Antifungal Activity in *Galleria mellonella*

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B.S. Biochemistry and Molecular Biology, B.A Spanish, University of Massachusetts Amherst, 2015

Thesis

Submitted in partial fulfillment of the requirements for the Degree of Master of Science in the Department of Molecular Pharmacology, Physiology and Biotechnology, and the Center of Biomedical Engineering at Brown University

Providence, Rhode Island

May 2017

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VITA

Shellyhan Gordon was born in Leominster, Massachusetts and grew up in Fitchburg, Massachusetts. In 2011 she graduated from Fitchburg High School in the top 10 of her class. Shellyhan is the recipient of the Horatio Alger Scholarship as well as the prestigious Gates Millennium Scholarship. She went on to complete a Bachelor of Science in Biochemistry and Molecular Biology as well as a Bachelor of Arts in Spanish from the University of Massachusetts Amherst in 2015. She is currently attending Brown University to complete her Master's degree in Biotechnology in May 2017. Shellyhan joined Dr. Mylonakis' laboratory in Infectious Disease, where she will apply her interest in Global Health. After completing masters work at Brown University, Shellyhan plans to move on to pursue an MD/PhD.

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who played a role in my success here at Brown University. I would like to thank the Graduate School for accepting me into the Biotechnology Master's Program. Thank you, Dr. Jacquelyn Schell, for your direction in the program. Thank you, Dr., Mylonakis for accepting me into your laboratory and for your continued support and willingness to work with me and offer me advise for projects in the lab as well as in my personal life and future academic goals including medical school plans. I extend my gratefulness to my committee members Dr. Sello and Dr. Belenky for being such a great help in a time of need for the process of writing my thesis. Lastly I want to thank the members of the Mylonakis Lab at Rhode Island Hospital as well as those visiting and who have visited from Brazil. Thank you, Dr. Beth Fuchs, for your availability and enthusiasm to answer my questions and offer your assistance. Thank you, Dr., Cleidi Martins for being a mentor, excellent company and supportive in my work in the laboratory. Again, thank you all for your support.

ABSTRACT

Cryptococcosis infection from *Cryptococcus neoformans (C. neoformans)* remains a global health threat. It is an encapsulated yeast saprophyte that mainly afflicts individuals with compromised immune systems. As current treatments require several months for clearance, and have toxicity and microbial resistance issues, there is a need for new antifungal treatments. This research employs the concept of *Galleria mellonella* (*G. mellonella*) as a useful model to understand host-pathogen interactions. Previous research (Mylonakis et al., 2005; Firacative et al., 2014; Trevijano-Contador et al., 2015; Tsai et al., 2015; García-Rodas et al., 2011) has already determined *G. mellonella* as a valuable model system to study *C. neoformans* pathogenesis as well as the pathogenesis of other microbes. This study expands on that idea and seeks to take advantage of this model system to study antifungal activity of novel compounds as well as their toxicity. This study investigates the behavior of *G. mellonella* in response to infection and compound doses as well as consistency in survival experiments when incorporating the anti-bacterial ampicillin.

1. INTRODUCTION

1.1 *C. neoformans* Natural Reservoirs

Less than 20 out of thousands of fungal species that have been discovered are considered pathogenic for humans (Carroll et al., 2007). Among these, *C. neoformans* is a fungus that can be found in bird guano and Eucalyptus trees. A 1990 report identified *Eucalyptus camaldulensis* as the first reported natural habitat of *C. neoformans* var. *gatti*, and its widespread incidence in Australian aborigines and world-wide distribution where it grows (Ellis et al., 1990). The global distribution of *E. camaldulensis* in Australia corresponded to the distribution of Cryptococcosis caused by *C. neoformans* var. *gatti*. Although this species of Eucalyptus is primarily native to Australia it has been reported in several countries including those in Europe, Asia, Middle East, Latin America and the United States (California, Hawaii, Arizona, New Mexico, Texas and Florida. *C. neoformans* patient isolates were prevalent in tropical and subtropical regions especially in southern California, southern Asia, central Africa and Brazil (Ellis et al., 1990). Infective yeast can also be found in wood, rotting fruits and vegetables, dairy products and soil (Randhawa et al., 2001). As an obligate aerobe, it can live in plants and animals in environments throughout the world. *C.neoformans* is able to spread as pigeons shed their droppings into the environment. For example, the accumulation of droppings from pigeons in urban areas has caused *Cryptococcus* to become established in Mexico's central region (Gonzales et al., 2013). Another example is with children living in the Bronx and urban areas. The abundant presence of pigeons in urban areas and elevated rates of childhood seroconversion of *C. neoformans* proteins, suggests a link between exposure to bird guano and infection with *C. neoformans* (Goldman et al., 2001).

1.2 Cryptococcosis

Cryptococcosis is the name of the infection caused by *C. neoformans* or *C. gattii* that affects humans. Cryptococcosis can be acquired by breathing in the spores of the fungus. The fungus will travel to the lungs and alveoli where it can manifest and cause pneumonia like symptoms. From there it can spread through the blood to the brain causing meningitis. Because it is common in the environment, people may inhale the microscopic fungus without realizing it. *C.neoformans* is an opportunistic fungus, as it is most often seen in those with compromised immune systems (especially those living with HIV/AIDS) compared to those with functioning immune systems. However, a study in Alabama found that in 302 cases of cryptococcosis, 36% of cases were in non-transplant and non-HIV patients (Brizendine et al., 2013). Apart from the few exceptions such as the last example, those with healthy immune systems are generally able to fight the fungus before contracting a serious infection. Since the initial pneumonia-like symptoms are so like those of other common illnesses it can often go unnoticed for what it really is, until such time the infection can spread to the brain where it causes major neurological damage and become lethal.

1.3 Virulence

It is believed that outside of the cell it is poorly encapsulated (Carrol et al., 2007). Once inside colonization occurs in distal alveolar spaces of the lungs (Idnurm et al., 2005). It can disseminate from the lungs through the blood to other organs in the body. Infections most often involve the lungs and central nervous system (CNS) and include cryptococcal meningitis, encephalitis, and meningoencephalitis (Rohatgi et al., 2015). One virulence factor promoting *neoformans* survival is growth at 37 degrees Celsius (Kwon-Chung et

al., 2006). Growth at this temperature facilitates proliferation thus causing infection and disease (Odom et al., 1997). The formation of an extracellular polysaccharide capsule (Chang et al., 1994) is another factor promoting survival against host defenses such as phagocytosis or opsonization (Idnurm et al., 2005). It may even use host phagocytes-the very cells that are supposed to ingest harmful particles- to facilitate its movement throughout one's body (Rohatgi et al, 2015). The production of melanin is a third virulence factor promoting survival that helps in resisting oxidative stress from phagocytes (Carrol et al., 2007). The secretion of degradative enzymes is a fourth factor (Chen et al., 1996). *C.neoformans* maintain their virulence in humans through the ability to survive in an intracellular environment. An example of a natural selection mechanism by which it overcomes this feat is phagocytic amoebae that ingest *C. neoformans* and yet the fungus survives in the amoebae's intracellular environment (Steenbergen et al., 2001).

1.4 History of AIDS and appearance of Cryptococcosis

Many factors have aided the emergence of diseases caused by Cryptococcus neoformans (Dixon et al., 1996). In the last couple decades, there has been a higher incidence of comorbid disease due to an aging population and advances in medical therapies (Cohen et al., 2000). For human mycoses, the options for therapy are scarce. Before immunosuppression by the human immunodeficiency virus (HIV), cryptococcosis was not a common disease. In fact, it was relatively rare. A documented example is the period between 1971 to 1980 where there was an incidence of less than 1 case per million Northern California (Freidman et al.,1983). To continue this timeline, the onset of HIV epidemic appeared in 1981, after which time cryptococcosis sprung up rapidly as an illness. This became a defining illness of Acquired Immunodeficiency Syndrome

(AIDS). Upon the launch of antiretroviral therapy years later in the 1990s, opportunistic fungal infections such as cryptococcosis began declining (Perfect et al., 1985 *Infect Immun*). Despite the introduction of antiviral therapy in North America, there were limitations in access to antiviral therapy (ART) in other geographic areas such as some countries in sub-Saharan Africa and Southeast Asia (Hakim et al., 2000). In Harare, Zimbabwe for example, in-hospital deaths were high from varying causes of meningitis (Hakim et al., 2000). The costs of health care delivery and providing antiretroviral therapy in Tanzania and Zambia were examined. It was confirmed that antiretroviral drugs make up half of the costs of care for those on ART (Kimaro et al., 2017). These limitations allow Cryptococcus to remain a leading cause of meningitis in populations afflicted by human immunodeficiency virus.

