Fungal spores were diluted in RPMI to 5 x 10^4 condida/mL. Subsequently, the fungal cells were treated with either POS at 0.25x MIC (0.06 µg/mL), ITC at 0.25x MIC (0.25 µg/mL), Elvit (16 µg/mL), or a combination of POS and LPV. To monitor the growth, the optical density (OD_{530}) of the samples was measured every 12 hours using a SpectraMax i3x microplate reader at 530 nm [35]. The experiment was performed in triplicate.

Germination assay.

To determine the efficacy of the drug combinations to prevent germination of *A. fumigatus* we incubated spores (5 x 10^4 spores/mL), with and without single, double, and no treatment. Five drugs were incubated alone at 16µg/mL, with penfluridol at 4 µg/mL, and with either POS at 0.125µg/mL or ITC at 0.25µg/mL and DMSO for 72 hours at 37ºC in 96-well plates. The plate

was then visualized with a Nikon ECLIPSE Ti2 at 0, 24, 48, and 72 hrs, and the images were stored in a .nd2 format for documentation, with duplicates stored as .jpeg files.

Discussion:

Patient mortality from azole-resistant *A. fumigatus* isolates increases a 40% mortality rate to beyond 80% nearly doubling the chances of a negative outcome[36]. Resulting from this tremendous loss of life due to increased resistance the WHO has designated *Aspergillus fumigatus* a critical priority pathogen[37]. Increasing resistance and decreasing novel drug development for *A. fumigatus* infections are contributing to a future in which antifungal options will be severely limited[38, 39]. We choose to respond to this set of challenges through drug repurposing, a novel tool allowing for FDA-approved or phase I/II drugs to undergo a new life or avenue as a therapeutic option for another disease wholly unrelated to its original purpose. This process allows one to readily identify known characteristics of drugs that synergy with our antifungal and reduces the time and cost associated with evaluating drug candidates for clinical settings[3]. In this article, we screened *A. fumigatus* CDC isolate #738 with the MedChemExpress drug repurposing library with ITC, a second-line azole for *Aspergillus spp.,* infection. Using a second-line treatment allows for one to determine possible drug combinations that will allow for their usage to become a frontline therapy thereby increasing clinical options for treating infections. As well ITC and POS are known to easily develop resistance through common mutations and by demonstrating the potential of the combinations to revert that resistance, we again open another avenue for treatment that was not available before. Our selection criteria post-initial screening allowed us to exclude a vast majority of isolates, primarily though setting the barrier as 90% observed growth inhibition. Once established we then identified and removed compounds classified as antiseptic and antifungal.

The list of compounds generated is listed in Table 1. After further reviewing the compounds for their known mechanisms of action and drug properties we selected Cobi, Elvit, LPV, Pen, Rila, and Rola. The HIV drugs Cobi, Elvit, and LPV are promising candidates due to agreeable pharmacokinetics [40, 41]. As well the patient population is highly susceptible to fungal infections demonstrating a promise to utilize already in place therapies with antifungals for effective treatment [40, 41]. It is even possible unrecognized co-administration of azoles and these HIV antivirals can have taken place and been included within a case report. Penfluridol an antipsychotic binding to D2 receptors and rolapitant an antinausea drug binding to NK1 receptors demonstrate favorable drug properties such as BBB penetration, which is observed in Pen and Rila[42, 43]. Furthermore, Pen when screened alone demonstrated a the lowest MIC against *A. fumigatus* of 4-16 μg/mL. Rilapladib a phase two clinical trial candidate demonstrated effective synergy across tested azoles. All drugs demonstrated acceptable plasma levels and half-life's with some up to 7 days [4]. Maximum plasma concentrations for the selected compounds are reached on average in 4 hours while Pen requires 12 hours [4]. We then sought to determine if our selected compounds were able to synergize with VRC a frontline therapy in order to compare different efficacious azoles against one another when co-applied with our selected compounds.

Table 2 details the MICs of the chosen *Aspergillus fumigatus* isolate panel to azoles and our selected compounds. In isolates 731-735 we observed high MICs for all azoles tested; accompanying this are the mutations that result in target changes, or overproduction of the drugs target. We then set out to determine if synergy or additive effects can be observed when the azoles and compounds are used in tandem. VRC synergized primarily with Cobi, Elvit, Rila, and Rola in sensitive strains, Tables 2.3, 2.4, 2.7, and 2.8. ITC synergized with all compounds in sensitive isolates, Tables 2.3-.8. However, Cobi, LPV, Pen, and Rila demonstrated synergy with one or

multiple heavily resistant strains, Tables 2.3, 2.5, 2.6, and 2.8. Of note, we observed Rila and ITC are able to revert three strains with MICs of 64 μg/mL to at or below 0.25 μg/mL. Representing a serious reversion from resistance to sensitive, in isolates that carry the hallmark mutation pattern (TR34 and L98H), Table 2.3 and 2.8. POS synergized with nearly all compounds and strains, apart from Rila in which there was 100% synergy observed, whether resistant or sensitive. Interestingly, we observe that Cobi, Elvit, LPV, Pen, and Rola synergize with strains containing the hallmark mutation, Table 2.3-.7. This synergy, however, becomes additive as more mutations are accumulated. Surprisingly, the combination of Rila and POS still retains synergy and even reverts the two remaining holdout isolates, #732 and #735. Demonstrating that this combination can overcome multiple point mutations that render previous singular and combinational therapies ineffective.

To evaluate the ability of our compounds to prevent fungal development over the course of 72 hours we employed a growth kinetics assay. In this assay we observe that all compounds and azoles, at sub-inhibitory MICs, fail to prevent fungal growth, Table 2.1 and Figure 2.2 A-C. Furthermore, when VRC and all compounds were co-applied only two combinations, Cobi and Rila, showed significance ($P > 0.001$, $P>0.0001$) from VRC alone, fig. 2.2B. Yet all compounds with VRC failed to prevent fungal growth. ITC when applied with LPV, Pen, and Rila demonstrated significance ($P>0.0001$) when compared to the ITC alone curve, yet only Rila of this group seemed to inhibit growth. Whereas Elvit, Rola, and Cobi demonstrated significance while maintaining minimal growth, fig. 2.2B. Most promising is the POS combinational curve, with only one compound, Pen, failing to prevent growth. Here we can readily observe the differences in synergy between azoles and the compounds. We see that VRC, a frontline therapy, at a sub-inhibitory dose does little to synergize with our compounds. We see a slight increase in the ability

of the compounds and ITC to synergize at sub-inhibitory doses and prevent growth. Most surprisingly we see that sub-inhibitory doses of POS and our compounds can effectively negate fungal growth over the course of 72 hrs in specific cases.

Given the impactful inhibition we are observing through the synergy values obtained from the checkerboard assay we sought to see if germination, swelling, and budding, and initial morphology would be impacted by sub-inhibitory azole doses when our compounds were co-administered. We utilized a Nikkon Ti2 with a 20X objective to visualize our spores and subsequent hyphae. We observe that VRC is unable to prevent growth with or without our selected compounds post 48 hours of incubation. While ITC has two combinations failing at 24 hr and only one more failing at 48 hours. POS in turn readily demonstrates its ability to synergize well with our compounds; over the course of 48 hours, only POS with Pen fails to prevent germination within that cohort. Furthermore, the CLSI MIC protocol states any observable growth at 48 hr is deemed a failure of that drug at that concentration; we sought to expand this criterion and observed our wells for an additional 24 hours. Surprisingly, we observe that ITC and POS with Elvit, and Rila and POS with Cobi prevent fungal germination. In comparing this to the growth kinetics assay we see that while OD presents itself as a useful tool, further investigation is required to fully determine the inhibition of germination. As previously stated, the Pen and POS/ITC combination while a failure, did result in distinct morphology not seen in any other group. An increase in branching density was observed alongside some angular discrepancy. *A.* fumigatus branches evenly at acute angles, 45^{o}, whereas these branches in ITC+Pen at 48 hr demonstrate 90^{o} angles in some cases[44]. Branching spacing is lessened as well. This can be suggested to arise from an interaction in the G-coupled protein receptors (GPCRs) of the fungus[45]. As fungi lack dopamine receptors, the known target for penfluridol, we can surmise that similarly functioning and structure receptors

would be susceptible to blockage by penfluridol, given dopamine receptors are part of a GPCR superfamily[14, 46].

We observe through the course of this library evaluation the promise drug repurposing holds. Previous research has shown that through screening of the Developmental Therapeutics Program of the National Institutes of Health (NIH), Pathogen Box, the NIH National Clinical Collection (NCC), and Prestwick Chemical Library, novel antifungal drugs and combinations with azoles can be determined the challenge *A. fumigatus*[47-49]. We have sought to build upon this knowledge base by evaluating the MCE Drug Repurposing Library with itraconazole and a clinical *A. fumigatus* isolate. We then sought to determine if we could translate the identified compounds to other azoles and develop a more robust candidate. Through this process we have identified six compounds capable of synergizing and to some extent re-sensitizing resistant isolates across ITC, POS, and VRC applications. Further investigations into proposed mechanisms of action are warranted such as evaluating efflux inhibition in the HIV class of drugs, GPCR activity of the neurologically active drugs, and differential gene expression analysis for all. We hope to further bolster our dwindling supply of antifungals through the introduction of novel combinational approaches that will aid clinicians in combating an ever-alarming fungal pathogen.

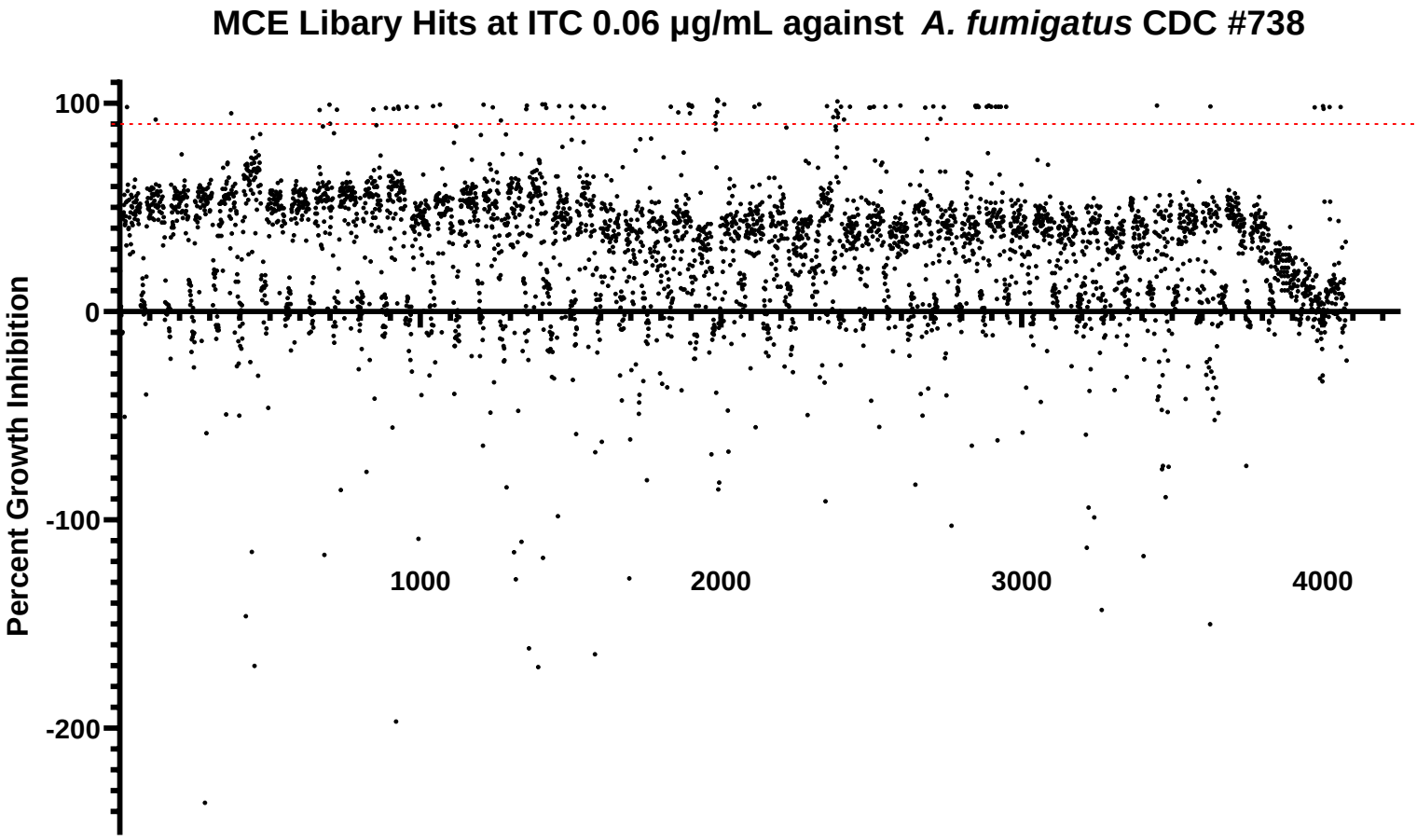


Figure 2.1: Percent growth inhibition of MCE library hits against *Aspergillus fumigatus* CDC #738, with itraconazole (ITC) at 0.06 µg/mL. The red dashed line indicates an sub-inhibitory ITC and co-drug combination that results in 90% growth inhibition. Recorded at 530nm, corrected with baseline.

Table 2.1: Description of MCE compounds identified in initial library screening and post-initial sieving for known agents.

Name	Category	Mechanism of Action (MOA)	Notes	Reference
Iniparib	Cancer	Suggested poly(ADP-ribose) polymerase inhibitor	Successfully completed Phase III trial with minimal toxicity, however mechanism of action is now in question	[50]
Diiodohydroxyquinoline	Amoebicidal	Chelation of ferrous iron	Shown effective in repurposing for *Clostridium difficult* infection	[51, 52]
Taurochenodeoxycholic acid	Inflammation & Immunology	Modulates TGR5 receptor to influence apoptotic events and immune response	Endogenous Metabolite	[53]
Leucomethylene blue (mesylate)	Neurological Disease	Tau aggregation inhibitor and mitochondrial protection via recycle	Derivative of methylene blue	[54]
DOTAP (chloride)	Liposomal transfection	Quaternary ammomium motif allows for nucleic acids and cell membrane interaction	N/A	[55]
Rilapladib	Inflammation Immunology; Neurological Disease	Possible Lipoprotein-associated phospholipase A2 inhibitor & Platelet Activating Factor Receptor antagonist	Recently acquired by SciNeuro to build upon for next generation inhibitors	[19-21]
MIK665	Cancer	Myeloid cell leukemia sequence 1 inhibitor	Undergoing initial clinical phase 1 trials for combinatorial usage	[56, 57]

Amcasertib	Cancer	Stemness kinases inhibitor targeting NANOG and other pathways	N/A	[58]
Amiselimod (hydrochloride)	Cancer	Sphingosine 1-phosphate receptor-1 modulator	Previous S1P modulators have been utilized to treat autoimmune diseases	[59]
Dronedarone	Cardiovascular Disease	Blocks potassium, sodium, and calcium channels	Short half-life and poor tissue accumulation	[60]
Visomitin	Inflammation/Immunology	Mitochondrial-targeted reactive oxidative species reduction	Ophthalmic formulation	[61]
Valnivudine	Infection	Pro-drug form of a nucleoside	Successful Phase I, II trials with a Phase III trial termination	[62]
Voclosporin	Inflammation/Immunology	Oral calcineurin inhibitor	Immunosuppressant	[63]
Cobicistat	Viral	Inhibition of human CYP3A proteins	Cytochrome P450; HIV	[7, 8]
Fimepinostat	Cancer	Inhibitor for phosphotidylinositol 3-kinase and histone deacetylase	Currently under review for usage as a HIV	[64, 65]
Mocravimod (hydrochloride)	Inflammation/Immunology	Sphingosine-1-phosphate receptor modulator to retain T-cell actions	Immune modulator	[66, 67]
Miltefosine	Infection	Unknown; speculated to be inhibition of cytochrome C and induction of cell death	Demonstrated *in vitro* ability for repurposing	[47, 68]
Elvitegravir	Viral	Integrase strand transfer inhibitor	Utilized in conjunction with Cobicistat	[69]
Febuxostat	Metabolic Disease	Inhibition of xanthine oxidase	Interest in repurposing for mycobacterium infection	[70, 71]

Rolapitant	Cancer; Anti-Nausea	Neurokinin-1 receptor agonist	Does not interfere with CYP3A4	[72]
Nilvadipine	Cardiovascular Disease	Selective blocking of calcium channels in smooth vascular muscle	N/A	[73]
Pericyazine	Neurological Disease	Central adrenergic blockade of the alpha adrenergic receptors	Approved in Canada and U.K.	[74]
Derazantinib	Cancer	Inhibits fibroblast growth factor receptor 2	N/A	[75]
Cefozopran (hydrochloride)	Infection	Binds to and inactivates penicillin-binding proteins	Effective against acute lung infections	[76]
Penfluridol	Neurological Disease; Cancer	Blockade of dopamine receptors, especially to postsynaptic D2 receptor, T-type calcium channel blocker	Potential drug repurposing candidate for cancer	[14, 15, 43]
Methylene blue (trihydrate)	Cancer; Infection; Neurological Disease	Electron acceptor and reversal of NADH inhibition	Precursor of Leucomethylene blue (mesylate)	[54]
Lopinavir	Antiviral	HIV protease inhibitor	Demonstrated synergy with azoles	[6, 12, 30]
Oleic acid	Cancer; Metabolic Disease	Inhibits lipid peroxidation and ferroptosis	Secreted by mammary adipocytes	[77]
Otilonium (bromide)	Neurological Disease	Blockage of calcium entry	Concentrates within intestinal region	[78]

Table 2.2: Description of the fungal strains utilized in this study and their minimum inhibitory concentration (MICs)

***Aspergillus* Strain**	**MIC [µg/mL] ITC**	**MIC [µg/mL] POS**	**MIC [µg/mL] VRC**	**MIC [µg/mL] Cobi**	**MIC [µg/mL] Pen**	**MIC [µg/mL] Elvit**	**MIC [µg/mL] Rila**	**MIC [µg/mL] Rola**	**MIC [µg/mL] LPV**	**Source & Characteristic & Resistance Mechanism**
CDC 731	64	1	2	>256	8	>256	>64	>256	>256	L98H, TR34
CDC 732	64	1	1	>256	8	>256	>64	>256	>256	F495I, L98H, S297T, TR34
CDC 733	64	1	2	>256	8	>256	>64	>256	>256	L98H, TR34
CDC 734	64	1	2	>256	8	>256	>64	>256	>256	L98H, TR34
CDC 735	64	1	1	>256	16	>256	>64	>256	>256	F495I, L98H, S297T, TR34
CDC 736	1	1	0.25	>256	4	>256	>64	>256	>256	USA
CDC 737	1	1	0.25	>256	4	>256	>64	>256	>256	USA
CDC 738	1	1	0.5	>256	4	>256	>64	>256	>256	USA
CDC 739	1	1	0.5	>256	8	>256	>64	>256	>256	USA
CDC 740	1	1	0.5	>256	8	>256	>64	>256	>256	USA

*Minimum Inhibitory Concentration (MIC), in which no observable growth was identified.

