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ABSTRACT

Invasive fungal infections account for nearly 1.5 million deaths a year and therefore are considered one of the leading causes of death worldwide. The first line of therapy towards these infections are antifungal drugs in the triazole class such as voriconazole, posaconazole, itraconazole, and isavuconazole. However, these antifungal drug therapies fail in a large number of patients and can produce toxic side effects, therefore increasing the risk of mortality worldwide. While prior research has demonstrated the efficacy of these drugs in mitigating fungal growth *in vitro*, it is unclear why these drugs fail to protect patients infected with fungal pathogens. Cytochrome P450 (CYP-P450) enzymes are involved in the metabolism of triazole antifungal drugs. Particularly, CYP3A4 and CYP1A are responsible for a large majority of triazole drug metabolism in the human body. I hypothesized that CYP3A4 and CYP1A proteins mediate triazole drug efficacy and toxicity in a whole organism. I utilized a larval zebrafish model to study how these genes affect the efficacy and toxicity of triazole drug treatments *in vivo.* I identified orthologs of the human *CYP3A4* and *CYP1A* genes in zebrafish using the Zebrafish Information Network (ZFIN): *cyp3c1* and *cyp1a*, respectively. I first tested if these genes are expressed in zebrafish larvae by performing RT-PCR of RNA extracted from zebrafish larvae. I find that both *cyp3c1* and *cyp1a* are expressed in the larvae at 2-, 3-, and 5-days post fertilization. To determine the effects of antifungal drug treatment on the expression of these genes, I treated zebrafish larvae with four antifungal drugs at 2 days post fertilization: voriconazole, posaconazole, itraconazole, and isavuconazole. The most notable difference was the significant upregulation of expression in the *cyp3c1* gene at one day post treatment with isavuconazole. However, there was a significant upregulation of this gene's expression at 2 days post treatment with itraconazole. To determine how this could impact the drug toxicity of the triazole antifungal drugs, CRISPR-Cas9 system was utilized to mutate the *cyp3c1* gene. To do so, I generated two gRNAs to target this gene as well as

forward and reverse primers to check that the gRNAs induce double strand breaks at the targeted regions of the gene. An injection mixture containing both gRNAs for the genes as well as Cas9 protein was injected into the zebrafish embryos at the one cell stage. At two days post injection the larvae were treated with the four drugs and survival of the larvae was tracked. The findings thus far demonstrate that the *CYP3A4* homolog in zebrafish, *cyp3c1*, protects against isavuconazole drug toxicity *in vivo*. In the future we will infect *cyp3c1* mutant larvae with *Aspergillus fumigatus* and treat infected larvae with triazole drugs to determine the role of *cyp3c1* in drug efficacy against a fungal infection.

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INTRODUCTION

Impact of fungal infections in humans

It has been estimated that more than 1.5 million invasive fungal infections resulting in death occur globally each year. These infections are primarily seen in immunocompromised patients, as their bodies are less equipped to fight off the infection [1]. These invasive fungal infections typically occur secondary to other health problems such as asthma, cancer, organ transplants, corticosteroid therapies, autoimmune diseases, and AIDS [2]. As the number of immunocompromised individuals increases worldwide, the incidence of invasive fungal infections will continue to rise as well.

Fungal infection treatment

The first line of defense towards these invasive infections are antifungal drugs. However, the development of new antifungal drugs is difficult due to the evolutionary close relationship of humans and fungi reducing the number of differences that antifungal drugs can target [3]. Currently there are three main classes of antifungal drugs prescribed to treat infections: polyenes, azoles, and echinocandins [4]. Due to the toxic effects of polyene amphotericin B, azole compounds have become the first-line therapy for treating cases of fungal infections [5].

The majority of antifungal drugs prevent spore germination. However, by the time patients are diagnosed with an infection and are provided with treatment, invasive hyphal growth is already present [6]. Therefore, nearly 50% of the patients provided with antifungal drug treatment following diagnosis still succumb to disease [2]. While prior research has demonstrated the efficacy of these drugs in mitigating this invasive hyphal growth *in vitro*, it is unclear why these drugs fail to protect patients infected with invasive fungal pathogens [7].

Triazole antifungal drugs and their mechanism of action

Azole drugs cause minimal side effects in patient treatment and exhibit broad-spectrum activity targeting a wide range of yeasts and filamentous fungi. This drug class is further categorized into imidazoles and triazoles based on the number of nitrogen atoms present in their azole ring. Imidazoles contain two nitrogen atoms while triazoles contain three [8]. Triazoles are newer antifungal drugs and have shown to be less toxic and more effective when compared to imidazoles. This drug class includes fluconazole, itraconazole, isavuconazole, ravuconazole, posaconazole, voriconazole, and terconazole [8].

Azoles function by preventing ergosterol synthesis. Ergosterol is the primary component of the fungal cell membrane, but it is not found in animal cell walls. Therefore, a drug targeting this component of the fungal cells would be ideal in treating invasive fungal infections [9]. Both groups of azoles, imidazoles and triazoles, have the same mechanism of action in which they inhibit ergosterol biosynthesis by blocking a cytochrome P450-dependent enzyme, lanosterol 14-alpha-demethylase. Blocking this CYP enzyme inhibits the conversion of lanosterol to ergosterol thereby increasing the membrane permeability allowing cell lysis and death [8].