Among other populations throughout the world, most cases of cryptococcal meningitis affect those in sub-Saharan Africa. This area carries a hefty part of the global burden of cryptococcal meningitis, a leading cause of death in HIV/AIDS patients. Even with antiretroviral therapy, it is still the cause of thousands of deaths worldwide. A 2009 report estimated 960,000 cases and 625,000 deaths occur globally each year from cryptococcal meningitis. Above 80% of that burden is carried by sub-Saharan Africa where 720,000/960,000 cases and 500,000/625,000 deaths occurring annually. It is implied to kill more of these patients each year than tuberculosis (Park et al., 2009). In the most recent global burden study Cryptococcus remains the leading cause of death in adults living with HIV in sub-Saharan Africa. Of the estimated global cases of cryptococcal meningitis cases in 2014, sub-Saharan Africa accounted for 73% with

162,500 cases. The estimated annual deaths from cryptococcal meningitis were 135,900 in sub-Saharan Africa compared to 181,100 worldwide (Rajasingham et al., 2017).

1.5 Current Treatments

Despite antifungal therapy, cryptococcal meningitis remains a common opportunistic infection among immunosuppressed people (Bicanic et al., 2005). Drugs that target the cell membrane, RNA/thymidine synthase, or the cell wall are used for the management of cryptococcosis. Prescription antifungal medication is needed for at least 6 months. Common drugs for the management of cryptococcosis are fluconazole, amphotericin B, and flucytosine. Fluconazole is used to treat mild to moderate pulmonary infections while amphotericin B coupled with flucytosine is used to treat severe infections in the lungs and CNS. Besides the long treatment schedule, other limitations such as toxicity and fungal resistance are of concern. Azoles such as fluconazole, have been used for long-term treatment of serious fungal infections, however long-term use increases the development of resistant fungal strains (Brant et al., 1996). The most common adverse event reported with fluconazole use has been hepatotoxicity (Egunsola et al., 2013). Although a new liposomal formulation of amphotericin B has a decreased toxicity, the original deoxycholate form was nephrotoxic (Mishra et al., 2013). Flucytosine, which has existed for over 50 years, is still limited in Africa and Asia (Loyse et al., 2013). These regions, mentioned previously, are where the infection burden is the greatest. If serum concentrations of flucytosine surpass 100mg/L it will cause haematological, gastrointestinal toxicity as well as bone marrow depression and hepatoxicity (Loyse et al., 2013). Other treatments include photodynamic therapy which is an antimicrobial

technology that works to inactivate *C. neoformans* by interfering with its cell wall (Fuchs et al., 2007).

1.6 Model Host Systems for Infection

Besides humans, *C. neoformans* naturally infects several invertebrate species and mammals including cats, dogs, cows, horses, and primates (Carrol et al., 2007). The guinea pig, rabbit, mouse and rat are among the animal models that have been developed for the study of *C. neoformans*. Symptoms that resemble human illness has contributed to the development of diverse model systems for the study of cryptococcosis.

Considerations

Each model system has their advantages and limitations per the relevant purposes of modeling human disease. Furthermore, host-pathogen interactions vary between models and objectives. Each model host confers a different level of response and severity of clinical manifestations in response to infection. For vaccine efficacy trails, an appropriate model would be one that is susceptible to infection in its natural environment. In this case the use of a naturally resistant animal such as the rabbit would not be useful for the study of infection for goal of creating a vaccine. One would have to perform several manipulations such as immunosuppression which is undesirable. When studying meningoencephalitis, larger animals are desirable as their brains are easier to access for experimentation. Lasting considerations are the cost and maintenance associated with larger animal models such as primates (Carrol et al., 2007).

Mouse

The mouse (*Mus musculus*), weighs 20-40g and is among the smallest of the models developed for *C. neoformans* study. It is also considered the most popular model for the study of cryptococcal infection (Singh et al., 2017). Advantages include low cost and maintenance. Plentiful inbred strains have been developed for the study of this pathogen that are susceptible to infection. These strains can produce clinically relevant results and can be used for genetic analysis. This model boasts several routes of infection including intravenous, intranasal, intracerebral, intraperitoneal and intratracheal. Immunosuppression is not required. A disadvantage includes the small size that limit certain procedures.

Rat

The rat (*Rattus norvegicus*), weighing in at 250-350 grams is the second largest animal model used to study *C. neoformans*. Routes of infection include intraperitoneal, intracisternal and intratracheal (Goldman et al., 1994; Graybill et al., 1983). With the larger size intratracheal infections can be carried out without surgery (Goldman et al., 1996). Inbred strains are also available (Mashimo et al., 2005) advantageous as well as characterizing the immune responses in the central nervous system from central spinal fluid samples (Goldman et al., 1996). No immunosuppression is required. A disadvantage is that the higher maintenance costs compared to the mice model (Carrol et al., 2007).

Guinea Pig

With an average weight of 350-450 grams the Guinea pig (*Cavia porcellus*) is the third largest animal model developed to study *C. neoformans*. Not too long after the discovery of Cryptococcus the guinea pig was the first model to study disease pathogenesis (Carrol et al., 2005). Its genome has been sequenced by the Broad Institute (Broad Institute, 2006). Routes of infection include intravenous, intraperitoneal, and intratracheal. Immunosuppression is not required. Guinea pigs are docile in nature (Bonner et al., 1976). Disadvantages are the limited number of inbred strains that make biological relevance limited (Festing et al., 1979). Immunologic agents and genetic tools are also insufficient.

Rabbit

(*Oryctolagus cuniculus*) is the largest among these developed animal models at 2000-3000g. Routes of injection include retinal via carotid artery (Fujita et al., 1983), intracisternal, intratracheal, and intratesticular. The large body size allows for repeated drug administration and fluid sampling of cerebral spinal fluid (Carrol et al., 2007). This allows for investigation of cryptococcal meningitis. Samples can also be taken from the lung to study pulmonary infection (Nessa et al., 1997). Their high body temperature of about 39.5 degrees Celsius gives them natural resistance to cryptococcosis as fungal replication is inhibited preventing spreading from the respiratory system (Carrol et al., 2007). This makes corticosteroids a required for immune suppression (Perfect et al., 1999) therefore requiring larger infectious doses compared to other animal models (Perfect et al.,1980). Immunosuppression although viewed as a disadvantage may also be an advantage as it mirrors the immunosuppression in humans that makes them susceptible

to infection by the opportunistic fungus. Another disadvantage is the high cost and maintenance. To continue genetic and immunologic information is limited.

G. mellonella as a model system to study cryptococcosis

G. mellonella is an invertebrate model used to study *C. neoformans* and a variety of other pathogens. This alternative model has been developed for the study of cryptococcal virulence. Currently there are over one thousand articles published on *G. mellonella* as an infection model. This model is inexpensive, low maintenance and easy to manipulate (Mylonakis et. al., 2005). *G. mellonella* can mimic mammalian host environment and be maintained at varying temperatures (15-37 degrees Celsius). The amounts of pathogen or drug being injected can be controlled. Mylonakis et al., also concludes that there is a positive correlation between the pathogenicity of *C. neoformans* in *G. mellonella* compared to mammalian and other invertebrate model systems. This study goes on to suggest that the *G. mellonella-C. neoformans* system can be used to identify *C. neoformans* genes that contribute to virulence and therefore lead closer to the development of new antifungal drugs. Disadvantages include limited genetic information, a non-adaptive immune system and limited number of injections that can be given before trauma is caused (Mylonakis et al., 2005). Notwithstanding, their ease of use and short life-span makes them an ideal candidate for large-scale studies (Tsai et al., 2015). Tsai et al., provides detail for the immune system of *G. mellonella* and points out similarities between their innate immune system and immune responses of vertebrate models. Not only does the use of *G. mellonella* relieves the cost, maintenance, and long reproduction periods in mammalian animal models, it may also relieve ethical concerns associated with the use of animal models. There is no requirement for ethical approval for

the use of *G. mellonella*. This thesis highlights the concept that such infection by *C. neoformans* can be studied in this model host as well as the effects of drugs regarding toxicity or possible antifungal properties against cryptococcosis.