Table 2.3: Synergistic activity of Cobicistat (COBI) and azole antifungals against different strains of *Aspergillus*.

A. fumigatus	Cobi/ITC Combination			Cobi/POS Combination			Cobi/VRC Combination		
	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode
CDC 731	8/4	0.125	SYN	8/0.5	0.313	SYN	32/2	0.625	ADD
CDC 732	8/64	1.063	IND	4/1	0.531	ADD	32/1	0.625	ADD
CDC 733	8/64	1.063	IND	8/0.5	0.313	SYN	32/4	1.125	IND
CDC 734	8/64	1.063	IND	8/0.5	0.313	SYN	32/4	1.125	IND
CDC 735	8/64	1.063	IND	4/1	0.531	ADD	32/1	0.625	ADD
CDC 736	32/0.125	0.375	SYN	16/0.06	0.245	SYN	32/0.25	0.625	ADD
CDC 737	32/0.125	0.375	SYN	32/0.00075	0.252	SYN	32/0.25	0.563	ADD
CDC 738	32/0.125	0.375	SYN	32/0.03	0.310	SYN	16/0.25	0.563	ADD
CDC 739	16/0.125	0.250	SYN	8/0.00075	0.064	SYN	32/0.125	0.375	SYN
CDC 740	32/0.125	0.375	SYN	16/0.00075	0.127	SYN	16/0.25	0.375	SYN

**ΣFICI (Fractional Inhibitory Concentration Index) utilized to quantify interactions between the tested combinations with the following definitions: Synergy (SYN) is an ΣFICI value ≤ 0.5, Indifference (IND) is an ΣFICI of > 0.5 to ≤ 4, and Antagonistic with an ΣFICI value of > 4.

*** Cobicistat (Cobi), Itraconazole (ITC), Posaconazole (POS), and Not Applicable (N/A)

Table 2.4: Synergistic activity of elvitegravir (Elvit) and azole antifungals against different strains of *Aspergillus*.

A. fumigatus	Elvit/ITC Combination			Elvit/POS Combination			Elvit/VRC Combination		
	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode
CDC 731	16/64	1.125	IND	4/0.25	0.156	SYN	32/4	1.125	IND
CDC 732	16/64	1.125	IND	8/1	0.563	ADD	16/1	0.563	ADD
CDC 733	16/64	1.125	IND	16/0.5	0.375	SYN	32/2	1.125	IND
CDC 734	16/64	1.125	IND	1/1	0.508	ADD	32/4	1.125	IND
CDC 735	16/64	1.125	IND	8/1	0.563	ADD	32/1	0.625	ADD
CDC 736	16/0.125	0.250	SYN	8/0.00075	0.064	SYN	16/0.25	0.563	ADD
CDC 737	8/0.125	0.188	SYN	8/0.00075	0.064	SYN	32/0.25	0.625	ADD
CDC 738	16/0.125	0.250	SYN	16/0.00075	0.127	SYN	32/0.125	0.375	SYN
CDC 739	32/0.25	0.500	SYN	8/0.00075	0.064	SYN	32/0.125	0.375	SYN
CDC 740	16/0.25	0.375	SYN	16/0.00075	0.127	SYN	16/0.25	0.563	ADD

**ΣFICI (Fractional Inhibitory Concentration Index) utilized to quantify interactions between the tested combinations with the following definitions: Synergy (SYN) is an ΣFICI value ≤ 0.5, Indifference (IND) is an ΣFICI of > 0.5 to ≤ 4, and Antagonistic with an ΣFICI value of > 4.

*** Elvitegravir (Elvit), Itraconazole (ITC), Posaconazole (POS), and Not Applicable (N/A)

Table 2.5: Synergistic activity of lopinavir (LPV) and azole antifungals against different strains of *Aspergillus*.

A. fumigatus	LPV/ITC Combination			LPV/POS Combination			LPV/VRC Combination		
	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode
CDC 731	32/16	0.313	SYN	16/0.5	0.281	SYN	0.25/4	1.001	IND
CDC 732	32/64	1.063	IND	32/1	0.563	ADD	0.25/1	1.001	IND
CDC 733	32/64	1.063	IND	32/0.5	0.313	SYN	0.5/4	1.002	IND
CDC 734	32/64	1.063	IND	32/0.5	0.313	SYN	0.25/4	1.001	IND
CDC 735	32/64	1.063	IND	32/1	0.563	ADD	0.25/1	1.001	IND
CDC 736	8/0.5	0.516	ADD	16/0.25	0.281	SYN	16/0.25	0.563	ADD
CDC 737	16/0.25	0.281	SYN	16/0.125	0.281	SYN	0.25/0.25	1.001	IND
CDC 738	16/0.25	0.281	SYN	16/0.25	0.281	SYN	16/0.25	0.563	ADD
CDC 739	16/0.25	0.281	SYN	16/0.125	0.156	SYN	32/0.25	0.625	ADD
CDC 740	8/0.25	0.266	SYN	16/0.125	0.156	SYN	16/0.25	0.563	ADD

**ΣFICI (Fractional Inhibitory Concentration Index) utilized to quantify interactions between the tested combinations with the following definitions: Synergy (SYN) is an ΣFICI value ≤ 0.5, Indifference (IND) is an ΣFICI of > 0.5 to ≤ 4, and Antagonistic with an ΣFICI value of > 4.

*** Lopinavir (LPV), Itraconazole (ITC), Posaconazole (POS), and Not Applicable (N/A)

Table 2.6: Synergistic activity of Penfluridol (PEN) and azole antifungals against different strains of *Aspergillus*.

A. fumigatus	PEN/ITC Combination			PEN/POS Combination			PEN/VRC Combination		
	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode
CDC 731	2/4	0.313	SYN	8/0.5	0.313	SYN	16/2	1.500	IND
CDC 732	16/64	1.500	IND	4/1	0.531	ADD	16/1	1.500	IND
CDC 733	16/64	1.500	IND	8/0.5	0.313	SYN	16/2	1.500	IND
CDC 734	4/4	0.188	SYN	8/0.5	0.313	SYN	16/2	1.500	IND
CDC 735	16/64	1.500	IND	4/1	0.531	ADD	16/1	1.500	IND
CDC 736	8/0.25	0.500	SYN	16/0.06	0.245	SYN	16/0.25	1.500	IND
CDC 737	8/0.25	0.500	SYN	32/0.00075	0.252	SYN	16/0.06	2.120	IND
CDC 738	2/0.5	0.563	ADD	32/0.03	0.310	SYN	16/0.25	2.500	IND
CDC 739	8/0.25	0.500	SYN	8/0.00075	0.064	SYN	8/0.25	1.500	IND
CDC 740	8/0.25	0.500	SYN	16/0.00075	0.127	SYN	16/0.25	2.500	IND

**ΣFICI (Fractional Inhibitory Concentration Index) utilized to quantify interactions between the tested combinations with the following definitions: Synergy (SYN) is an ΣFICI value ≤ 0.5, Indifference (IND) is an ΣFICI of > 0.5 to ≤ 4, and Antagonistic with an ΣFICI value of > 4.

*** Penfluridol (PEN), Itraconazole (ITC), Posaconazole (POS), and Not Applicable (N/A)

Table 2.7: Synergistic activity of rilapladib (RILA) and azole antifungals against different strains of *Aspergillus*.

A. fumigatus	RILA/ITC Combination			RILA/POS Combination			RILA/VRC Combination		
	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode
CDC 731	8/0.5	0.258	SYN	8/0.25	0.375	SYN	8/4	1.125	IND
CDC 732	16/4	0.563	ADD	8/0.25	0.375	SYN	8/0.5	0.375	SYN
CDC 733	8/0.125	0.252	SYN	8/0.25	0.375	SYN	8/2	0.625	ADD
CDC 734	8/0.125	0.252	SYN	8/0.25	0.375	SYN	8/2	0.625	ADD
CDC 735	16/4	0.563	ADD	8/0.25	0.375	SYN	8/0.5	0.375	SYN
CDC 736	8/0.0015	0.252	SYN	4/0.0035	0.132	SYN	8/0.25	0.563	ADD
CDC 737	8/0.0015	0.252	SYN	8/0.0035	0.257	SYN	8/0.125	0.375	SYN
CDC 738	8/0.06	0.310	SYN	8/0.0075	0.265	SYN	8/0.125	0.375	SYN
CDC 739	8/0.0015	0.252	SYN	8/0.0035	0.257	SYN	8/0.125	0.375	SYN
CDC 740	8/0.06	0.310	SYN	8/0.0015	0.253	SYN	8/0.125	0.375	SYN

**ΣFICI (Fractional Inhibitory Concentration Index) utilized to quantify interactions between the tested combinations with the following definitions: Synergy (SYN) is an ΣFICI value ≤ 0.5, Indifference (IND) is an ΣFICI of > 0.5 to ≤ 4, and Antagonistic with an ΣFICI value of > 4.

*** Rilapladib (RILA), Itraconazole (ITC), Posaconazole (POS), and Not Applicable (N/A)

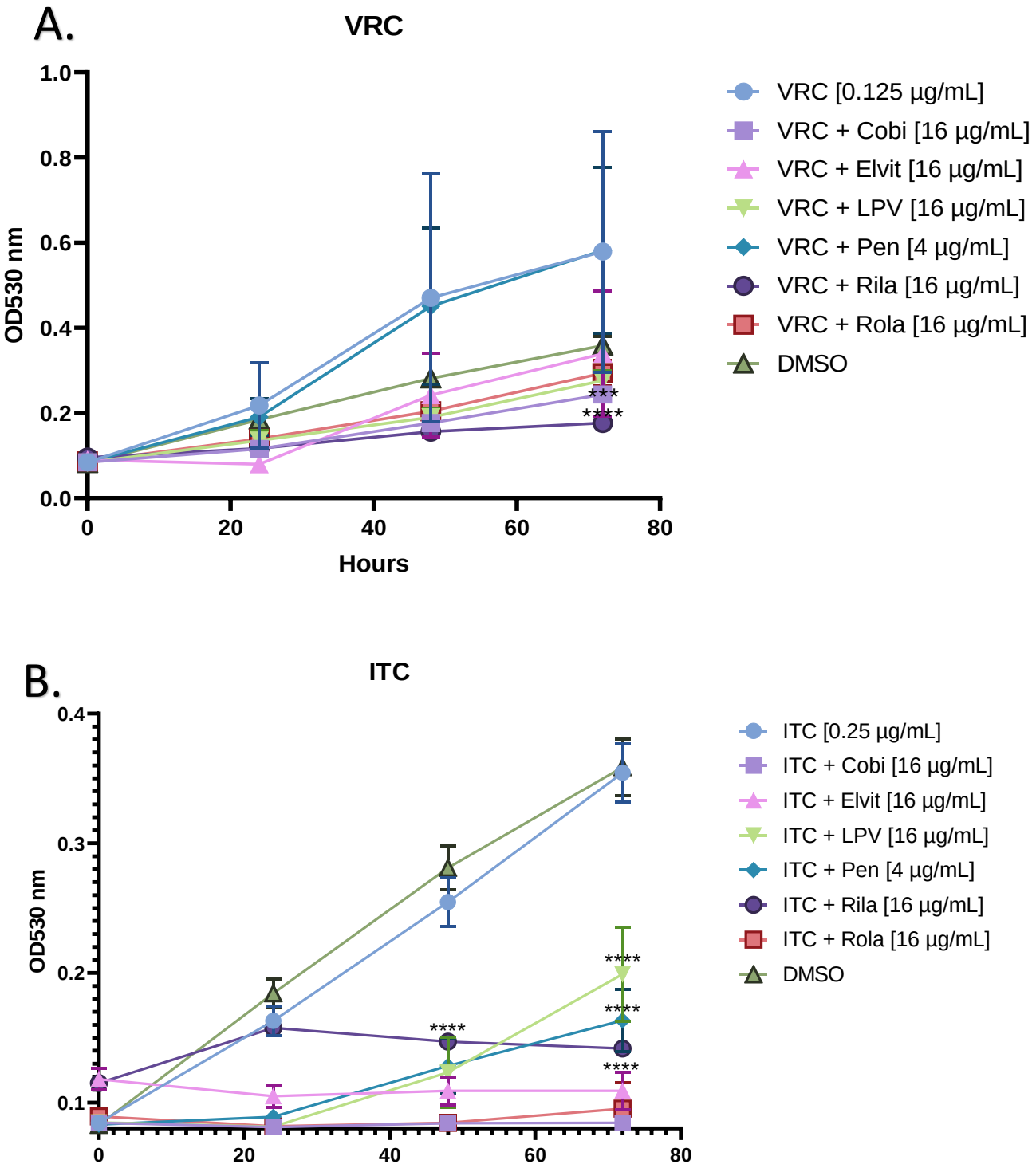
Table 2.8: Synergistic activity of Rolapitant (ROLA) and azole antifungals against different strains of *Aspergillus*.

A. fumigatus	ROLA/ITC Combination			ROLA/POS Combination			ROLA/VRC Combination		
	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode
CDC 731	16/64	1.125	IND	4/0.25	0.156	SYN	32/4	1.125	IND
CDC 732	16/64	1.125	IND	8/1	0.563	ADD	32/2	1.125	IND
CDC 733	16/64	1.125	IND	16/0.5	0.375	SYN	32/4	1.125	IND
CDC 734	16/64	1.125	IND	1/1	0.508	ADD	32/4	1.125	IND
CDC 735	16/64	1.125	IND	8/1	0.563	ADD	32/2	1.125	IND
CDC 736	16/0.125	0.250	SYN	8/0.00075	0.064	SYN	32/0.5	1.125	IND
CDC 737	16/0.125	0.188	SYN	8/0.00075	0.064	SYN	32/0.5	1.125	IND
CDC 738	16/0.125	0.250	SYN	16/0.00075	0.127	SYN	32/0.5	1.125	IND
CDC 739	32/0.25	0.500	SYN	8/0.00075	0.064	SYN	32/0.125	0.375	SYN
CDC 740	16/0.25	0.375	SYN	16/0.00075	0.127	SYN	32/0.5	1.125	IND

**ΣFICI (Fractional Inhibitory Concentration Index) utilized to quantify interactions between the tested combinations with the following definitions: Synergy (SYN) is an ΣFICI value ≤ 0.5, Indifference (IND) is an ΣFICI of > 0.5 to ≤ 4, and Antagonistic with an ΣFICI value of > 4.

*** Rolapitant (ROLA), Itraconazole (ITC), Posaconazole (POS), and Not Applicable (N/A)

Figure 2.2: Growth kinetics of A. fumigatus CDC #738 in the presence of azoles and MCE selected drugs.



C.