Cytochrome P450 enzymes

Much like fungal cells, all other eukaryotes also have cytochrome P450 enzymes as well. These CYP 450 enzymes function to metabolize any chemicals or drugs that get into the human body to help prevent their toxic side effects. It has been estimated that 90% of human metabolism of azole drugs can be attributed to six main cytochrome P450-enzymes. Genetic polymorphisms in these enzymes can impact a person's ability to metabolize these drugs in the liver [8]. The effect that these human cytochrome P450 enzymes may have on triazole antifungal drugs *in vivo*, impacting drug efficacy and toxicity, is currently unknown.

Zebrafish as whole organism model

Current research is interested in determining why antifungal drug efficacy decreases in human patients as compared to results obtained *in vitro*. *Danio rerio*, commonly known as zebrafish, can be used to help study this question. Zebrafish are small, freshwater fish that are relatively inexpensive to maintain and produce a large number of progeny. These fish have an immune system that is largely conserved with humans and the pathogenesis of fungal infections in larval zebrafish replicates that of humans [3]. Zebrafish are also genetically tractable, meaning their genome can be readily modified to examine the effects of antifungal drugs. The triazole antifungal drugs in question can be delivered through addition to larval water in which they are then absorbed through the larval skin [11]. For these reasons, zebrafish can be used as a test model to study antifungal triazole drugs *in vivo*.

Prior research concluded that antifungal drugs may work against invasive fungal infections *in vivo* when specific immune cells, such as macrophages, are present at the site of infection [12]. In this study, I examined why triazole antifungal drug efficacy decreases *in vivo* using larval zebrafish as a test model. It was hypothesized that CYP450 enzymes mediate triazole drug efficacy and toxicity in a whole organism model. I specifically tested this question using four of the known triazole drugs: isavuconazole, itraconazole, posaconazole, and voriconazole. I found that CYP450 may be important to mediate triazole drug toxicity *in vivo*.

RESEARCH OBJECTIVE

Triazole antifungal drug treatments are a first line defense against invasive fungal infections. However, the efficacy and toxicity of these drugs are a primary concern for patients who have fungal infections. CYP450 enzymes are responsible for metabolizing drugs in the body, hence may affect the triazole drug efficacy and toxicity in a whole organism. Therefore, the aim of this study is to determine how CYP450 genes affect the efficacy and toxicity of triazole antifungal drug treatment *in vivo* using a zebrafish animal model.

MATERIAL AND METHODS

Zebrafish lines and maintenance.

Animal care and use protocol was approved by the Clemson University Animal Care and Use Committee. Prior to performing any experimental manipulations on larvae, they were anesthetized in E3 medium containing 0.2 mg/mL tricaine. All fish used in this study were wild-type AB zebrafish.

Zebrafish spawning.

Zebrafish breeding tanks were set up the evening before breeding. The tank has a grated insert to ensure embryos cannot be eaten by the zebrafish. Water from the system was first added to each individual breeding tank. A divider was then added, allowing males and females to be separated. Male and female zebrafish were characterized based on body shape and color. Male zebrafish typically are slimmer, more active, and have a yellow/pink cast with dark blue stripes. Female zebrafish are easily distinguished as they have enlarged bellies and are duller in color. After sex of each fish was determined, two males and three females were placed on opposite sides of the tank. Fish were left undisturbed overnight, in the dark. When it was time to spawn fish, typically within the first 30 minutes of exposure to light, the dividers were removed so males and females could interact. Spawning was allowed to occur undisturbed for at least one hour or until sufficient embryos were seen at the bottom of the tank, usually until early afternoon.

Table 1. Zebrafish lines used throughout the duration of experiments.

Line	Serial Number (SN)	Experiment
AB	206 and 226	*cyp* gene expression in zebrafish larvae
AB	283, 354, 206, 197	*cyp* expression when treated with antifungal drug
AB	206, 354, 400, 283, 197	gRNA injections for drug toxicity

Embryo Collection and Maintenance.

First, fish were removed and returned to their tank on the system. After being added back to their tank, fish were fed ample food. Embryos were then collected using a strainer. The water containing the embryos was poured through the strainer and gently washed to ensure no excess waste was present. Embryos were then transferred to a Petri dish by rinsing the strainer with E3 until all embryos had been removed. Dead embryos were then be removed via pipette and the plate was incubated at 28.5° C overnight. At 1-day-post fertilization, dead embryos were again be removed and new E3 was added to the Petri dish. Embryos were again incubated overnight at 28.5° C. At 2 days post fertilization, dead embryos were again removed and then larvae were dechorionated. Chorions were removed from the larvae using two forceps. One forcep was used to hold the chorion and the other to make a small tear in the chorion allowing the larvae to escape.

Drug treatments.