1.7 Novel Compounds used for Toxicity and Survival

Table 1. displays a list of all compounds used in this study by code name and common group. Novel compounds included butenafine analogs, a benzimidazole analog, 14 aldimine analogs as well as a quinolinol analog. In brief butenafines can be classified as allylamine antifungals that inhibit the squalene epoxidase enzyme which results in a malfunction of fungal membrane bound proteins (Castellano et al., 2000; Nahm et al. 1999). The lone benzimidazole compound "IM 25 5D4" can also be classified as an azole which may have antimicrobial, antiviral, and anti-inflammatory properties. Benzimidazole fungicides bind to microtubules and block division of fungal cells (El-Gohary et al., 2017). It is also indicated as an antiparasitic to treat parasitic worm infections in humans and animals (Kenchappa et al., 2016). The aldimine compounds are composed of heterocyclic compounds joined in the middle by an aldimine group. Heterocyclic compounds play a role in biological processes of the living cell. Many synthetic heterocycles are used as pharmaceutical drugs such as antimicrobial, anticancer and antifungal (Pitucha et al., 2013). The quinolinol compound with common name "STK509423" is hypothesized to be immunomodulatory. Gershon et. Al 1963 describes some quinolinol compounds including copper(II) 8-quinolinate to have antimicrobial properties: fungistatic, fungicidal, bacteriostatic, bactericidal.

2. Materials and Methods

2.1 Compounds

Compounds from Table 1.a were synthesized by the Grupo de Estudos em Química Orgânica e Biológica (GEQOB), headed by Prof. Dr. Ângelo de Fátima from the Federal University of Minas Gerais (UFMG). STK509423 compound from Table 1.b was synthesized by Vitas-M Laboratory ChemDiv4 library, plate 1615 Well # F18. All compounds were stocked in 100% DMSO solution and stored at -20 or -80 degrees Celsius. (Sigma Aldrich, St. Louis, MO, USA). Compounds were prepared in filter-sterilized phosphate buffered saline (PBS) for injection in *G. mellonella* larvae. The concentration of DMSO in all compound preparations were less than 10% to avoid toxicity passed that level.

2.2 Minimum Inhibitory Concentration (MIC)

Using a 96-well plate, serial dilutions of compound were performed starting from 64 µg/ml and decreasing by half each time until 0.125µg/ml. RPMI media was used in the wells as well as to suspend KN99α cells. The final concentration of KN99α was 2.5 x 10^3 cells/ml. The first column was reserved for the sterility control where only 50µl of RMPI per well was placed. The last column (column 12) was reserved for the growth control where 2.5 x 10^3 cells/ml KN99α was left to grow. Columns 2-11 were reserved for the 10 different dilutions of 64,32,16,8,4,2,1,0.5,0.25,0.125 µg/ml. The setup goes as follows: 50µl were put in columns 2-11, however column 2 starting with 64 µg/ml was given 100µl of RPMI and compound was placed in column 2 such that the concentration is 128 µg/ml, or twice the intended concentration of 64 µg/ml. Then 50 µl is taken out of

the 100 µl in well 2 and placed in well 3 and this pattern should continue down the columns until row 12 where the 50 µl taken out of column 12 and discarded. Next 50 µl of inoculum doubled to 5.0 x 10^3 cell/ml is added to every column except for the sterility control in column 1. This should result in each column being restored to their proper concentrations as listed previously as well as the final concentration of KN99α at 2.5 x 10^3 cell/ml. After dilutions plates secured and stored at 35 degrees Celsius.

2.3 Strains and media.

C.neoformans wild-type strain KN99α was obtained from frozen stock maintained on yeast peptone dextrose (YPD) agar. Overnight cultures of fungal cells are also grown in YPD for 48 hours at 30 degrees Celsius. Cells were suspended in PBS and washed at least twice by means of centrifuge at 3600rpm. Cells were then diluted at 1:10, 1:100 and 1:1000. 10µl out of the 1:1000 dilution was pipetted in a hemocytometer counted, and appropriate dilutions are made in PBS.

2.4 *G. mellonella* killing and survival assays. *G. mellonella* (Vanderhorst, Inc., St. Mary's, Ohio) larvae were used at the 6-instar larval stage. Larvae were used within 7 days of shipment. 16 larvae per group were selected relatively the same size and light in color. A 10 µl Hamilton syringe was used to inject 10µl of either inoculum or compound. Before and during experiments syringes were washed with the following sterilization washes: 10% bleach, 100% ethanol, ddH_2O, and 1X PBS. Needles were sterilized every 6 injections by plunging up and down several times. When ampicillin was used in experiments (indicated in the test) it was administered at 20 mg/kg body weight to prevent unwanted bacterial infection. Appropriate Inoculum concentrations of 10^4, 10^5, 10^6 and 10^7 cfu/larva were prepared in PBS. Besides the Kaplan-Meier survival

curve in Fig. Each injection whether inoculum or compound is given in different pro-leg of the larva starting from the bottom left and going counter-clockwise. Fluconazole at 14mg/kg body weight (Toronto Research Chemicals) + KN99α was used as a control that is expected to prolong larvae life against fungal challenge. A placebo group PBS or PBS+PBS (depending on number of injections that are given in an experiment) is necessary to compare death due to physical injury. PBS + KN99α controlled for death due to fungal infection. Larvae were incubated at 37 degrees Celsius after infection or just injection of compound. Groups were checked daily for 7 days and observations on the number of larvae dead were noted. Larvae that exhibited no movement at the response to touch were declared dead and removed from plate. Kaplan-Meier survival curves were plotted and P values were obtained using GraphPad Prism software.

3. Results

3.1 *G. mellonella* Killing Assay

G. mellonella was infected at varying concentrations of KN99α strain. These concentrations including 10^4, 10^5, 10^6 and 10^7 cfu/larva (Fig.1a-b) compares the progress and speed of infection over the course of 7 days. The Kaplan-Meier Curve Fig.1a as well as the pictorial grid (Fig1.b) both show that infection at 10^4 cfu/larva results in death by day 5-6, 10^5 cfu/larva shows death by day 5, 10^6 cfu/larva shows death starting between 48 and 72 hours and finally 10^7 cfu/larva displays death within 24 hours ($P<0.0001$). Also shown is a pupa formation that the larvae occasionally transform into during an experiment. These pupae were counted as alive.

3.2 Minimum Inhibitory Concentration (MIC)

Table 2. list the MICs of the compounds used in this study. Most of the MICs (measured in µg/ml) were under the 2mg/kg body weight dose. Toxicity in *G. mellonella* was tested at the minimum concentrations at which the drug inhibited fungal growth *in vitro*. Preparation of drugs and injection methods are as described in the Materials and Methods section. Figure 2.a-h shows the toxicity survival with compounds on the left side as well as their comparison in response to fungal challenge on the right side. In general, the compounds at these extremely low MICs were not found to be toxic ($P<0.0001$), except for aldimine analog 3D10 (Fig 2.g) which displayed death within 24 hours (an abnormal observation explained in discussion). In brief any larvae that die within 24 hours after injection, be it infection or administration of drug or both, may have died as a result of physical trauma toxicity or other microbial infection confounding results. Because 3D10 was later tested at a much higher dose of 3mg/kg (Fig 3.d) and found to have no death within 24 hours and a high >90% survival rate ($P<0.0001$) it is not observed as being toxic to larvae. The fungal challenges displayed on the right side did not rescue larvae at their current low concentration from MIC.