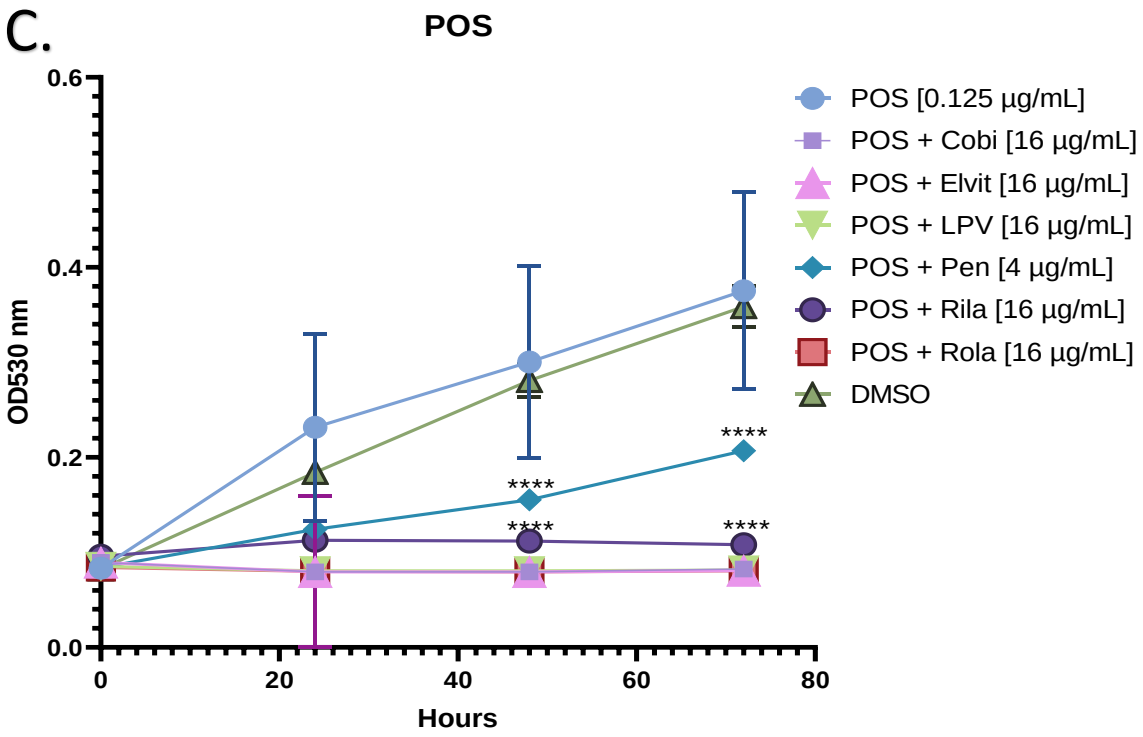
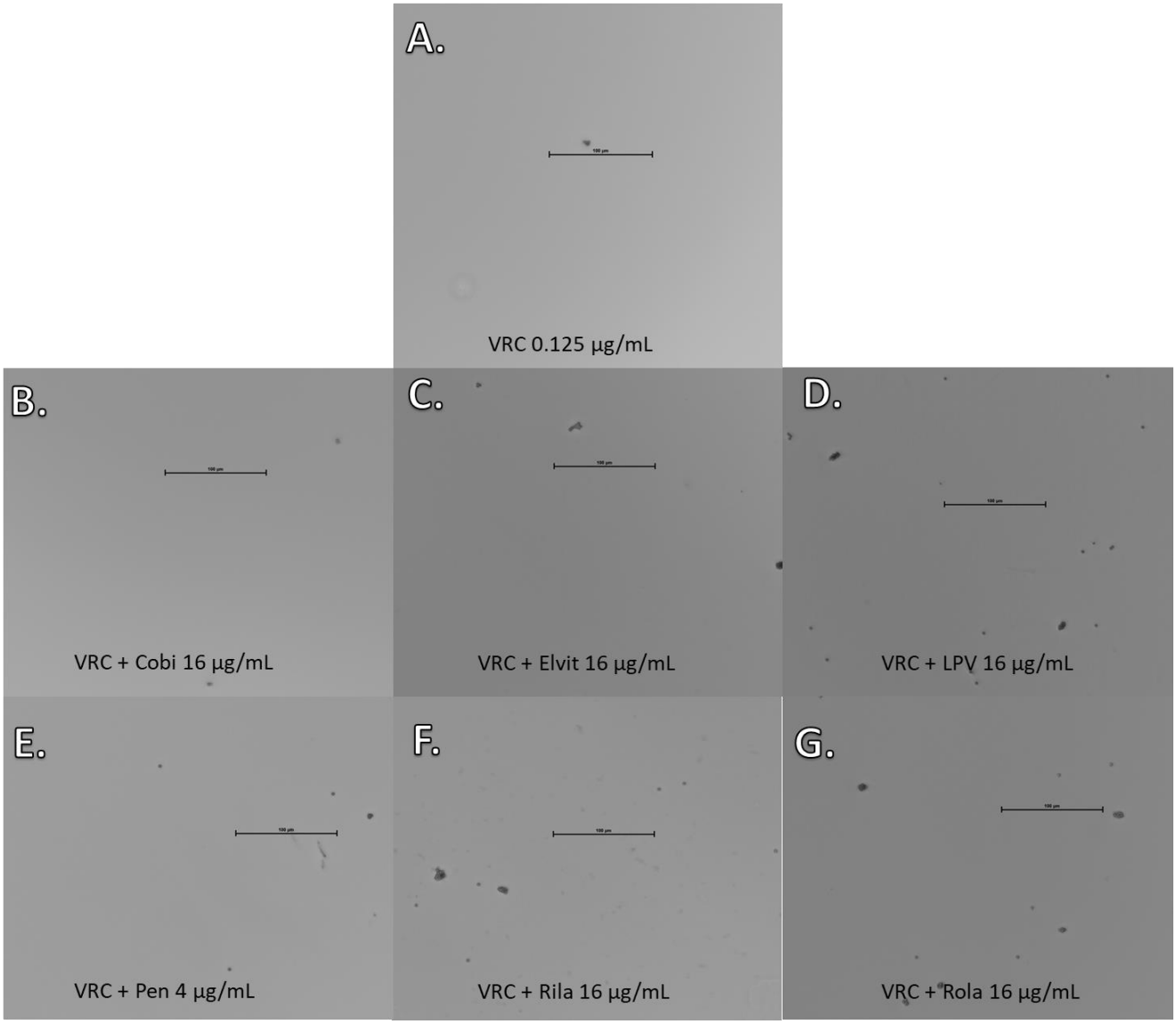
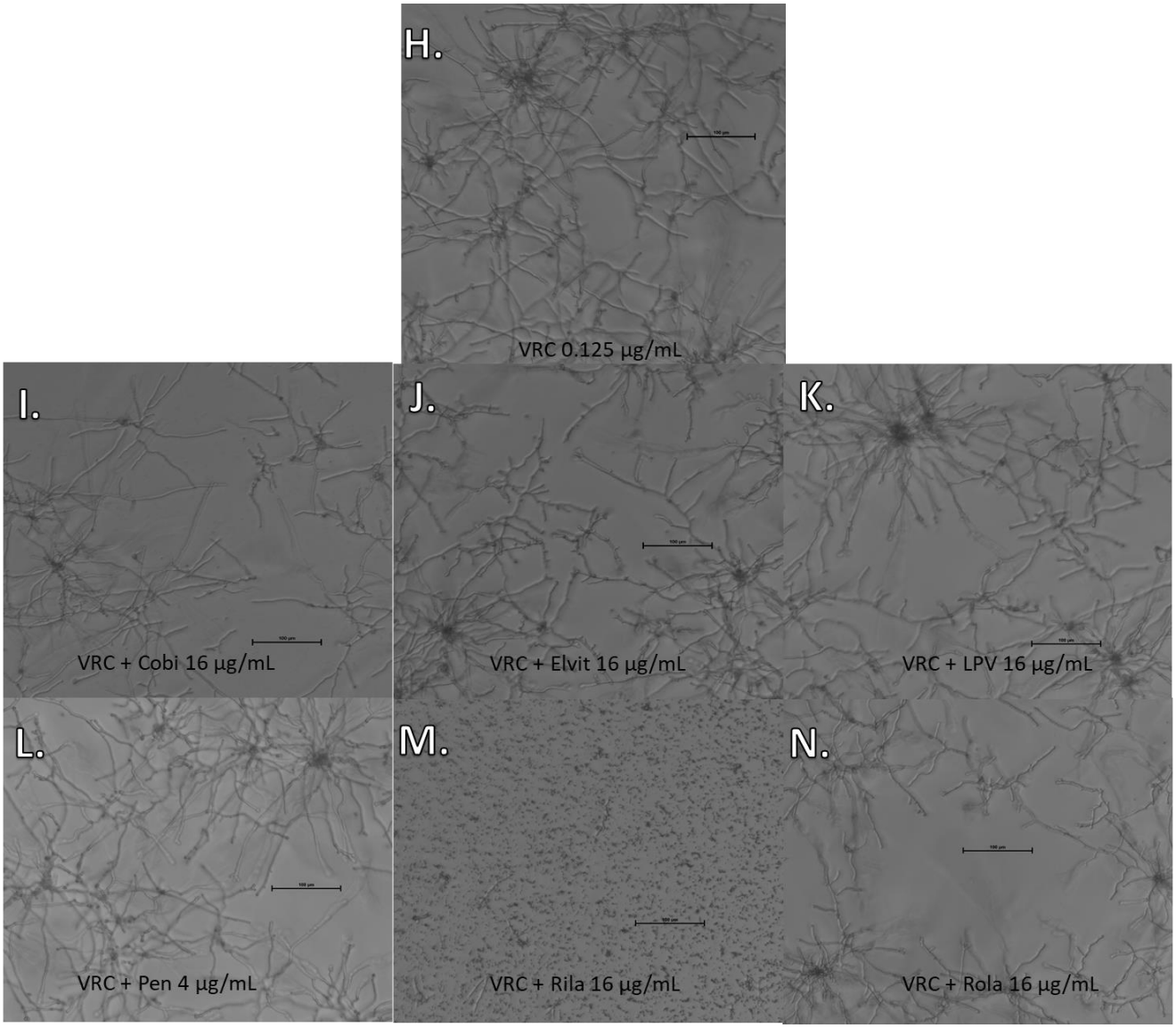
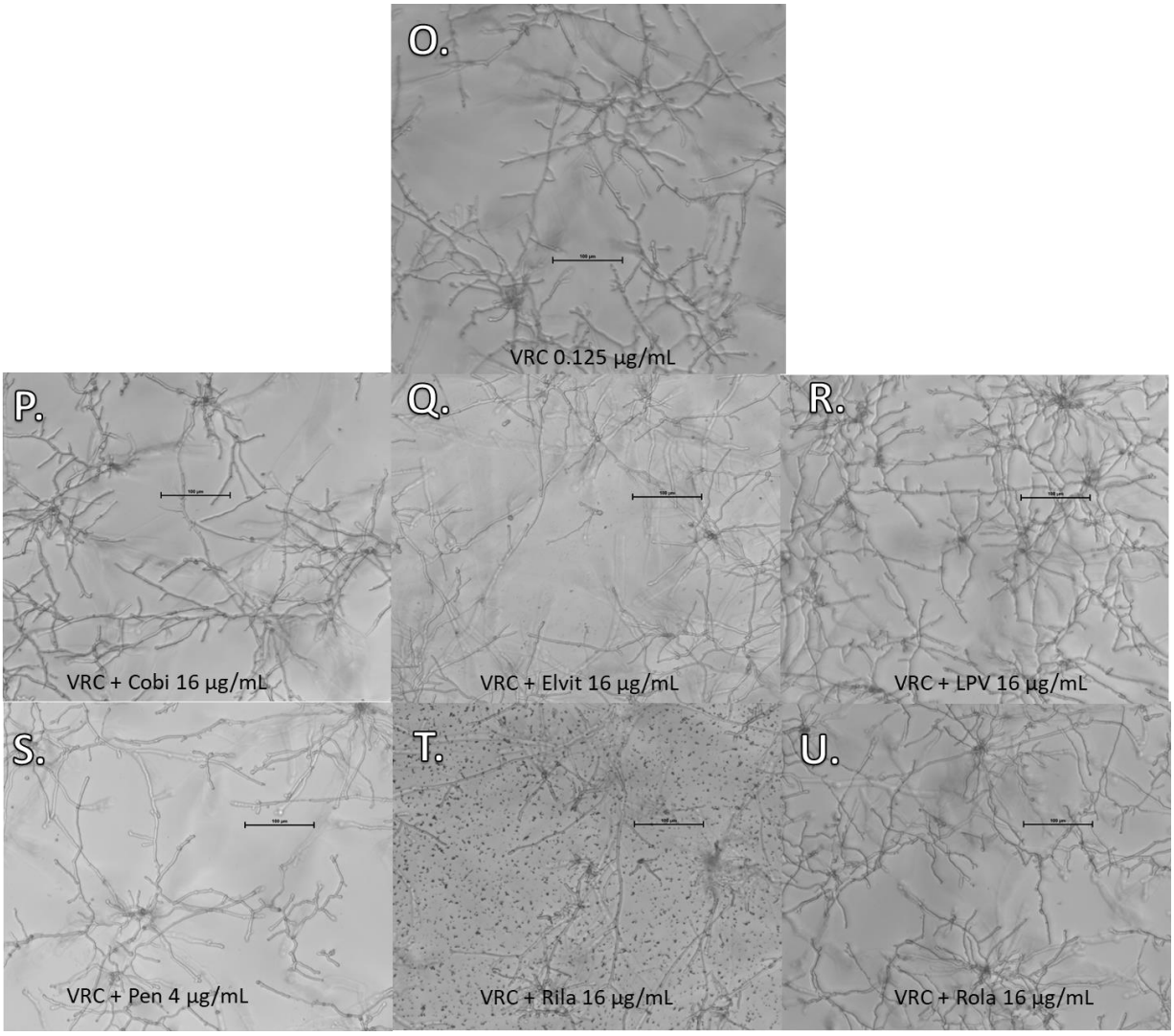

POS
OD530 nm
Hours
0.0
0.2
0.4
0.6
0
20
40
60
80
****
****
****
****
POS [0.125 µg/mL]
POS + Cobi [16 µg/mL]
POS + Elvit [16 µg/mL]
POS + LPV [16 µg/mL]
POS + Pen [4 µg/mL]
POS + Rila [16 µg/mL]
POS + Rola [16 µg/mL]
DMSO

Figure 2.3: A-AB: Germination Assay of *A. fumigatus* CDC #738 with VRC (0.125 µg/mL) and MCE compounds imaged from 0hr to 72hr. A-G images are at 0hr. H-N images are at 24 hr. O-U images are at 48hr. V-AB images are at 72hr




H.
VRC 0.125 µg/mL
I.
VRC + Cobi 16 µg/mL
J.
VRC + Elvit 16 µg/mL
K.
VRC + LPV 16 µg/mL
L.
VRC + Pen 4 µg/mL
M.
VRC + Rila 16 µg/mL
N.
VRC + Rola 16 µg/mL


O.
VRC 0.125 µg/mL
P.
VRC + Cobi 16 µg/mL
Q.
VRC + Elvit 16 µg/mL
R.
VRC + LPV 16 µg/mL
S.
VRC + Pen 4 µg/mL
T.
VRC + Rila 16 µg/mL
U.
VRC + Rola 16 µg/mL

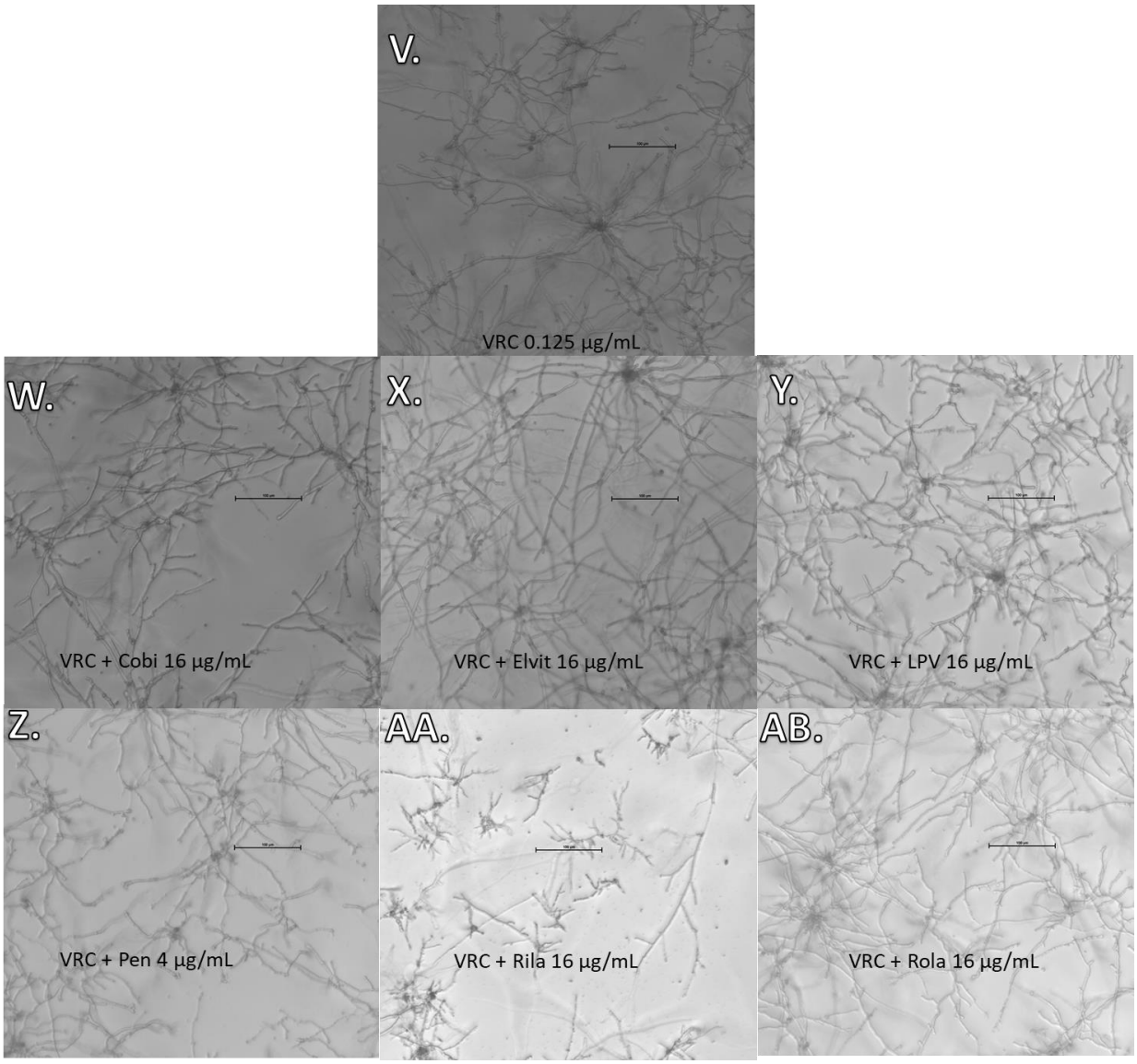
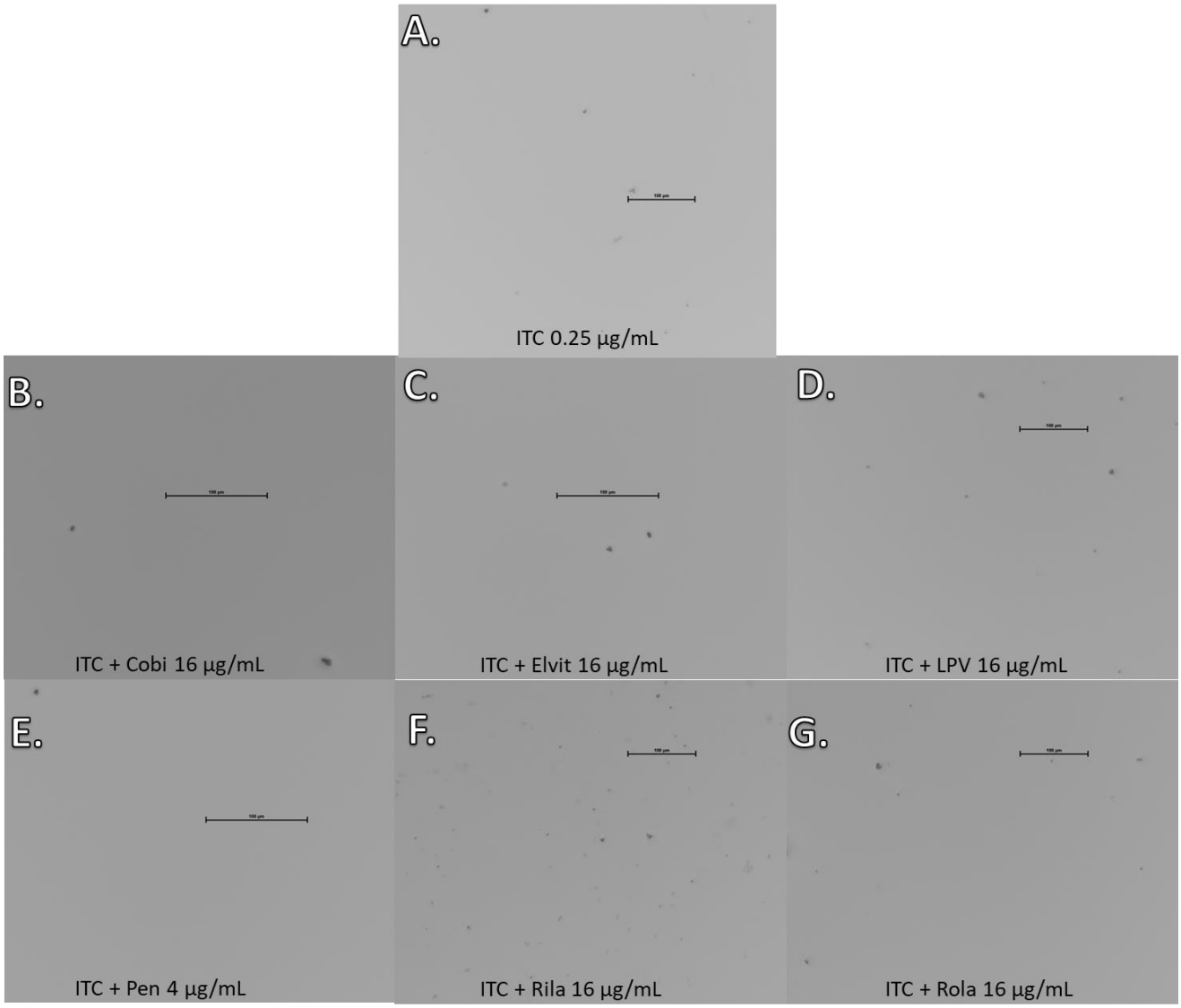
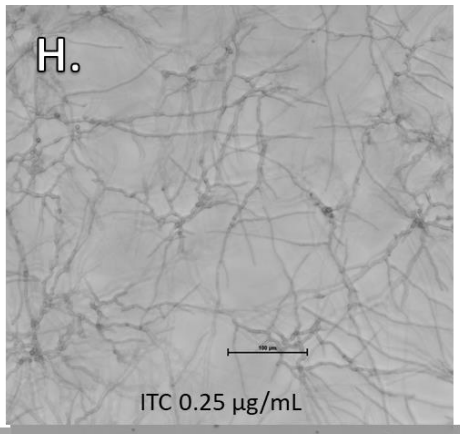
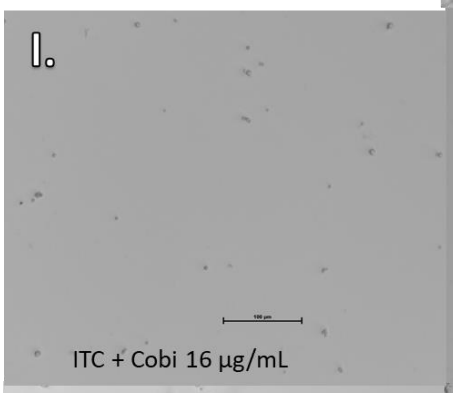

V.
VRC 0.125 µg/mL
W.
VRC + Cobi 16 µg/mL
X.
VRC + Elvit 16 µg/mL
Y.
VRC + LPV 16 µg/mL
Z.
VRC + Pen 4 µg/mL
AA.
VRC + Rila 16 µg/mL
AB.
VRC + Rola 16 µg/mL

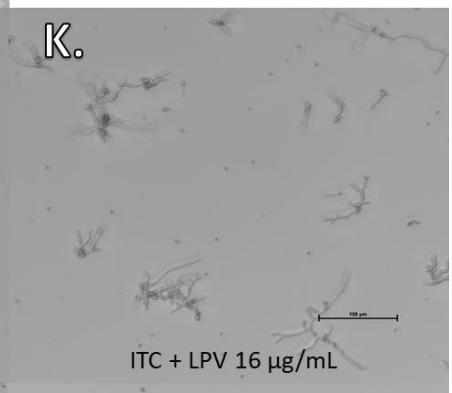
Figure 2.4: A-AB: Germination Assay of *A. fumigatus* CDC #738 with ITC (0.25 µg/mL) and MCE compounds imaged from 0hr to 72hr. A-G images are at 0hr. H-N images are at 24 hr. O-U images are at 48hr. V-AB images are at 72hr.

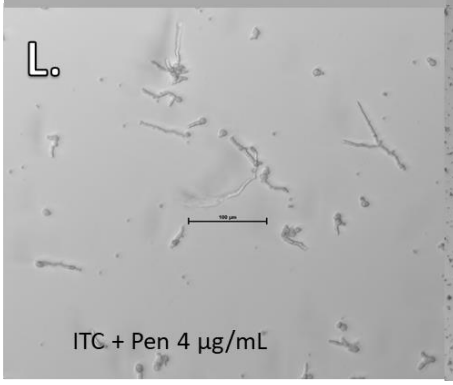


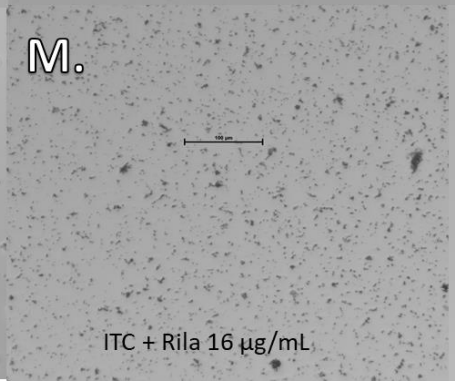

H.
ITC 0.25 µg/mL

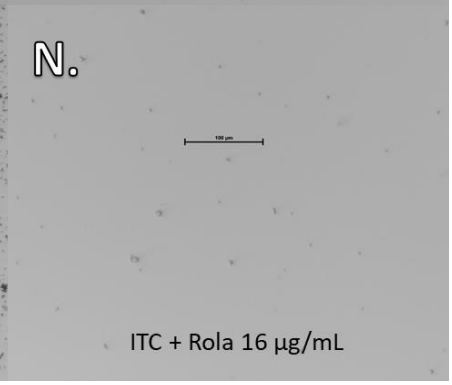

I.
ITC + Cobi 16 µg/mL

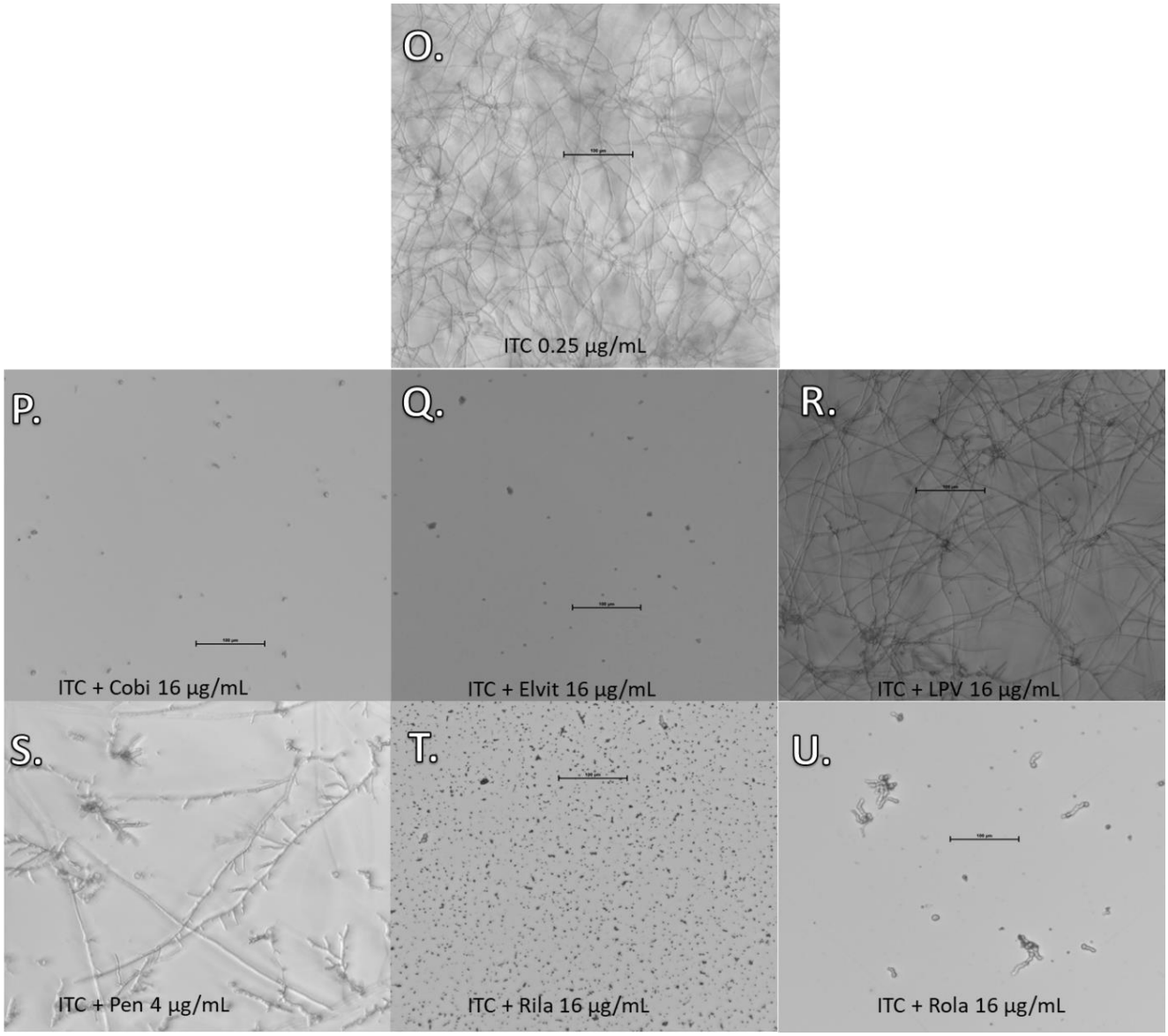

J.
ITC + Elvit 16 µg/mL


K.
ITC + LPV 16 µg/mL


L.
ITC + Pen 4 µg/mL


M.
ITC + Rila 16 µg/mL


N.
ITC + Rola 16 µg/mL


O.
ITC 0.25 µg/mL
P.
ITC + Cobi 16 µg/mL
Q.
ITC + Elvit 16 µg/mL
R.
ITC + LPV 16 µg/mL
S.
ITC + Pen 4 µg/mL
T.
ITC + Rila 16 µg/mL
U.
ITC + Rola 16 µg/mL