3.3 Comparison of Compound Toxicity and KN99α challenge with or without ampicillin

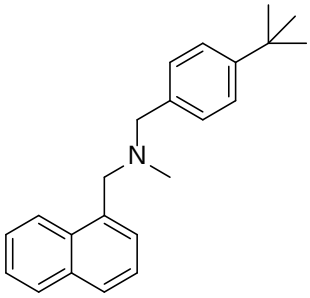
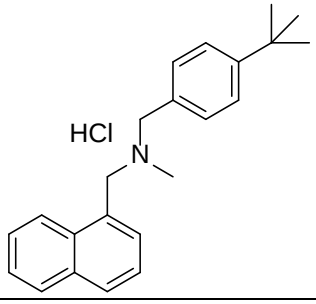
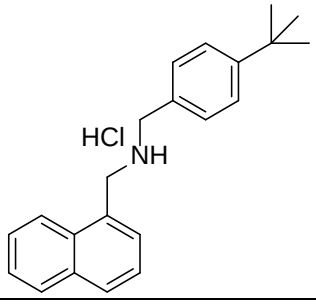
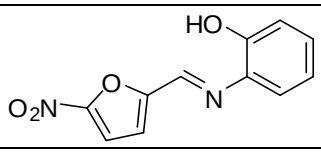
Figures 3-5 show *G. mellonella* survival at higher concentrations ranging from 3mg/kg to 15mg/kg. Specific concentrations are depicted in graph legends. Due to time constraints, only a select few compounds were chosen to test for toxicity and antifungal activity in larvae.

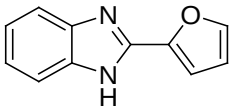
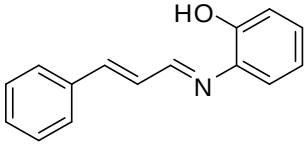
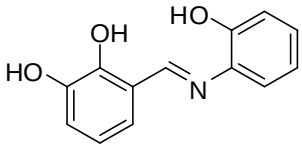
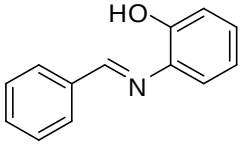
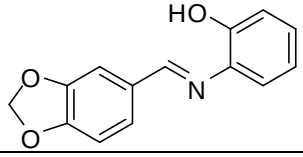
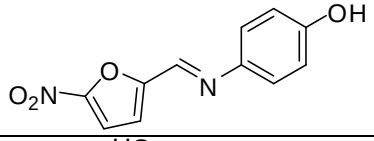
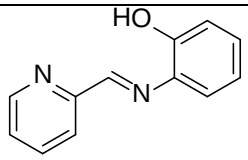
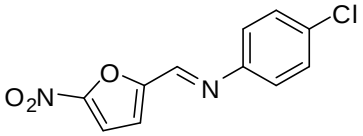
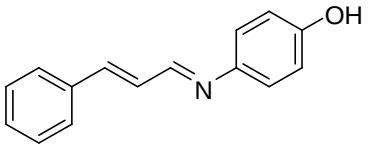
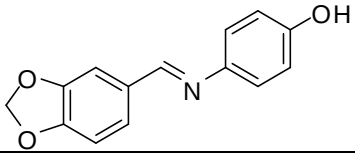
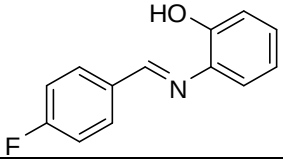
It was observed in some survival experiments (Fig 2b,d,e,f.g, Fig 4a-f) cases of death within the first day of injection. Based on the possibility of other microbial infections, the antibiotic ampicillin was given in some cases and withheld in others. Figure 3 a-d incorporated ampicillin when testing for toxicity and resulted in no pre-mature death of larvae but rather high survival by the end of 7 days. Figure 4 a-f showing survival with compound and fungal challenge did not incorporate ampicillin. In these figures including some controls death was seen in high amounts within the first 24 hours. Figure 5 a-c further zooms in on a more select few compounds that are compared side to side. The comparison shown here is between KN99α challenge + compound without AMP versus KN99α challenge + compound +AMP. The results imply that the incorporation of ampicillin resulted in consisted and expected high survival results ($P<0.0001$) given the fact that the compounds were not found to be toxic and death from fungal challenge occurs within 48-72 hours.

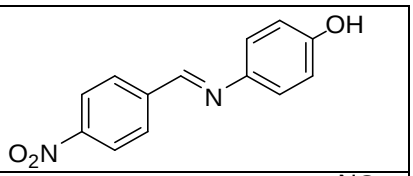
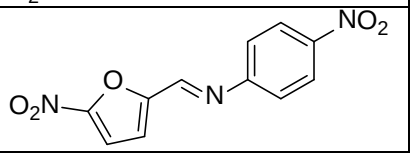
Overall, no compounds have yet demonstrated antifungal activity at the current administered concentrations.

3.4 Tables and Figures

Table 1. a-b. Compounds used in *G. mellonella* Toxicity and Survival Testing. (a.) These compounds were synthesized by the Grupo de Estudos em Química Orgânica e Biológica (GEQOB), headed by Prof. Dr. Ângelo de Fátima from the Federal University of Minas Gerais (UFMG). (b.) STK509432 (7-[(3-hydroxy-4-methoxyphenyl) [(4-methylpyridin-2-yl) amino] methyl] quinolin-8-ol) was synthesized by Vitas-M Laboratory ChemDiv4 library, plate 1615 Well # F18.

CODE	GROUP	STRUCTURE
GEQOB 15.31	ANALAGOUS OF BUTENAFINE	
GEQOB 15.55	ANALAGOUS OF BUTENAFINE	
GEQOB 15.43	ANALAGOUS OF BUTENAFINE	
GEQOB 3E3	ALDIMINE	

IM 25 5D4	BENZIMIDAZOLE Analog	
GEQOB 3D5	ALDIMINE	
GEQOB 3B7	ALDIMINE	
GEQOB 3A3	ALDIMINE	
GEQOB 3C3	ALDIMINE	
GEQOB 3E4	ALDIMINE	
GEQOB 3E9	ALDIMINE	
GEQOB 3E7	ALDIMINE	
GEQOB 3D6	ALDIMINE	
GEQOB 3C4	ALDIMINE	
GEQOB 3D3	ALDIMINE	

GEQOB 3D10	ALDIMINE	
GEQOB 3F10	ALDIMINE	

b.

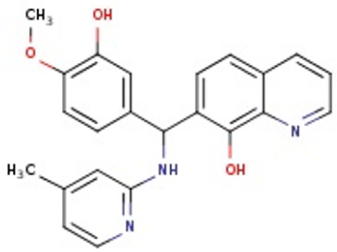
Code	Group	Structure
STK509423	Quinolinol	

Table 2. Minimum Inhibitory Concentration (MIC) of compounds after 48 and 72 hours.

Compound	MIC (µg/mL) 48 h	MIC (µg/mL) 72 h	MIC (mg/mL)	MIC (mg/kg)
STK509423	0.5	**0.5**	0.0005	0.016
15.31	0.5	**0.5**	0.0005	0.016
15.55	0.5	**1**	0.001	0.033
3E3	4	**4**	0.004	0.133
5D4	4	**4**	0.004	0.133
3D5	8	**8**	0.008	0.266
3B7	8	**8**	0.008	0.266
15.43	8	**8**	0.008	0.266
3A3	16	**16**	0.016	0.533
3C3	16	**16**	0.016	0.533
3E4	16	**16**	0.016	0.533
3E9	8	**16**	0.016	0.533
3E7	16	**16**	0.016	0.533
3F10	16	**16**	0.016	0.533
3D6	32	**32**	0.032	1.066
3C4	32	**64**	0.064	2.133
3D3	32	**64**	0.064	2.133
3D10	64	**64**	0.064	2.133

Figure 1a-b. Killing of *G. mellonella*. (a.) Kaplan-Meier survival curve demonstrating death of larvae at varying concentrations in central fungal units (cfu) of *C. neoformans* wild-type strain KN99α. (b.) Pictorial grid depicting characteristic changes in *G. mellonella* larvae at different stages of infection.