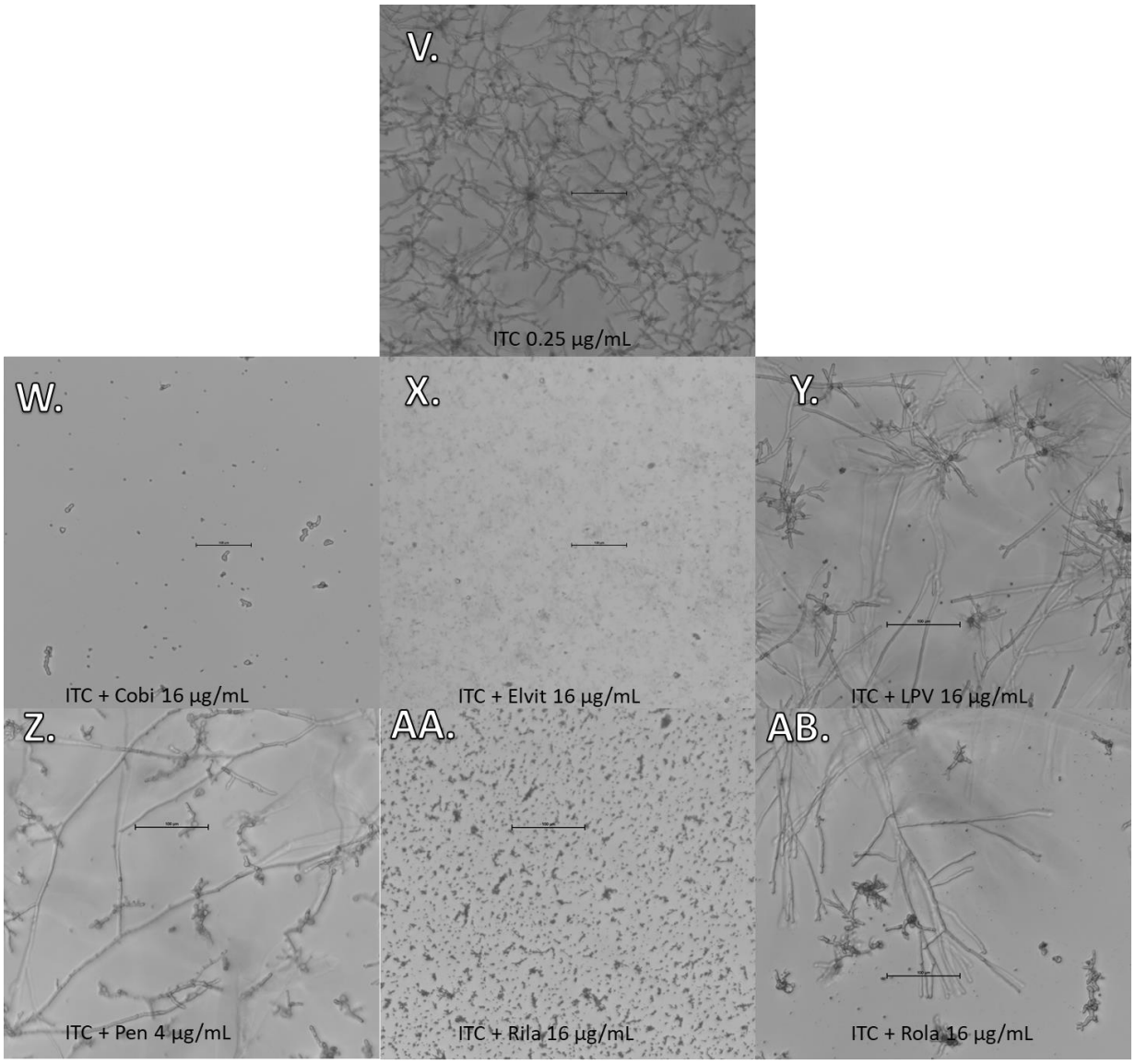
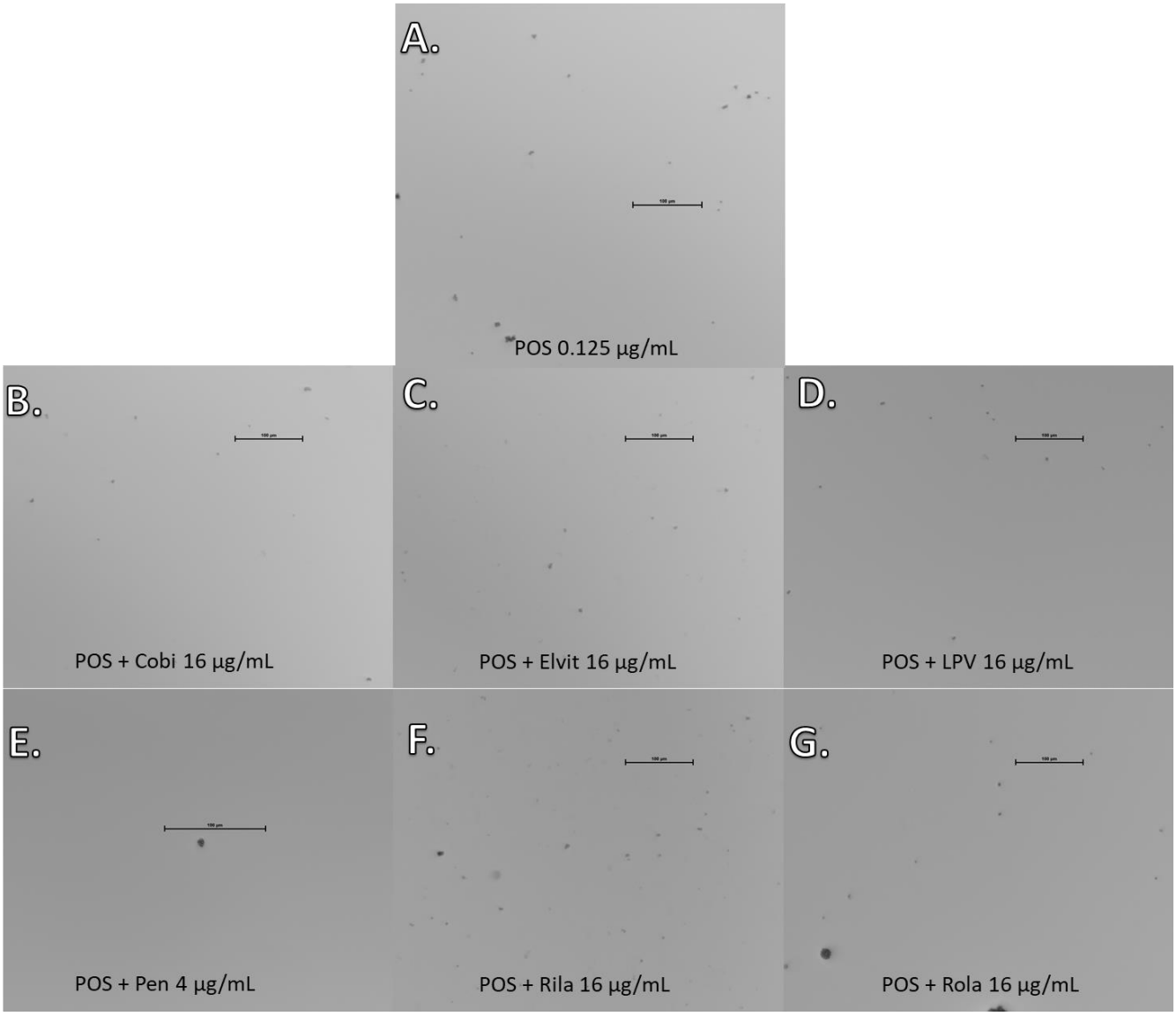
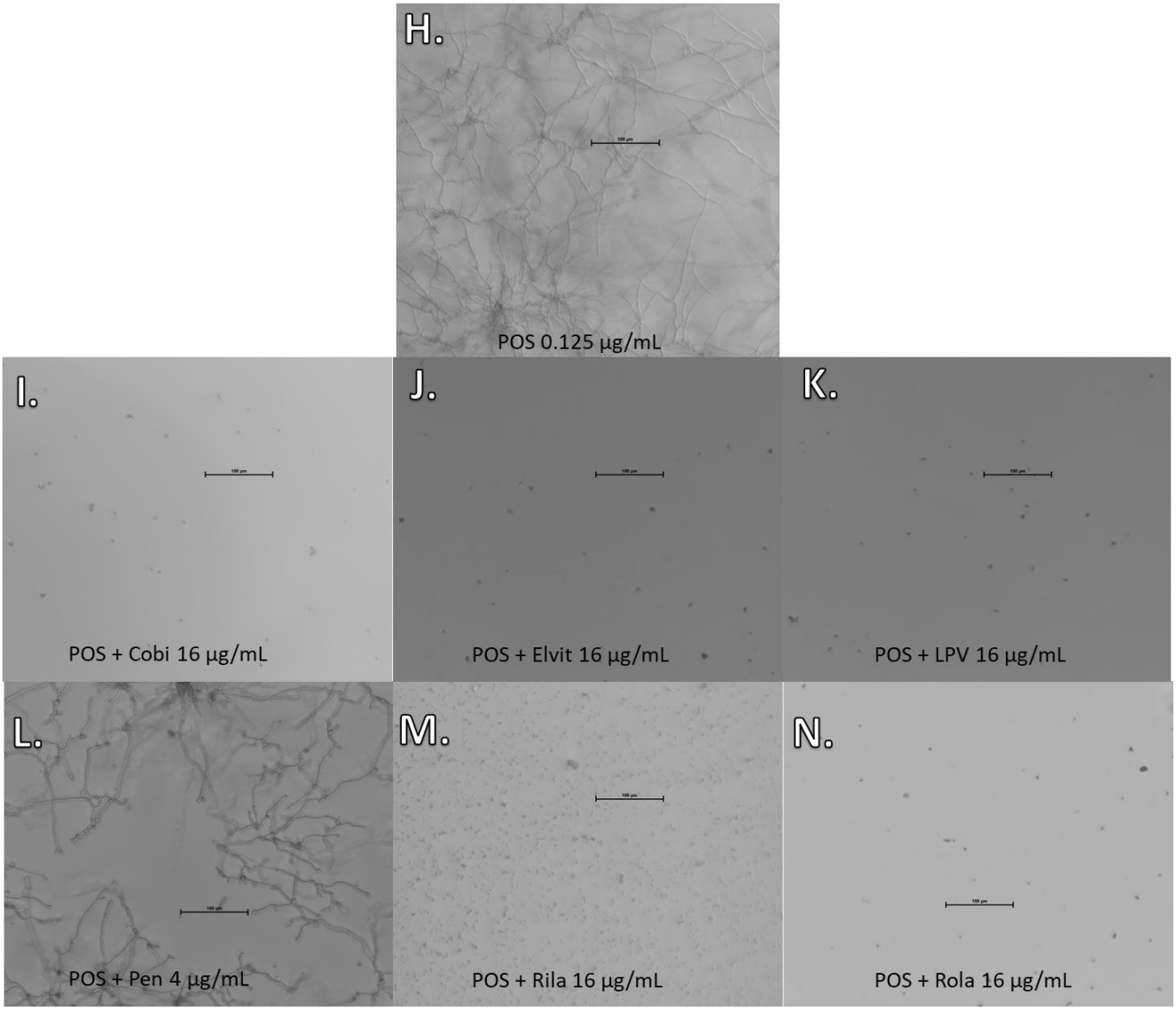
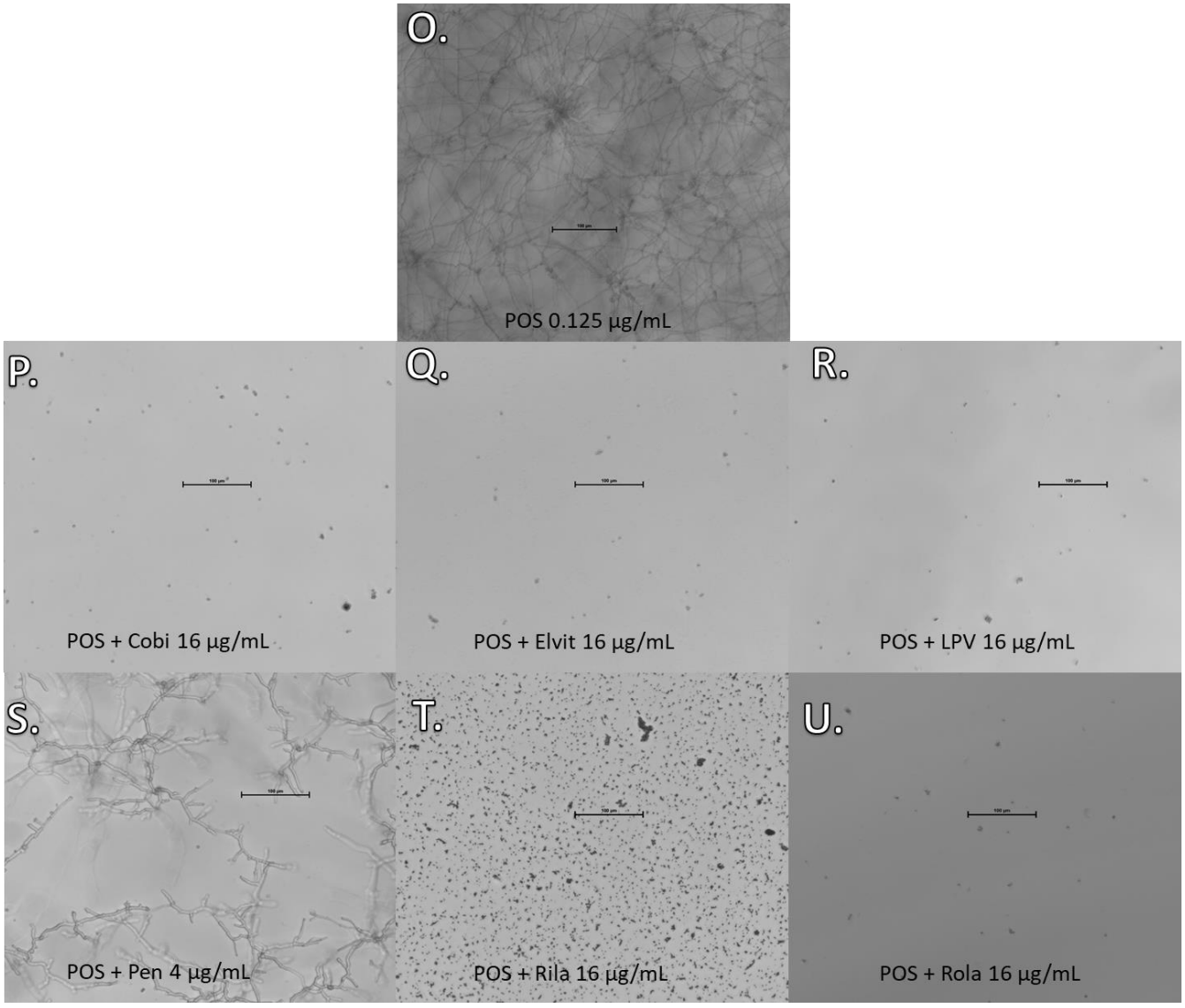
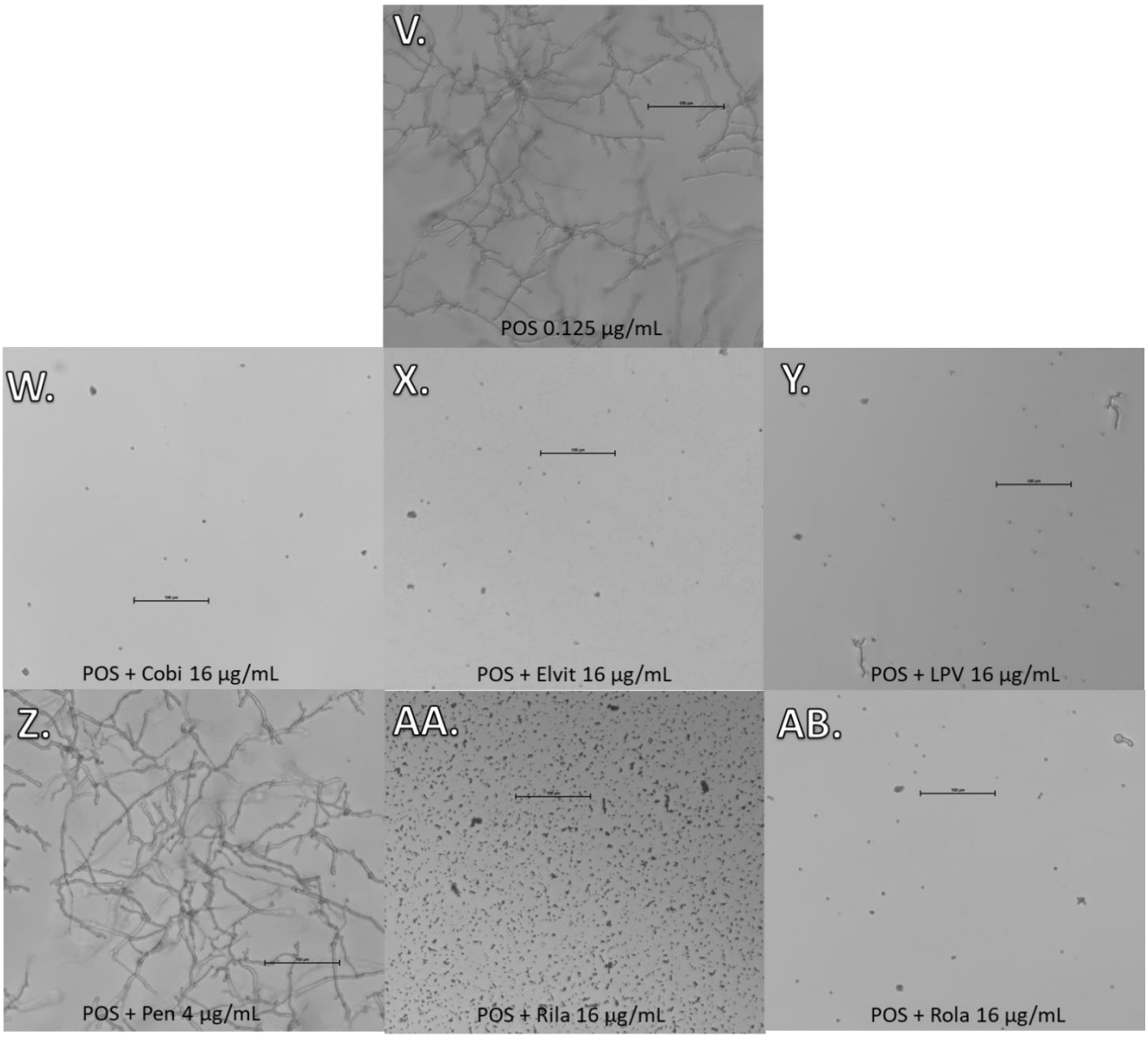

V.
ITC 0.25 µg/mL
W.
ITC + Cobi 16 µg/mL
X.
ITC + Elvit 16 µg/mL
Y.
ITC + LPV 16 µg/mL
Z.
ITC + Pen 4 µg/mL
AA.
ITC + Rila 16 µg/mL
AB.
ITC + Rola 16 µg/mL

Figure 2.5: A-AB: Germination Assay of *A. fumigatus* CDC #738 with POS (0.125 µg/mL) and MCE compounds imaged from 0hr to 72hr. A-G images are at 0hr. H-N images are at 24 hr. O-U images are at 48hr. V-AB images are at 72hr.




H.
POS 0.125 µg/mL
I.
POS + Cobi 16 µg/mL
J.
POS + Elvit 16 µg/mL
K.
POS + LPV 16 µg/mL
L.
POS + Pen 4 µg/mL
M.
POS + Rila 16 µg/mL
N.
POS + Rola 16 µg/mL


O.
POS 0.125 µg/mL
P.
POS + Cobi 16 µg/mL
Q.
POS + Elvit 16 µg/mL
R.
POS + LPV 16 µg/mL
S.
POS + Pen 4 µg/mL
T.
POS + Rila 16 µg/mL
U.
POS + Rola 16 µg/mL


V.
POS 0.125 µg/mL
W.
POS + Cobi 16 µg/mL
X.
POS + Elvit 16 µg/mL
Y.
POS + LPV 16 µg/mL
Z.
POS + Pen 4 µg/mL
AA.
POS + Rila 16 µg/mL
AB.
POS + Rola 16 µg/mL

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Chapter 3. Evaluation of MCE Screening Identified Compound Lopinavir

Synergistic potential of lopinavir and azole combinational therapy against clinically important *Aspergillus species*

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Abstract:

Aspergillus fumigatus is a widely distributed pathogen responsible for severe infections, particularly in immunocompromised individuals. Triazoles are the primary treatment options for *Aspergillus* infections; however, the emergence of acquired resistance to this antifungal class is becoming a growing concern. In this study, we investigated the potential of the antiviral drug, lopinavir (LPV) to restore the susceptibility of *A. fumigatus* strains to a set of azoles, while also reducing the required azole dosage for treatment of susceptible isolates. The combination of LPV with either itraconazole (ITC) or posaconazole (POS) demonstrated potent synergistic interactions against 16 out of 21 (~76%) and 19 out of 21 (~90%) *A. fumigatus* isolates, respectively. Moreover, the combination showed synergistic activity against other clinically important *Aspergillus* species, including *A. niger*, *A. flavus*, and *A. brasiliensis*. The fractional inhibitory concentration index (FICI) for the combinations ranged from 0.18 to 0.313 for ITC and 0.091 to 0.313 for POS, indicating strong synergistic effects. Further investigation revealed that efflux pump inhibition contributed to the synergy observed between azole and LPV. Morphological examination of the fungal cells subjected to this combinational therapy at sub-inhibitory doses showed the presence of carbohydrate granules/patches. The identification of LPV as a promising adjunct therapy holds promise for addressing the emerging challenge of azole resistance in *Aspergillus* species and improving treatment outcomes for patients.

Introduction:

Aspergillus fumigatus is a filamentous, spore-forming fungus posing a significant threat to immunocompromised individuals across a variety of settings (1, 2). The fungus itself is ubiquitous in nature and human society (3). Widely distributed in nature and human environments, *A. fumigatus* can cause various infections, including allergic bronchopulmonary aspergillosis, cutaneous aspergillosis, chronic pulmonary aspergillosis, aspergilloma (fungal ball), and invasive aspergillosis. Chronic pulmonary aspergillosis alone affects an estimated 3 million people globally (4). With an increasing number of immunocompromised patients, the incidence of *A. fumigatus* infections has risen significantly, resulting in over 200,000 infections annually, with mortality rates reaching as high as 90% (5, 6).