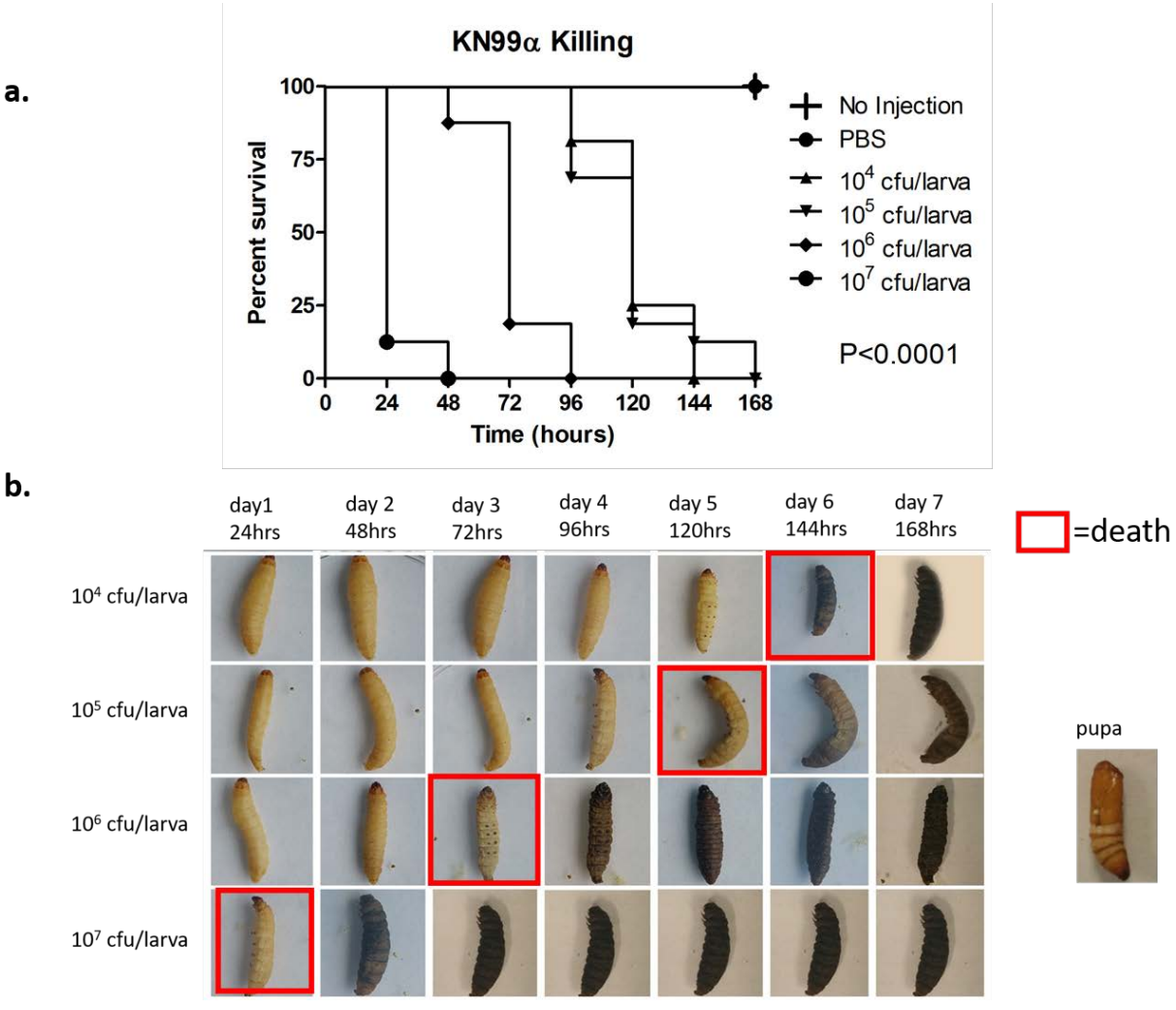
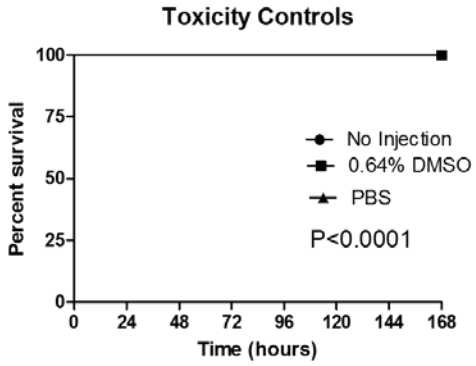
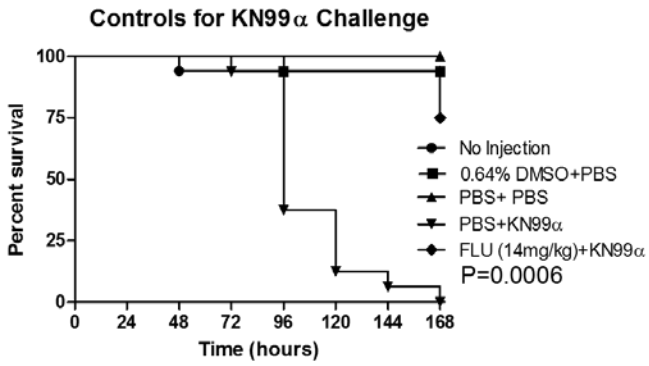


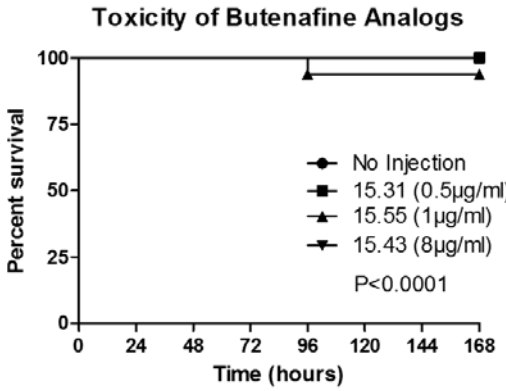
Figure 2 a-h. Kaplan-Meier Survival Curve of Compound Toxicity as compared to KN99α challenge with compound. Each set **a-h** displays toxicity survival of compounds on the left and *G. mellonella* survival given compound and KN99α on the right. KN99α in all cases is injected at 10^6 cfu/larvae. Compounds were prepared based on the MIC determined previously (Table 3) in filter-sterilized Phosphate Buffered Saline (PBS) solution.

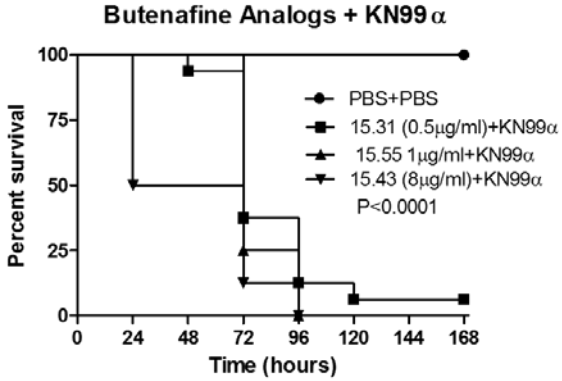
a.