Addressing the urgent need for effective treatments, there is a demand for drugs capable of restoring the activity of azoles against *A. fumigatus*. Combinational therapies, like voriconazole and anidulafungin, have shown success in treating severe fungal infections (7, 8). However, novel antifungal development is hampered due to the structural similarities between fungal drug targets and human proteins, leading to limited viable options and potential off-target effects. Although antifungals such as amphotericin B are effective, patients often endure severe side effects and may discontinue treatment.

Aspergillus species frequently encounter multiple azole fungicides in agriculture and clinical settings, contributing to the development of azole resistance (9, 10). In *A. fumigatus,* azole resistance commonly occurs with a combination of alterations in the cyp51A gene, including tandem repeats (TR34 or TR46) and nonsynonymous point mutations (L98H/Y121F/T289A) (8,

11, 12). Additionally, efflux pump machinery plays a significant role in triazole resistance, and investigations have revealed its concerning ability to readily adapt (13-15). Non-Cyp51A triazole resistance, driven by efflux pump overactivity, is a growing concern, as direct overexpression has been shown to increase drug resistance (15, 16). These mounting challenges, combined with limited novel antifungals options, have become a paramount concern for health organizations such as the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) (17).

In this study, our main objective was to identify new drugs that can be repurposed from their originally approved therapy to treat *Aspergillus* species infections. Building on our previous work, screening libraries for compounds/drugs that enhance the activity of azole drugs against Candida (18-21), we focused on LPV and antiviral agent as an enhancer of azoles against *Aspergillus* species. Lopinavir, a protease inhibitor originally developed as an antiretroviral drug for treating HIV/AIDS, has garnered significant attention as a potential repurposed therapeutic in various other medical contexts. Its FDA approval highlights its established profile, enhancing its potential as a co-drug candidate for managing invasive fungal infections. Additionally, we explored the impact of the LPV/azole combination on the hyphal formation, a critical step for fungal dissemination. To gain insights into the mechanism of action, we investigated the ability of LPV, known to be poor ABC substrate, to interfere with the efflux pump machinery of *A. fumigatus* (22). Our research revealed that LPV can effectively inhibit fungal efflux pumps, a crucial mechanism for antifungal resistance, permitting the synergy observed between POS/ITC and LPV. The combination of potent *in vitro* synergy with azoles and the ability to interfere with fungal efflux pump makes LPV an exceptionally promising drug of high interest in this study. These findings represent a significant step towards identifying alternative treatments for *Aspergillus*

species infections, and the repurposing of LPV as a potential adjunct therapy with azoles offers a new avenue to combat the challenge of antifungal resistance in these pathogens.

Results:

Minimum inhibitory concentration (MIC) and microdilution checkerboard

To determine the MICs for ITC, POS, VRC and LPV against 25 different *Aspergillus* isolates, we perfromed three independent assays following CLSI protocol M38 (23). The antifungal activity, complete growth inhibition, of itraconazole ranged from 0.25 to 64 µg/mL, while posaconazole and voriconazole ranged from 0.25 to 2 µg/mL (24). Notably, five isolates displayed resistance to azoles, with MICs of 64, 1, and ~1 µg/mL for ITC, POS and VRC, respectively. LPV, on the other hand, did not exhibit antifungal activity up to 256 µg/mL, Table 3.1.

To identify synergistic interactions between azole antifungals (ITC, POS, and VRC) and LPV, a checkerboard assay was performed. We observed synergy between ITC and LPV in 16/21 strains with ΣFICI values ranging from 0.137-0.313 (Table 3.2). Similarly, for POS, we observed synergy in 19/21 strains with ΣFICI values ranging from 0.091-0313, Table 3.2. However, the interaction between VRC and LPV was indifferent against all tested strains, with ΣFICI values ranging from 0.563-1.125, Table 3.2. Surprisingly, we observed synergy in three POS-resistant strains, CDC #731 #733 and #734 with ΣFICI values of 0.281, 0.312, and 0.312, Table 3.2. Furthermore, were examined *A. brasiliensis, A. flavus and A. niger* via checkerboard assay to evaluate the efficacy of LPV in enhancing antifungal activity of azoles against other *Aspergillus* species. The results demonstrated synergy for all the strains treated with POS/ITC in combination with LPV, ΣFICI values of 0.265-0.281 and 0.091-0.312, respectively. Additionally, we investigated the possibility of reducing the necessary azole concentration further by applying a fixed concentration of LPV

(16 µg/mL) in the media. When 16 µg/mL of LPV was utilized in the media, 50% of the CDC isolates screened with ITC and LPV demonstrated reduced MICs, Figure 3.1. VRC displayed no significant reductions. Moreover, all strains screened with POS and LPV showed a reversion from resistant profiles, Figure 3.1.

Efficacy of LPV and azole combinational treatment on the growth kinetics of *A. fumigatus* clinical isolate CDC #738.

To investigate the effect of azole/LPV combination on the growth kinetics of *A. fumigatus*, we conducted a growth kinetics assay using *A. fumigatus* strain CDC #738. The fungal strain, 2.5 x10^5, was treated with LPV (16 µg/mL), ITC (0.25 µg/mL), VRC (0.125 µg/mL) and POS (0.125 µg/mL), and their combinations. As shown in Figure 2, LPV, POS, VRC and ITC alone did not have a significant impact on fungal growth and closely resembled the untreated control. Fungal growth was not inhibited through the co-application of LPV and VRC. However, when treated with ITC and LPV, fungal growth was arrested for nearly 30 hr until the azole was ineffective. Similarly, the combination of POS and LPV resulted in arrested fungal growth for 48 hr.

Effect of LPV on efflux machinery.

Efflux pumps play a critical role in microorganisms by facilitating the removal of undesirable products, including antifungal drugs, thereby contributing to drug resistance (14). To investigate the mechanism by which LPV enhances azole activity, we evaluated the ability of LPV to inhibit efflux in Aspergillus. Using a Rhodamine-6G efflux assay with the addition of glucose, we observed significant differences between untreated and treated groups. As depicted in fig. 3, LPV significantly hindering *A. fumigatus* efflux machinery up to 70% when compared to the untreated

cells (P<0.001). These results provide strong evidence that the enhanced antifungal efficacy of azole in combination with LPV is attributed to the inhibition of efflux.

Impact of LPV on formation of cell-wall carbohydrate patches induced by azoles.

We aimed to investigate the impact of LPV on the formation of cell-wall carbohydrate patches induced by azoles When fungal cells are challenged with effective azole levels, they undergo cellular dysregulation, resulting in the formation of patches that can be easily stained by calcofluor-white (25). Untreated cells showed no aberrant morphology, including the absence of carbohydrate patches, Figure 4A. Cells challenged with LPV alone displayed similar properties to the untreated group, Figure 4B. Similarly, cells challenged with sub-inhibitory doses of either azole alone also showed minimum to no patch formation, Figure 4C-D. However, when challenged with ITC and LPV, patches were readily observed along the interior of hyphae, Figure 4E. This observation was consistent with cells treated with POS and LPV, Figure 4F. Interestingly, a decrease in septa was observed around the patches. Demonstrating ineffective azoles doses when applied alone results in negligible patch formation while through the co-application of LPV large groups can be observed.

Discussion:

Clinical outcomes of azole-resistant Aspergillosis are frequently unfavorable, resulting in treatment failures and elevated mortality rates (26). In recognition of this significant concern, the WHO has designated Aspergillosis as a critical priority pathogen (26). The scarcity of new antifungal options further accentuates the need for innovative treatment strategies. In response to these challenges, our study has delved into alternative therapeutic avenues, with a specific focus on drug repurposing. The concept of repurposing drugs has gained prominence in antifungal drug

discovery, proving particularly effective in identifying co-drugs that complement existing antimicrobial agents (18-20, 27, 28). In a whole-cell screening assay of approximately 1500 FDA approved drugs we previously identified LPV, HIV protease inhibitor, as a promising candidate against *Candida* (20). The rationale behind repurposing LPV as an antifungal agent lies in its capacity to effectively inhibit the efflux pump in *Candida*, which addresses a significant concern in the context of azole-resistant aspergillosis (18). As shown by enhanced drug retention within the pathogen, we can effectively increase the availability of inhibitory concentrations of azole drugs at the target site. However, when considering modified drug targets, particularly for CDC isolates #731-735, a mixed effect is observed, as indicated in Table 1 and 2. Our findings show that in the case of ITC isolate #731, synergy can be achieved with cyp51A mutations, specifically TR34 and L98H. Upon investigating the synergy of A. fumigatus #731, 733, and 734 with POS in combination with LPV, we observe that only TR34 and L98H mutants exhibit synergy, with one reverting to a susceptible profile. However, as we delve deeper into the analysis, we find that the emergence of additional mutations, such as F495I and S297T, leads to a failure in combination efficacy. This discovery prompted a comprehensive investigation into the potential synergistic effects of LPV in combination with three azoles against a diverse array of isolates from both resistant and susceptible *Aspergillus* species. Checkerboard data clearly illustrates the ability of LPV to synergize with ITC and POS, against various *Aspergillus* isolates. However, unlike its efficacy against *Candida*, the combination of LPV with VRC does not demonstrate the same degree of synergistic activity against *Aspergillus* isolates (18). This disparity could be attributed to the unique interaction between the side chains of ITC and POS, which occupy a specific channel within CYP51(28, 29). This interaction, absent with VRC, is believed to bolster the stability of

binding to CYP51 proteins, potentially explaining the differential response observed between the three azoles (28, 29).

Our initial checkerboard data demonstrated that the co-application of LPV enhances azole activity against susceptible strains, as well as two CDC-resistant strains Surprisingly, LPV was able to revert azole resistance in two resistant strains, CDC #731 and CDC #733, specifically for POS. These findings underscore the promising role of LPV as a synergistic agent in combating azole resistance in *Aspergillus*.

Growth kinetic assays are crucial for understanding the ability of the microorganism to survive and proliferate in their environment. We demonstrate that both the azoles, ITC, VRC and POS, and LPV alone fail to inhibit fungal proliferation at sub-MIC concentrations. When POS and LPV are combined, we observe a significant reduction in fungal proliferation at sub-MIC concentrations over a 48-hour period. ITC and LPV, on the other hand, exhibited substantial inhibition of fungal growth up to 36 hours, as shown in Figure 2. In contrast, VRC and LPV failed to inhibit fungal growth at the same time points when ITC/POS and LPV were able to effectively curb growth. This underscores the immense promise of synergistic combinations as innovative therapeutic strategies in combating multidrug-resistant fungal infections.

The checkerboard data and growth kinetic assays have provided compelling insights into the enhanced activity of azoles against Aspergillus, igniting the exploration of underlying mechanisms. Among these mechanisms, efflux systems have emerged as pivotal players in triazole resistance within *Aspergillus* species (16). These efflux mechanisms operate through the active expulsion of azoles from fungal cells, a process that diminishes intracellular drug concentrations and diminishes azole efficacy. Efflux pump genes, such as Cdr1B efflux transporter, ABC transporters (AtrF, AtrI), and a major facilitator superfamily transporter (MdrA) have been

documented to exhibit elevated activity in azole-resistant clinical isolates of *Aspergillus* (30). Furthermore, cellular efflux assays have shown that LPV is unable to be effluxed via efflux pumps, including ABC transporters, within MCF-7/Dox40 and MCF-7/MR cells lines (22). Additionally, LPV has been found to inhibit efflux activity of *C. auris* (20). Given the similarity in functionality of ABC transporters to *Candida species*, which have been shown to contribute to resistance, we hypothesized that LPV application reduces the ability of *Aspergillus* to efflux azoles. Interestingly, our experiments have shown that cells treated with LPV retain an impressive retention rate of nearly 70% of Rhodamine-6G. This impressive retention rate has been observed when clorgyline, a proposed efflux pump inhibitor, was applied by Esquivel et al., (31). Our results and those of Esquivel et al., collectively indicate that the Aspergillus efflux system can be effectively inhibited by the addition of LPV or another efflux inhibitor, even with the addition of glucose (31). While ITC and POS can be enhanced by efflux inhibition VRC demonstrated little increased efficacy. Literature suggests that VRC is not significantly impacted by efflux mechanisms, although it acts as a substrate and its presence can increase efflux activity(31, 32). The intricate interplay between LPV and efflux mechanisms holds promise for the development of novel strategies to counteract azole resistance in Aspergillus, thereby advancing our understanding of potential avenues for antifungal intervention.

Research findings suggest that azole antifungals exert their fungicidal activity in *Aspergillus* by inducing the creation of cell wall carbohydrate patches (25). These patches, composed of both toxic and nontoxic sterol intermediates, trigger the demise of the fungus (25). Typically, exposure to effective azole concentrations triggers the generation of carbohydrates patches (25). Intriguingly, our observations indicate that patch formation can also be prompted by challenging isolates with sub-inhibitory levels of both azoles and LPV. This potential to enhance azole activity,

even at sub-inhibitory concentrations, highlights the promising role of LPV as a co-drug. The results demonstrate the efficacy of LPV and azoles in combination and provide valuable insights into potential therapeutic strategies against *Aspergillus* infections.

The investigation of additional Aspergillus species, including *A. brasiliensis, A. flavus,* and *A. niger*, using the checkerboard assay provided valuable insights into the broader applicability of LPV's synergistic effect in combination with azoles. The observed synergy, reflected by ΣFICI values ranging from 0.13 to 0.38 for POS/ITC in combination with LPV, further strengthens the potential of LPV as a co-drug to enhance the antifungal activity of azoles across different *Aspergillus* strains.

In conclusion, our study highlights the ability of LPV to revive the antifungal activity of azoles by inhibiting efflux mechanisms. The combination of sub-inhibitory doses of azoles with LPV leads to the formation of carbohydrate patches, signifying enhanced retention of azoles and the accumulation of toxic intermediates. Furthermore, the usage of LPV is well tolerated in a population highly susceptible to Aspergillus fumigatus infections (33, 34). The potential to apply an FDA approved drug to enhance current azole therapies in a clinical setting is readily apparent. Overall, our findings support the use of LPV as a promising antifungal co-drug. Further investigations are warranted to explore the clinical potential of this combination therapy in treating *Aspergillus* infections and combating the emerging challenge of antifungal resistance.

Materials and Methods:

Fungal Strains, Media, Reagents and Chemicals.

Fungal strains utilized in this study, table 1, were obtained from the CDC (Atlanta, GA, USA), the American Type Culture Collection (ATCC), and BEI Resources (Manassas, VA, USA). RPMI

media components were purchased from Thermo Fisher Scientific (Waltham, MA), RPMI 1640, Sigma-Aldrich (St. Louis, MO) 3-(N-Morpholino) propane sulfonic acid (MOPS), Becton, Dickinson and Company (Franklin Lakes, NJ), Potato Dextrose (PD) broth and agar. Itraconazole and lopinavir were purchased from Acros Organics (New Jersey, USA). Voriconazole was purchased from TCI Ltd., (Tokyo) and posaconazole was purchased from Biosynth Carbosynth (San Diego, CA USA). Rhodamine 6G was procured from Alfa Aesar (Tewksbury, MA USA). Reagents and dyes for fungal cell imaging are as follows: formaldehyde (Sigma Aldrich, St. Louis MO.), triton X-100 (Acros Organics, New Jersey, USA), and calcofluor-white (Sigma-Aldrich, St. Louis, MO).

Minimum inhibitory concentration (MIC) and microdilution checkerboard assays.

To assess the activity of LPV and azoles (ITC, POS and VRC) against a variety of *Aspergillus* species, we conducted a standard broth microdilution checkerboard assay(35, 36). The MIC and Checkerboard readings were determined at 48 hours post incubation, wells selected for displayed 100% growth inhibition (23). The fractional inhibitory concentration indexes (ΣFICI) were calculated to determine drug interactions between the drugs (37). Drug interactions were determined to be synergistic (SYN) when ΣFICI ≤ 0.5, indifferent (IND) at >0.5 to ≤4 and antagonistic (ANT) when values were > 4 (38-40). Each assay was independently performed in duplicate; all values were then compared to the published ranges.

Growth kinetics of *A. fumigatus* clinical isolate CDC #738.

To assess the effect of the combinational therapies on *A. fumigatus* strain CDC #738, a growth kinetic was performed (41, 42). Fungal spores were diluted in RPMI to 5 x 10^4 condida/mL. The fungal cells were then treated with either POS at 0.125x MIC (0.125 μg/mL), ITC at 0.25x MIC

(0.25 μg/mL), LPV (16 μg/mL) or a combination of POS and LPV. To monitor the growth, the optical density (OD_{530}) of the samples was measured at 0, 6 24, 36 and 48 hours using a SpectraMax i3x microplate reader at 530 nm (42). VRC at 0.25x MIC (0.125 μg/mL) in combination with LPV at 16 μg/mL was utilized to challenge isolate #738 spores diluted in RPMI at 5 x 10^4 condida/mL and read at 0, 24 and 48hrs using a SpectraMax i3x microplate reader at 530 nm (42). This allowed us to track the growth progression over 48 hr under the different treatment conditions. The experiment was independently performed in triplicate.

Efflux Pump Inhibition in *A. fumigatus* by Lopinavir.

To investigate the mechanism by which LPV enhances azole activity, we performed a rhodamine 6G (R6G) efflux assay (20, 31, 43, 44). Briefly, mid-log phase cells cultured at 37ºC were harvested, washed thrice with PBS, and starved by incubating them in PBS for 8 hr. The starved cells were then incubated with R6G at a final concentration of 7.5 μM for 30 minutes at 37ºC and then washed twice with PBS. Fungal cells were treated with LPV (16 μg/mL) then transferred to an opaque 96-well plate, in 100μL aliquots. Glucose at 100mM was added in 25μL amounts to initiate efflux in fungal cells. Over a period of 30 minutes, the fluorescence intensity of R6G was measured at 430/485 nm using a SpectraMax i3x microplate reader (Molecular Devices, San Jose, CA). The fluorescent values were averaged and compared to the control (untreated) wells using unpaired two-tailed *t*-test. This experiment was performed in triplicate.

Impact of LPV on formation of cell-wall carbohydrate patches induced by azoles.

To assess the morphological changes induced by LPV and azole therapy, fungal cells were visualized using calcofluor-white staining as described before (25). Briefly, *A. fumigatus* strain (CDC #738) was cultured overnight in RPMI 1640 at 37ºC in a shaking incubator. The cells were

then exposed to either LPV (16 µg/mL), POS (0.125 µg/mL), ITC (0.25 µg/mL), azole/LPV combination, or left as a negative control. After incubating for 3 hr at 37 ºC, 200 uL aliquots of the cells were placed onto a slide and allowed to adhere for 3 hr at 37 ºC. Subsequently, cells were fixed with a solution of 4% formaldehyde and 0.2% Triton X-100, prepared in 1X PBS and incubated at 25ºC for 30 minutes. The cells were then washed twice with PBS. For staining, the cells were exposed to 50-100uL calcofluor-white for 10 minutes at room temperature and then washed again twice with PBS. Finally, the stained samples were visualized with a Zeiss LSM 880 confocal laser scanning microscope, and the images were stored in a CZI format for analysis and documentation.

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Transparency Declaration:

None to declare.

Data Availability:

The datasets generated during this work and those analyzed in the current study are available from the corresponding author on reasonable request.

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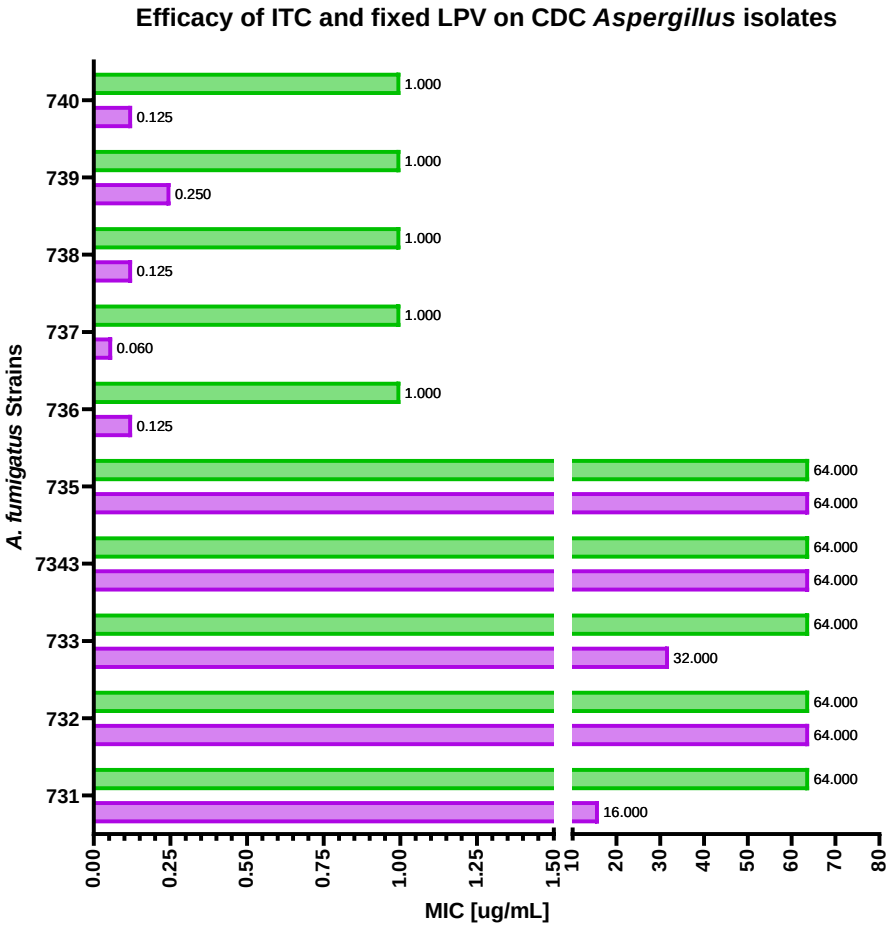
Efficacy of ITC and fixed LPV on CDC Aspergillus isolates
A. fumigatus Strains
740
739
738
737
736
735
7343
733
732
731
1.000
0.125
1.000
0.250
1.000
0.125
1.000
0.060
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0.125
64.000
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0.00
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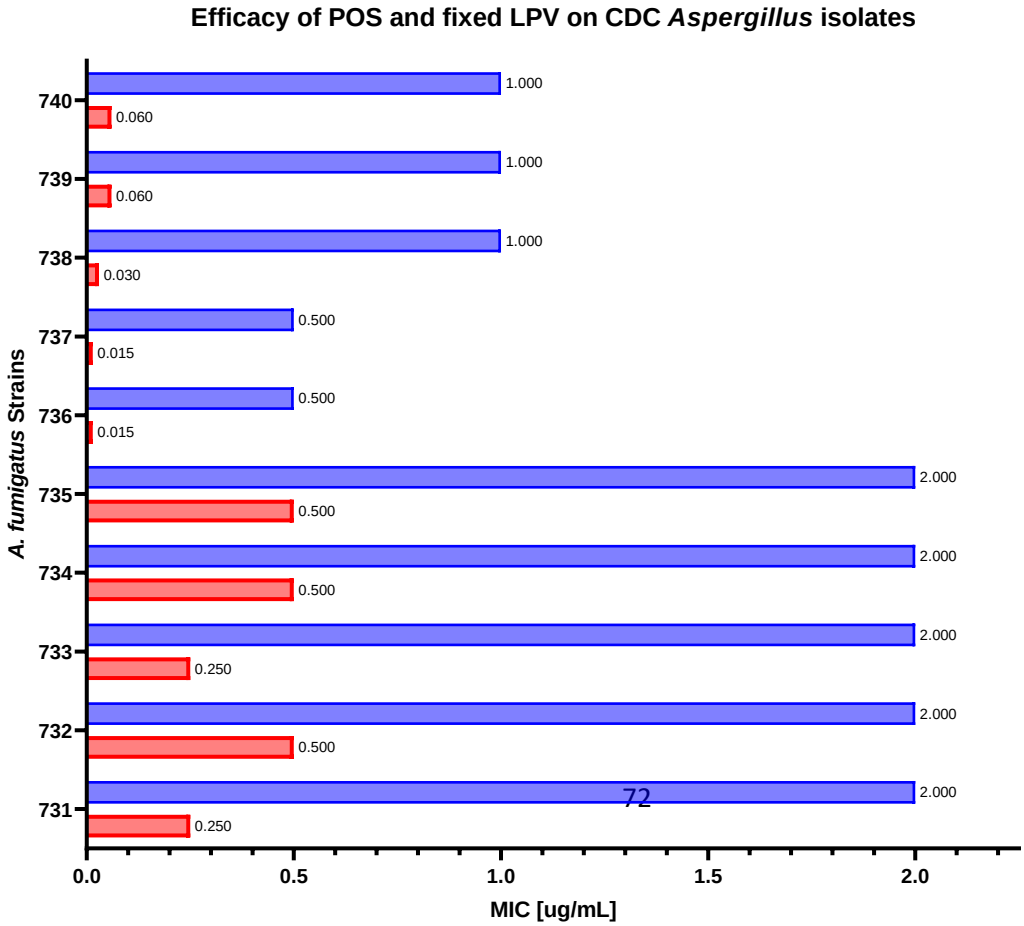
MIC [ug/mL]


Efficacy of POS and fixed LPV on CDC Aspergillus isolates
A. fumigatus Strains
740
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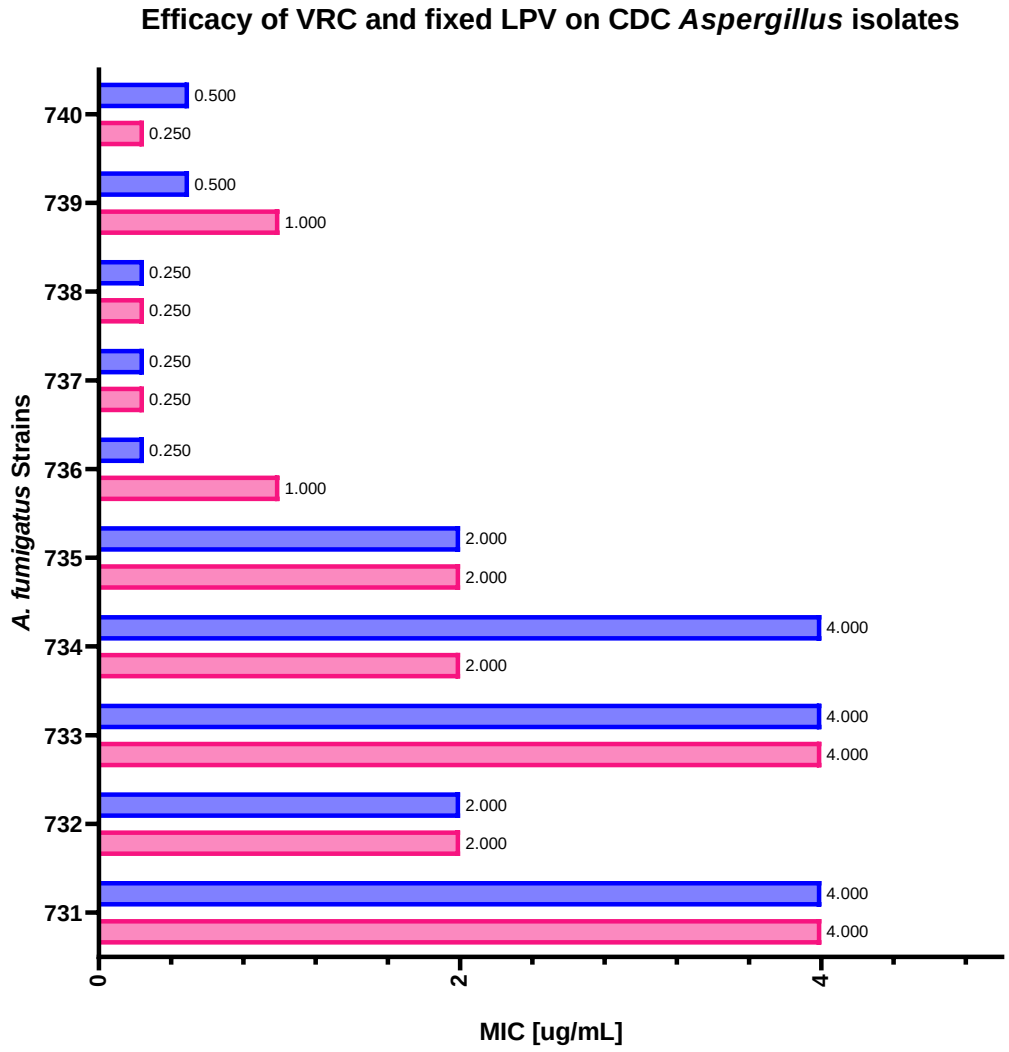

Figure 3.1: **Efficacy of fixed LPV combinational treatment on the susceptibility of *A. fumigatus* clinical isolates to posaconazole/itraconazole/voriconazole.** Lopinavir at a fixed concentration of 16 µg/mL with serially diluted posaconazole and itraconazole displays potent reduction in MIC when present. Red/Purple/Magenta contain LPV, while Blue/Green/Blue lack LPV.

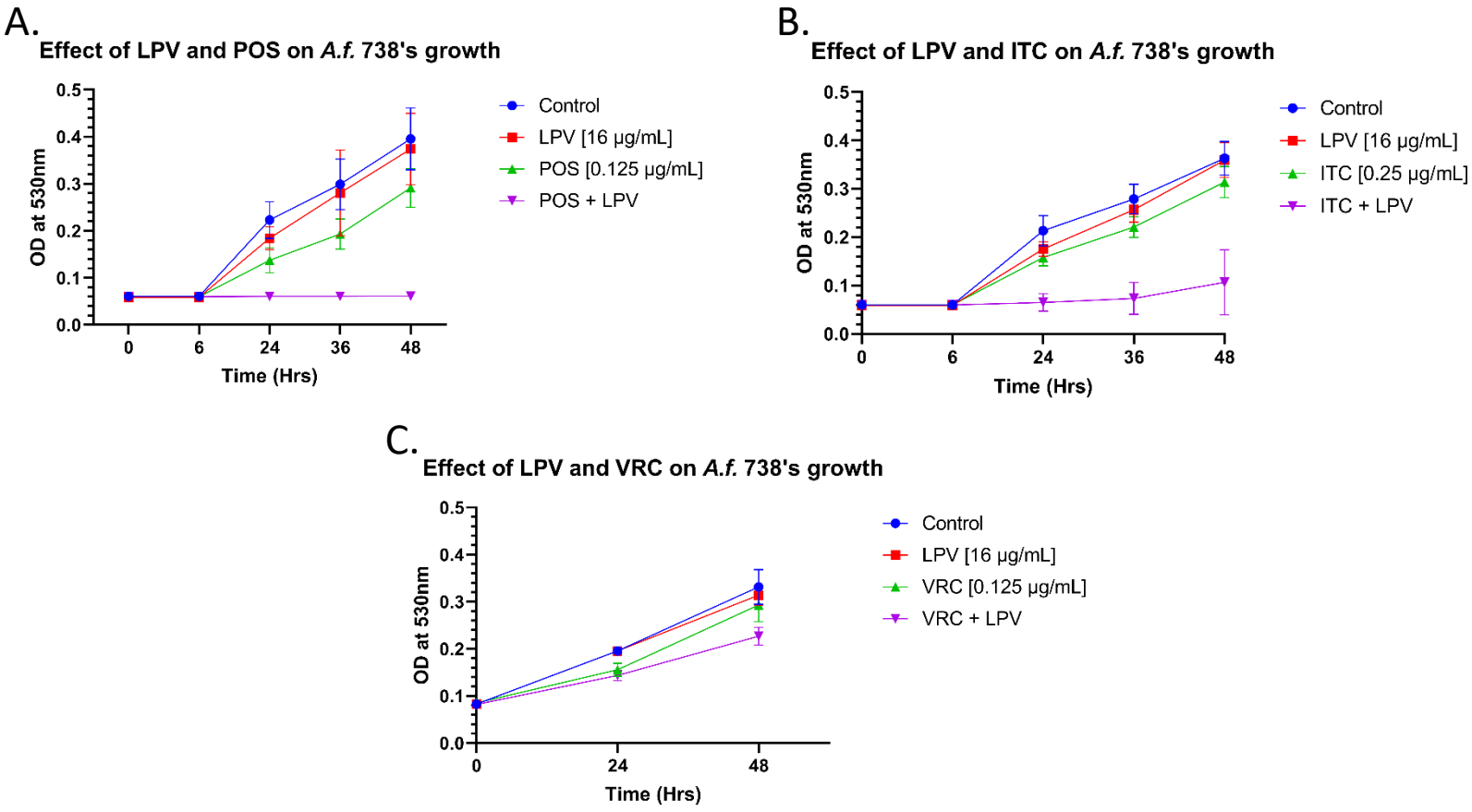

Figure 3.2: **Effect of POS/LPV and ITC/LPV on the growth Kinetics of *A. fumigatus* strain CDC #738. (A)** Growth kinetics of *A. fumigatus* CDC #738 in RPMI 1640 at 37° C with or without LPV, POS, or both present in the media, OD at 530 nm was recorded over 48 hr. **(B)** Growth

kinetics of isolate CDC #738 in RPMI 1640 treated with LPV, ITC or both present in the media. **(C)** Growth kinetics of isolate CDC #738 in RPMI 1640 treated with LPV, VRC or both present in the media. Data represents the means and standard deviations of triplicate determinations.

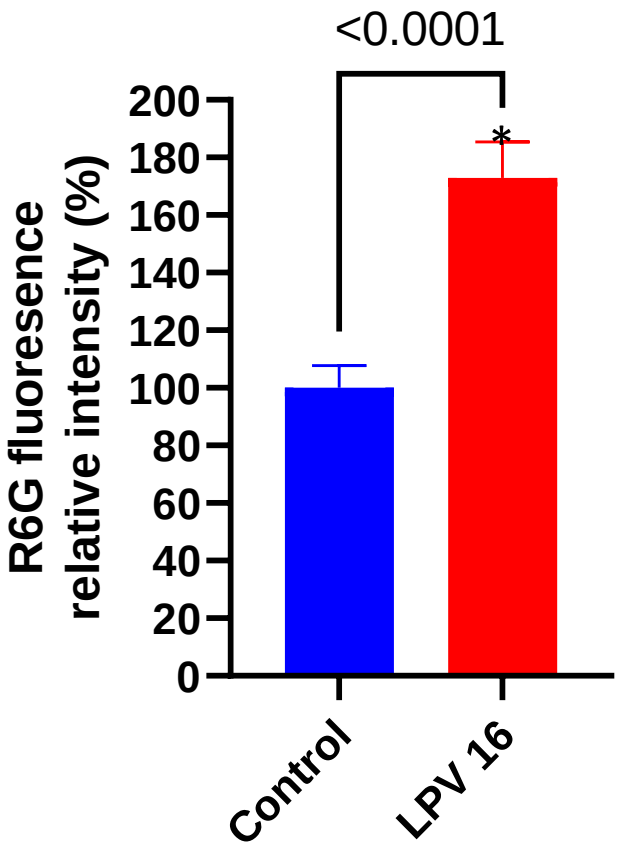

Figure 3.3: **Efflux Pump Inhibition in *A. fumigatus* by Lopinavir.** R6G relative fluorescence in the presence or absence of LPV using *A. fumigatus* strain CDC #738. The relative change in the effluxed rhodamine as compared to the untreated, positive, control was determined as percentage. Data represents the means and standard deviations of triplicate determinations. * Indicates a significant difference between LPV and the untreated control ($P < 0.0001$) as determined by unpaired two-tailed *t*-Test.