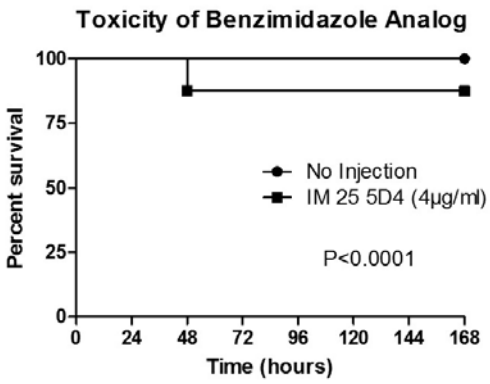


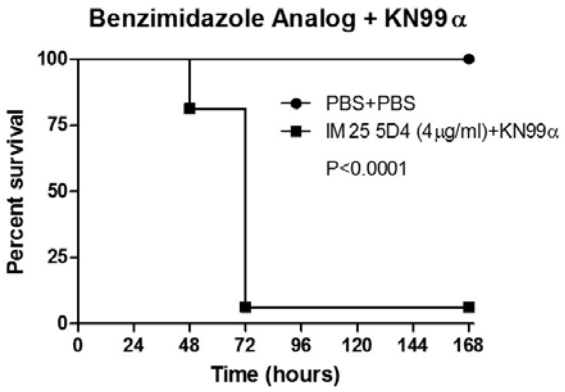
b.



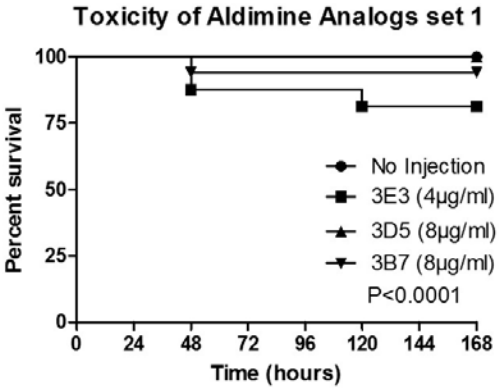


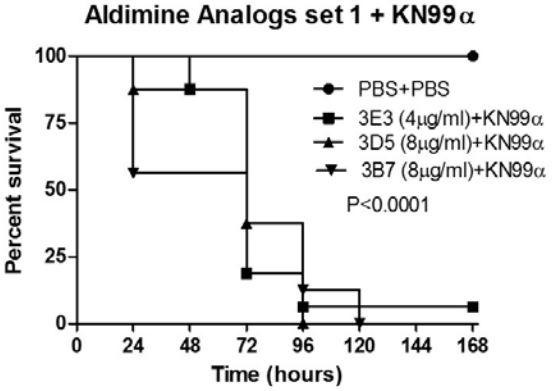
c.


Toxicity of Benzimidazole Analog
Percent survival
Time (hours)
No Injection
IM 25 5D4 (4µg/ml)
P<0.0001

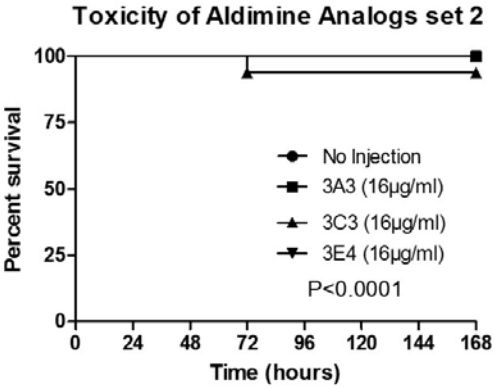

Benzimidazole Analog + KN99α
Percent survival
Time (hours)
PBS+PBS
IM 25 5D4 (4µg/ml)+KN99α
P<0.0001

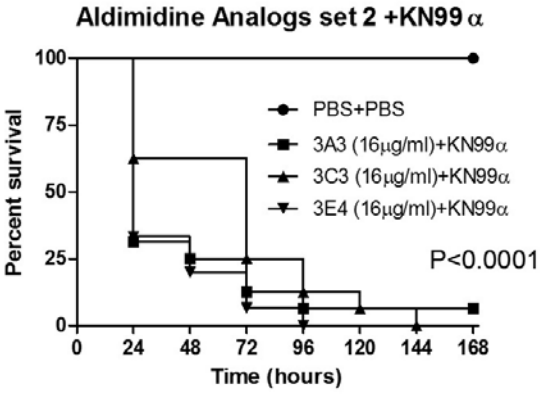
d.


Toxicity of Aldimine Analogs set 1
Percent survival
Time (hours)
No Injection
3E3 (4µg/ml)
3D5 (8µg/ml)
3B7 (8µg/ml)
P<0.0001

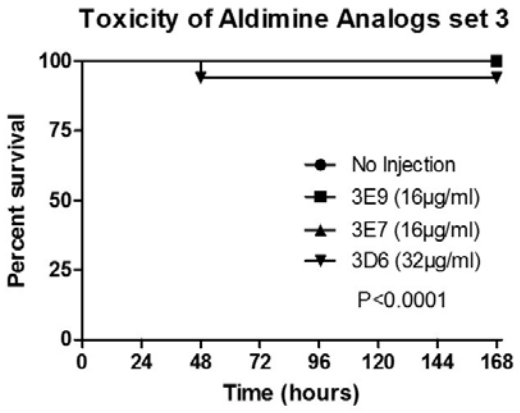

Aldimine Analogs set 1 + KN99α
Percent survival
Time (hours)
PBS+PBS
3E3 (4µg/ml)+KN99α
3D5 (8µg/ml)+KN99α
3B7 (8µg/ml)+KN99α
P<0.0001

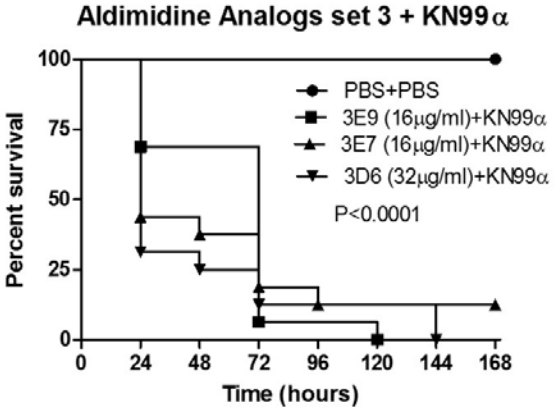
e.


Toxicity of Aldimine Analogs set 2
Percent survival
Time (hours)
No Injection
3A3 (16µg/ml)
3C3 (16µg/ml)
3E4 (16µg/ml)
P<0.0001

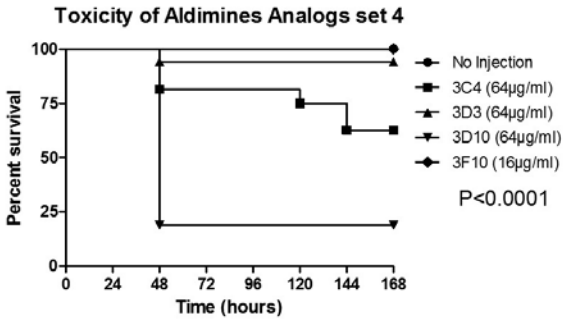

Aldimidine Analogs set 2 +KN99α
Percent survival
Time (hours)
PBS+PBS
3A3 (16µg/ml)+KN99α
3C3 (16µg/ml)+KN99α
3E4 (16µg/ml)+KN99α
P<0.0001

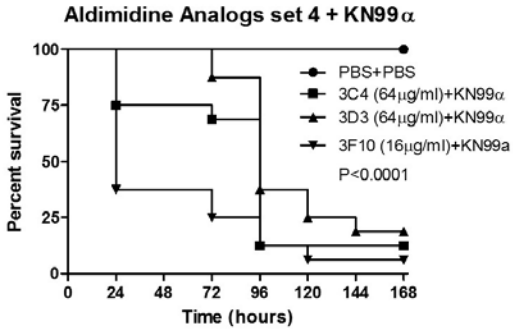
f.


Toxicity of Aldimine Analogs set 3
Percent survival
Time (hours)
No Injection
3E9 (16µg/ml)
3E7 (16µg/ml)
3D6 (32µg/ml)
P<0.0001

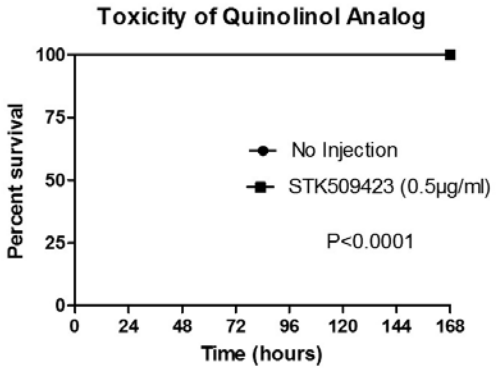

Aldimidine Analogs set 3 + KN99α
Percent survival
Time (hours)
PBS+PBS
3E9 (16µg/ml)+KN99α
3E7 (16µg/ml)+KN99α
3D6 (32µg/ml)+KN99α
P<0.0001

g.