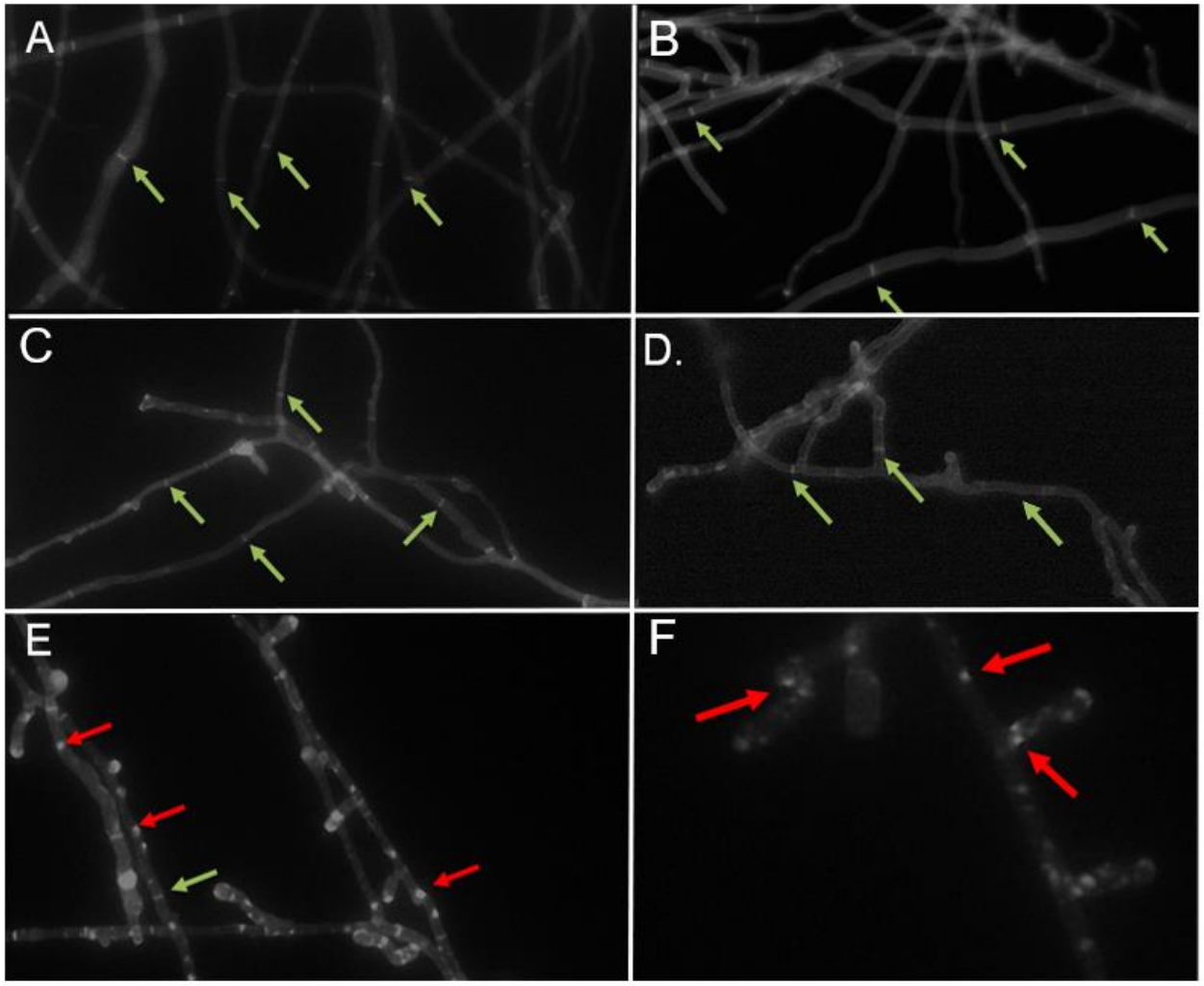

Figure 3.4 A-F: **Determination of toxic patch formation within hyphae of *A. fumigatus* arising from combinational therapy.**

Figures A-F represent *A. fumigatus* grown (20hr) under different conditions. RPMI alone **(A)**, LPV [16 µg/mL] **(B)**, POS [0.125 µg/mL] **(C)**, ITC [0.25 µg/mL] **(D)**, LPV [16µg/mL] + POS [0.125 µg/mL] **(E),** and LPV [16 µg/mL] + ITC [0.25 µg/mL] **(F)**. The presence of septa, identified by green arrows, can be observed as bars spanning across the sides of the fungi. **(A)** & **(B)** show no other morphological oddities within the fungal cells, indicative of healthy unimpeded growth. These are also observed withing **(C)** & **(D)** when sub-inhibitory of azole doses are applied to the fungus. Figures **(E)** & **(F)** represent *A. fumigatus* grown with a combination of LPV+POS **(E)**,

LPV+ITC **(F)**; in these figures, green arrows can be observed to point out septa, a common motif. The red arrows point out morphological oddities not present in normal fungal cells. These granules or patches are conglomerations of carbohydrates resulting from dysregulation within the cell wall synthesis.

Table 3.1: Description of the fungal strains utilized in this study and their minimum inhibitory concentration (MICs)

Aspergillus **Strain/Designation**	**MIC [µg/mL] ITC**	**MIC [µg/mL] POS**	**MIC [µg/mL] VRC**	**MIC [µg/mL] LPV**	**Source & Characteristic & Resistance Mechanism**
NR-35301	1	0.5	0.125	>256	Human abdominal tissue (1998)
NR-35302	1	0.5	0.25	>256	Human peritoneal fluid (1998)
NR-35303	1	0.5	0.25	>256	Human sputum-tracheal suction(1998)
NR-35304	1	0.5	0.25	>256	Human sputum-tracheal suction(1998)
NR-35305	1	0.5	0.25	>256	Hospital Environment (1998)
NR-35307	1	0.5	0.25	>256	Hospital Environment (1998)
NR-35310	1	0.5	0.25	>256	Environmental Isolate (2002)
NR-35311	1	0.5	0.25	>256	Environmental Isolate (2002)
NR-35312	1	0.5	0.25	>256	Environmental Isolate (2002)
NR-41311	1	0.5	0.5	>256	Human Sputum
NR-41312	1	0.5	0.125	>256	Human Sputum
CDC 731	64	1	2	>256	L98H, TR34
CDC 732	64	1	1	>256	F495I, L98H, S297T, TR34
CDC 733	64	1	2	>256	L98H, TR34
CDC 734	64	1	2	>256	L98H, TR34
CDC 735	64	1	1	>256	F495I, L98H, S297T, TR34
CDC 736	1	1	0.25	>256	USA
CDC 737	1	1	0.25	>256	USA
CDC 738	1	1	0.5	>256	USA
CDC 739	1	1	0.5	>256	USA
CDC 740	1	1	0.5	>256	USA
Aspergillus niger 6275	2	2	ND	>256	Isolate from leather
Aspergillus niger 16888	2	1	ND	>256	
Aspergillus flavus 9643	0.5	2	ND	>256	Isolate from New Guinea
Aspergillus brasiliensis 16404	8	2	ND	>256	Isolate from blueberry, NC, USA

* Minimum Inhibitory Concentration (MIC), in which no observable growth was identified.

** Lopinavir (LPV), Itraconazole (ITC), Posaconazole (POS), Voriconazole (VRC), and Not Determined (N.D.)

Table 3.2: Synergistic activity of lopinavir (LPV) and azole antifungals against different strains of *Aspergillus*.

A. fumigatus	LPV/ITC Combination			LPV/POS Combination			LPV/VRC Combination		
	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode	MIC [µg/mL]	ΣFICI	Mode
NR-35301	16/0.125	0.156	SYN	16/0.06	0.151	SYN	0.25/0.25	1.001	IND
NR-35302	16/0.125	0.156	SYN	32/0.06	0.183	SYN	0.25/0.5	1.001	IND
NR-35303	8/0.125	0.141	SYN	16/0.03	0.091	SYN	32/0.25	0.625	IND
NR-35304	8/0.125	0.141	SYN	32/0.06	0.183	SYN	0.25/0.5	1.001	IND
NR-35305	16/0.125	0.156	SYN	8/0.125	0.266	SYN	32/0.25	1.125	IND
NR-35307	32/0.125	0.188	SYN	8/0.125	0.266	SYN	0.25/0.5	1.001	IND
NR-35310	32/0.125	0.188	SYN	8/0.125	0.266	SYN	32/0.25	1.125	IND
NR-35311	16/0.125	0.156	SYN	16/0.125	0.281	SYN	32/0.25	1.125	IND
NR-35312	32/0.125	0.188	SYN	16/0.125	0.281	SYN	16/0.5	1.063	IND
NR-41311	16/0.125	0.156	SYN	16/0.125	0.281	SYN	0.25/0.25	1.001	IND
NR-41312	16/0.125	0.156	SYN	16/0.125	0.281	SYN	32/0.25	1.125	IND
CDC 731	32/16	0.313	SYN	16/0.5	0.281	SYN	0.25/4	1.001	IND
CDC 732	32/64	1.063	IND	32/1	0.563	IND	0.25/1	1.001	IND
CDC 733	32/64	1.063	IND	32/0.5	0.313	SYN	0.5/4	1.002	IND
CDC 734	32/64	1.063	IND	32/0.5	0.313	SYN	0.25/4	1.001	IND
CDC 735	32/64	1.063	IND	32/1	0.563	IND	0.25/1	1.001	IND
CDC 736	8/0.5	0.516	IND	16/0.25	0.281	SYN	16/0.25	0.563	IND
CDC 737	16/0.25	0.281	SYN	16/0.125	0.281	SYN	0.25/0.25	1.001	IND
CDC 738	16/0.25	0.281	SYN	16/0.25	0.281	SYN	16/0.25	0.563	IND
CDC 739	16/0.25	0.281	SYN	16/0.125	0.156	SYN	32/0.25	0.625	IND
CDC 740	8/0.25	0.266	SYN	16/0.125	0.156	SYN	16/0.25	0.563	IND
Aspergillus niger 6275	8/0.5	0.266	SYN	32/0.125	0.125	SYN	NA	NA	NA
Aspergillus niger 16888	8/0.5	0.266	SYN	16/0.06	0.091	SYN	NA	NA	NA
Aspergillus flavus 9643	16/0.125	0.281	SYN	16/0.25	0.156	SYN	NA	NA	NA
Aspergillus brasiliensis 16404	8/2	0.266	SYN	16/0.5	0.281	SYN	NA	NA	NA

***FICI (Fractional Inhibitory Concentration Index) utilized to quantify interactions between the tested combinations with the following definitions: Synergy (SYN) is an ΣFICI values ≤ 0.5, Indifference (IND) is an ΣFICI of > 0.5 to ≤ 4, and Antagonistic with an ΣFICI value of > 4.

**** Lopinavir (LPV), Itraconazole (ITC), Posaconazole (POS), Voriconazole (VRC), and Not Applicable (N/A)

Chapter 4. Bright Horizons

Looking Ahead for Antifungal Therapies

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Fungal infections and resistance are rising [1]. This work in conjunction with others demonstrates our ability to not only develop novel compounds but to identify safe and available drugs[2-5]. While identifying novel compounds is crucial for the advancement of the field, the high cost and time associated with such developments to yield a fraction of drugs is unstainable[6-8]. The other side of the drug discovery process coin is drug repurposing, utilization of known drugs for alternative therapies and pathogens[7]. Drug repurposing has been evaluated within the field of cancer research [6]. One such drug, penfluridol, is currently under review for its anti-cancer properties[9]. Synthesized analogs of this compound have been tailored to combat cancer [9]. Penfluridol was identified in our screening process (Ch. 2, Figure 1) demonstrating a robust ability for potential anticancer and antifungal properties. Can these previously developed penfluridol analogs be even more effective at combating *A. fumigatus*? Additionally, we have observed the history of Miltefosine —a compound determined by Dos Reis et al. (2021), repurposed from an anti-*Leishmania* compound to a novel antifungal agent for recurrent and initial Candida sp., infections[2, 3, 10]. These efforts represent a crucial step towards the recognition of

a whole region of known compounds that can be investigated for their ability to enhance or become novel antifungals[3].

We are also witnessing the development and implementation of several novel antifungal agents, ibrexafungerp (IBX), rezafungin (RZF), isavuconazole (ISV), and fosmanogepix (FMGX) [11]. These novel agents and next-generation antifungals, while incredibly fortunate, are the result of a long and costly process. IBX and RZF work through glucan synthase inhibition targeting cell wall integrity. Ibrexafungerp demonstrates the drive of researchers to generate a first-in-class oral inhibitor of glucan synthase[12]. IBX is the first of the triterpenoid antifungals to undergo clinical testing and development[12, 13]. Furthermore, this compound has been approved by the FDA for invasive candidiasis and aspergillosis – the most severe forms of fungal infections[11]. Rezafungin presents itself as a more stable analog of another known agent, anidulafungin[14]. This design, as well, demonstrates increased safety. Research has demonstrated that RZF can be considered a single-agent prophylactic and warrants further evaluation through a phase 3 trial[15]. Isavuconazole is a newer generation of triazoles, derived from voriconazole the leading agent of the previous generation[11, 16]. By developing ISV from VRC researchers are observing increased tolerability, favorable pharmacokinetics, and antifungal activity[16]. Fosmanogepix is one of the more important developments in the field of antifungals due to a novel mechanism of action, inhibition of glycosylphosphatidylinositol (GPI) through binding and inactivation of Gwt1[17]. FMGX represents an important step to novel routes of developing antifungals. This is important due to the current program of derivatization we are witnessing with azoles. It cannot be understated the enhanced safety, efficacy, and tolerability we are seeing with antifungal derivatives. Yet, we must factor in the impact previous resistance traits developed against parent compounds can do[16].

The discovery of novel antifungal agents and mechanisms presents us with a bright, but difficult future. Drug repurposing allows us to screen and identify novel functions of compounds/drugs that have been intensively studied. While a recently novel application for antifungals, drug repurposing has been successful over its decades-long work[18, 19]. Miltefosine – identified from the Pathogen Box and NIH Clinical Collection libraries, demonstrated through screening potent inhibition of *A. fumigatus*[2, 3]. The work discussed here seeks to utilize this process with azoles, beginning with itraconazole, at a known ineffective dose to identify enhancers. The screening process with itraconazole resulted in over 30 compounds/drugs, that when further evaluated –for cost, serum concentration, bioavailability, and drug class, resulted in six prime candidates: cobicistat, elvitegravir, penfluridol, lopinavir, rolapitant and rilapladib, Table 2.1. These candidates were then evaluated through *in vitro* assays to determine intra-ranking amongst the six prime candidates.

Penfluridol has the most potential as a base template for the development of analogs to be evaluated for their antifungal properties. Yet, this does not carry over to its ability to synergize effectively with azoles, marking penfluridol as the least effective enhancer. Rolapitant, another CNS active compound has increased synergy over penfluridol with ITC and VRC, Table 2.6 & 2.8. Lopinavir –another effective synergizer and additive agent for ITC and VRC, can be considered close to its equal in rank, Table 2.5. Both demonstrate enhancing effects of subeffective azoles applications over a 72-hour period, Figure 2.2. When visualized at 24 and 48 hours we observe increased fungal hyphae present in the LPV and ITC group compared to ROLA and ITC, Figure 2.4 D & K, G & N, respectively. Rilapladib presented 100% synergy when utilized with ITC and POS, Table 2.7. A growth kinetics assay of RILA with ITC and POS resulted in no

germination over the course of 72 hours, Figure(s), 2.3, 2.4, 2.5 AA. This compound while an effective synergizer has a serious drawback, cost; 100mg of the compound is priced at or above 2,400 USD rendering it one of the most expensive of those evaluated[20, 21]. The second most effective compound observed was Elvitegravir, an integrase strand transfer inhibitor [22]. When co-applied we observed the third highest level of synergy across azoles, Table 2.4; this synergy was carried over to a growth kinetics assay and held for POS and ITC combinations resulting in total growth inhibition, Figure 2.2 B-C. Cobicistat an HIV drug enhancer resulted in our best potential candidate. We would like to note the ability of these HIV antivirals to readily synergize with azoles irrespective of their original purpose. Cobicistat demonstrated the second highest synergy that readily carried over to the growth kinetics assay, Table 2.3 and Figure 2.2. What places cobicistat above the other HIV antivirals and identified compounds is its usage as a drug enhancer[23, 24]. This ability to have a well-known safety profile, alone and in combination with other drugs places cobicistat first[25-27]. Our work represents the hidden potential of the MedChem Express library for antifungal enhancers[5, 28]. Previous reports have provided a robust of other compounds for a diverse group of libraries, we seek to add to this growing repository. Drug repurposing is an important and crucial step that needs to be undertaken to combat our increasing fungal pathogen resilience.

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