Toxicity of Aldimines Analogs set 4
Percent survival
Time (hours)
No Injection
3C4 (64µg/ml)
3D3 (64µg/ml)
3D10 (64µg/ml)
3F10 (16µg/ml)
P<0.0001


Aldimidine Analogs set 4 + KN99α
Percent survival
Time (hours)
PBS+PBS
3C4 (64µg/ml)+KN99α
3D3 (64µg/ml)+KN99α
3F10 (16µg/ml)+KN99a
P<0.0001

h.


Toxicity of Quinolinol Analog
Percent survival
Time (hours)
No Injection
STK509423 (0.5µg/ml)
P<0.0001

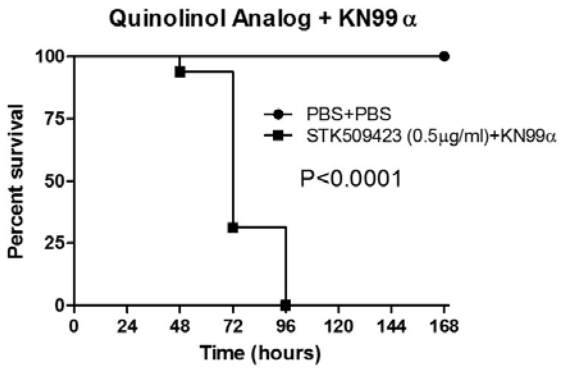

Quinolinol Analog + KN99α
Percent survival
Time (hours)
PBS+PBS
STK509423 (0.5µg/ml)+KN99α
P<0.0001

Figure 3 a-d. Toxicity of compounds at higher concentrations (3mg/kg to 15mg/kg). Kaplan-Meier Survival Curve of *G. mellonella* with the addition of ampicillin.

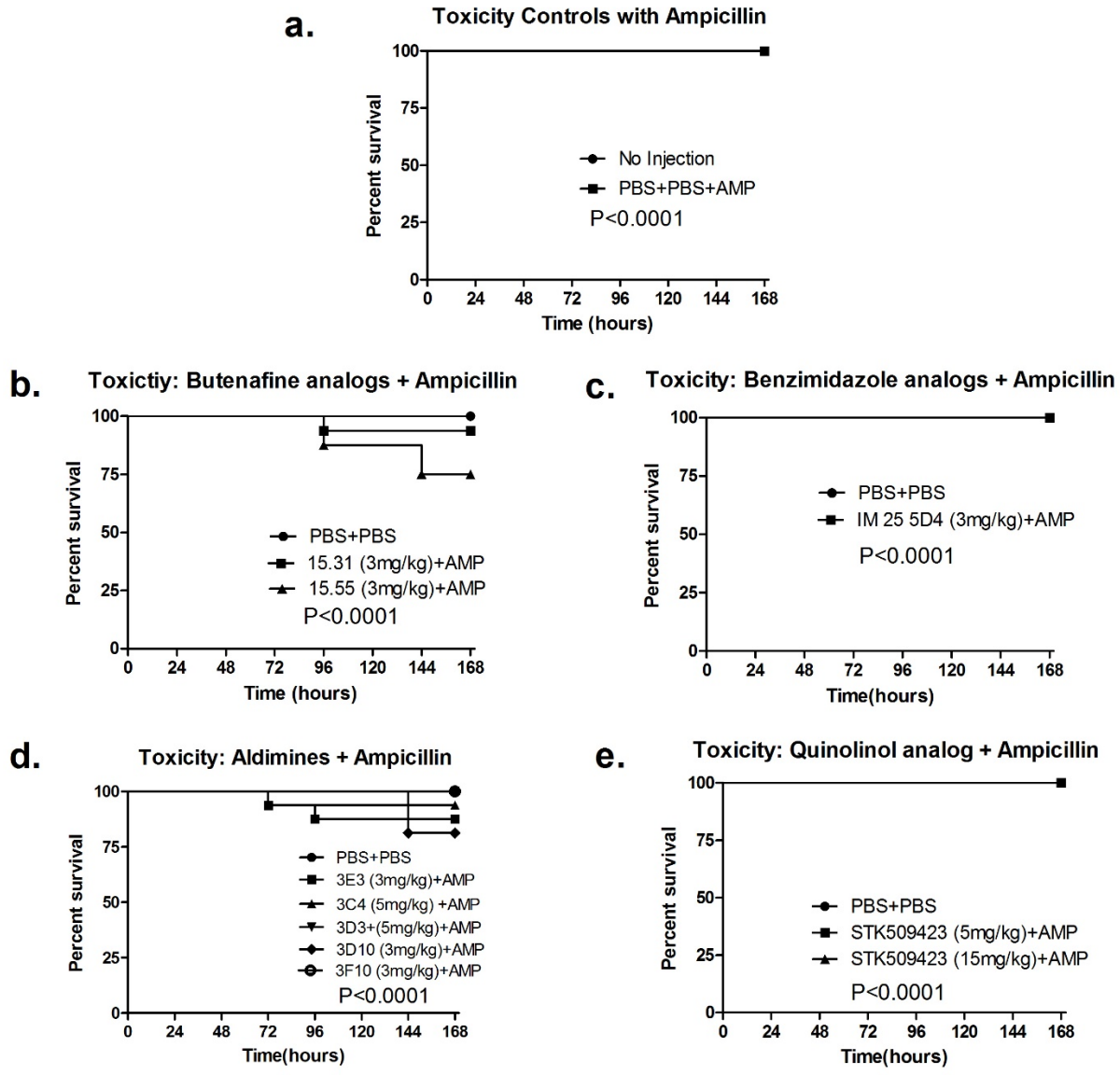


Figure 4 a-f. Kaplan-Meier Survival Curve Comparison of *G. mellonella* with KN99α challenge without ampicillin.

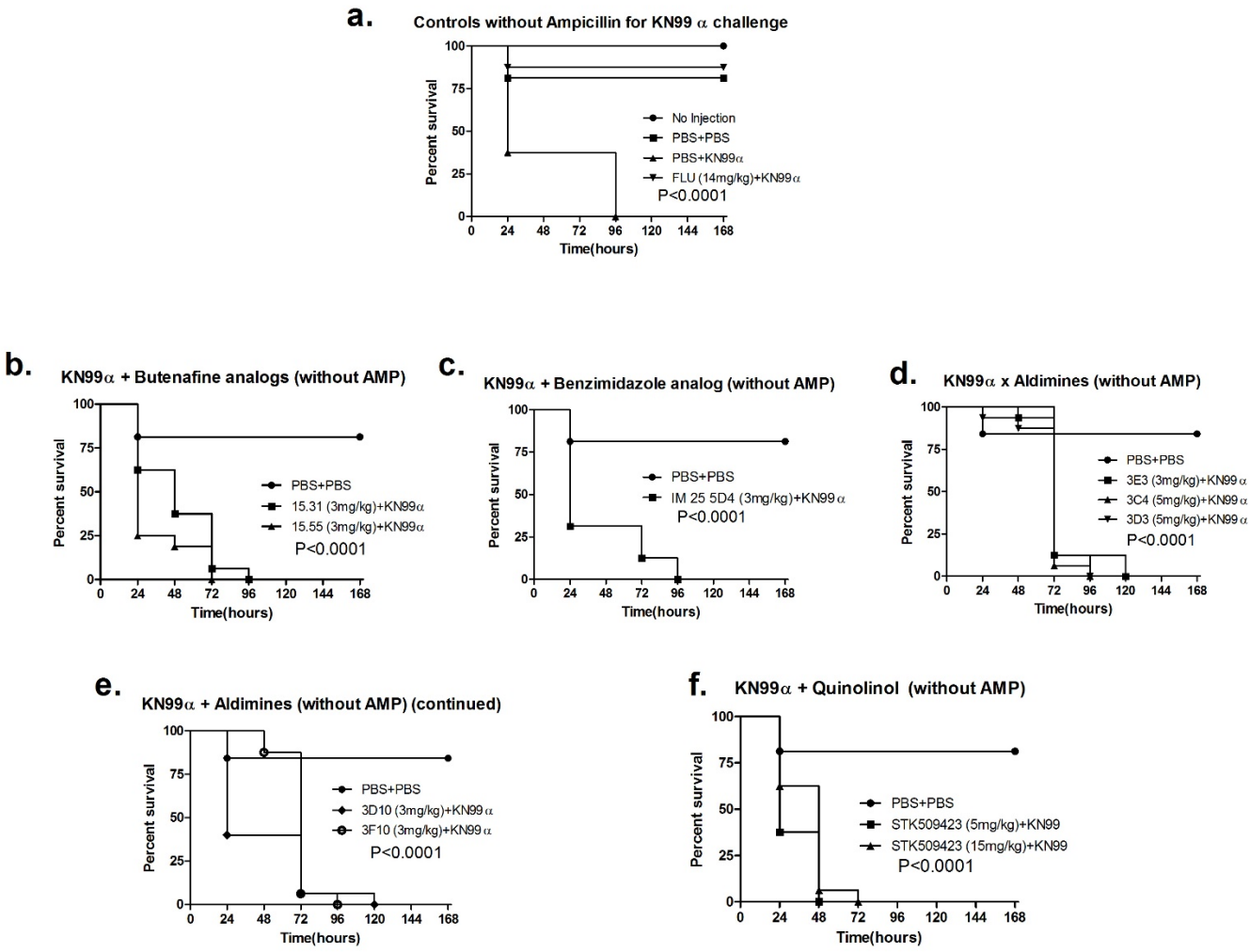
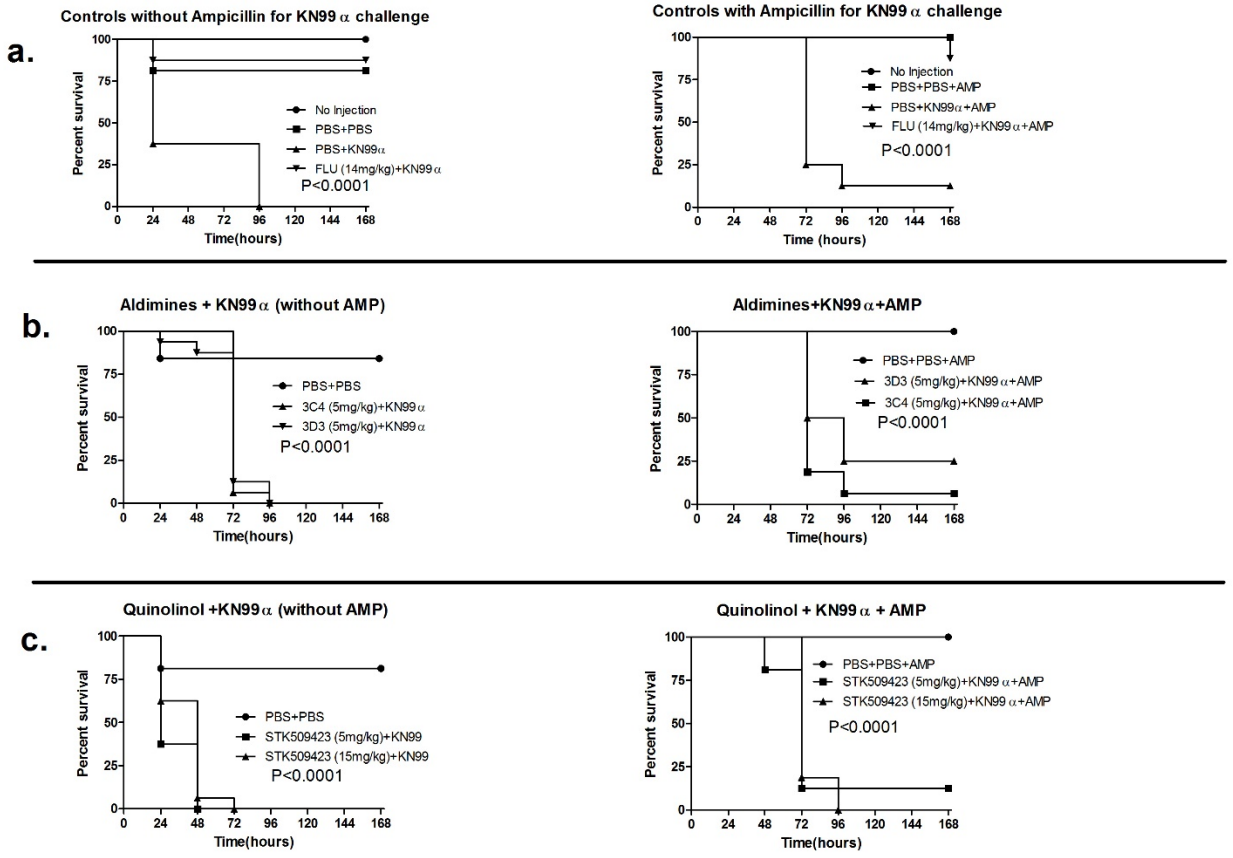


Figure 5 a-c. Kaplan-Meier Survival Curve Comparison of *G. mellonella* with KN99α challenge, compound and with or without ampicillin. The left column displays survival without ampicillin while the right column incorporates ampicillin.



4. DISCUSSION

4.1 Efficacy

Initial toxicity testing and fungal challenge were done at the concentrations determined by the MIC in Table 2. Given that the compounds were not found to be toxic at these levels they could have been used in fungal challenge at much higher concentrations. The survival curves with KN99α challenge with compound in the right column (Fig 2 a-h) were extremely under dosed due to the misconception that the MIC value should suffice in an *in-vivo G. mellonella* survival experiment. Obviously, this set of data did not show any signs of antifungal activity.

The interest in incorporating ampicillin to buffer against unwanted bacterial infections came to be when death of larvae within 24 hours of injection (either by actual infection of larvae or injection of compound) was observed. This observation of death within 24 hours is abnormal considering that *G. mellonella* should only begin to die from infection at 10^6 cfu/larva between 48 and 72 hours after infection as indicated in Fig 1a-b. It is also abnormal given that the compounds tested at the concentrations shown in any the figures were not toxic to the larvae. This leaves the other option of trauma due to physical injury. Given the many examples of multiple injections especially the triple injections per larvae at a time (Fig 5 a-c right column), and the 100% survival rate, it is unlikely that the death observed within 24 hours in some experiments were due to physical injury from injection. This now leaves other microbial infection as a possible explanation as to why so much death was being observed even in the controls. To investigate this hypothesis, a select few compounds were administered to larvae in addition to both cryptococcal challenge and ampicillin and these results were compared

to a group with all the categories except for ampicillin (Fig 5 a-c). One can observe from these graphs that the incorporation of ampicillin allowed for consistency among the experiments. Here there is no longer death within the first 24 hours of injection.

4.2 Conclusion

These results give rise to the possible advantage of using ampicillin as another control measure when giving fungal infections to *G. mellonella.* This circumstance in comparisons with or without ampicillin came about as an unexpected topic of discussion. Nevertheless, it sheds light on improving injection techniques that apply to *G. mellonella* survival with future fungal challenges or toxicity assays. It is understood that drug discovery takes several years even decades to get a hit and be seen through clinical trials until acceptance by the FDA. This study has provided a sliver of insight into the process of drug discovery. It is my hope that new efficient drugs will be discovered for the management of cryptococcosis and cryptococcal meningitis to relieve or prevent the illness that thousands across the globe are still inflicted with.

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