All drugs used in the experiment were taken from 1000X stocks previously made and added to E3 to make 1:1000 dilutions to result in the proper final concentrations. All of these stocks were dissolved in DMSO and stored at 20°C. All drugs were dissolved in E3 for zebrafish larvae exposure. Voriconazole was resuspended to a final concentration of 1 μg/mL, posaconazole was resuspended to 2.5 μg/mL, itraconazole was resuspended to 1 μg/mL, isavuconazole was resuspended to 0.5 μg/mL, 1 μg/mL, and 10 μg/mL. 0.1% DMSO was used as the vehicle control. At 2 dpf, larvae were dechorionated and transferred to 30 mm petri plates. Drug solutions were made in 50 mL conical tubes. E3 from petri plates were removed and the E3 containing the drugs were added. Drugs were left on the larvae for the duration of experimentation.

RNA Extraction Protocol.

Using ~25-50 larvae, 1 ml of tricaine was added to anesthetize the larvae. All liquid was then removed and 500 μL TRIzol (Invitrogen) was added in microcentrifuge tube and vortexed for

15 min. While this was vortexing, all workspaces and equipment were sprayed with 70% EtOH and RNase away. Following the vortexing, 100 µl chloroform was added to each tube and they were vigorously shaken by hand for 15 sec to mix. They were then left on the benchtop to incubate at room temperature for approximately 5 minutes. They were then centrifuged at 14,000 g for 20 minutes at 4ºC. The aqueous layer was then pipetted off into a fresh, RNA-free microcentrifuge tube. The RNA was then isolated using the following steps. 2 µl of 2 mg/mL glycogen was added to the aqueous layer. Then, 250 µL of isopropanol was added, mixed by inverting the tube, and incubated at room temperature for 10 minutes. Following incubation, the samples were centrifuged at 12,000 rpm for 10 minutes at 4ºC. The supernatant was poured off, avoiding disruption of the pellet. This pellet was then washed using 500 µl 75% EtOH and quickly vortexed to dislodge the pellet. The dislodged pellet was then centrifuged at 7,500 rpm for 5 minutes at 4ºC. The supernatant was again poured off and the washing step was repeated. The supernatant was again poured off and sample was spun again at room temperature. The remaining liquid was pipetted off and the sample was allowed to dry open on ice for 15 minutes. At the conclusion of the 15 minutes, the pellet was resuspended in 30 µL of RNase free water. The sample was then incubated at 55ºC for 15 minutes then returned to ice. The RNA is then stored at -80ºC until cDNA synthesis.

cDNA Synthesis Protocol.

cDNA synthesis is performed using iScript Reverse Transcription Supermix (Bio-Rad) protocol. First, the work area and lab equipment being used was cleaned with 70% EtOH and RNase away. The RNA was retrieved from the -80ºC freezer and placed on ice to defrost. Into PCR tubes, 16 µl of RNA template + nuclease free water was added as well at 4 µl of iScript RT Supermix for a total of 20 µl in each PCR tube. The PCR tubes were then spun down and placed in the PCR machine. The PCR reaction run was 5 minutes at 25 ºC for priming followed by 20

minutes at 46ºC for reverse transcription and lastly 1 minute at 95 ºC for RT inactivation. Following cDNA synthesis, gel electrophoresis is utilized to visualize the results.

To determine the expression of *cyp3c1* and *cyp1a in vivo*, zebrafish were spawned, embryos were collected and maintained until 2 days post fertilization as described above. Total RNA from 25 pooled larvae were extracted using TRIzol as described above. Undiluted cDNA was used for PCR amplification of *cyp3c1*, *cyp1a*, and the control gene *rps11*. PCR products were run on a 2% gel. Primers used for this study are listed in Table 2.

Table 2. Primers used to determine the expression of *cyp3c1 and cyp1a*.

rps11_1	TAAGAAATGCCCCTTCACTG
rps11_2	GTCTCTTCTCAAAACGGTTG
cyp1a_1	CTTCCCTTCACCATTCCTCA
cyp1a_2	GGTTGACTTGCCACTGGTTT
cyp3c_1	CACTGGTTGGAATTCCTTTG
cyp3c_2	GGCACACTGGTTGATCTCTG

Gel Electrophoresis

1, 2, and 2.5% gels were used for the duration of the experiments performed. Agarose and TAE buffer were added to a 500 mL Erlenmeyer flask. The mixture was heated up for approximately 3 minutes and halfway through, it was spun to mix and placed back in the microwave for the remaining time until it was a homogenous mixture. The gel was then placed in a 60ºC water bath to cool down for 15 minutes. Once taken out of the water bath, 2 µL of ethidium bromide was added to the mixture and mixed thoroughly. The gel was then poured into a casting tray with a well comb. After the gel was left to solidify for ~45 minutes, the gel was moved to the electrophoresis chamber and the comb was removed. TAE was added until the gel was fully submerged, and the wells were flushed with TAE. The wells were then loaded with 5 µl of each sample and 5 µL of ethidium bromide was added to the positive end of the electrophoresis chamber.

The gel was run for ~1-1.5 hours at 80 to 100 mV. After completed, the gel was removed from the chamber and imaged.

RT-qPCR

To test the effects of triazole drug treatment on the expression of *cyp3c1* and *cyp1a*, RT-qPCR was performed. Zebrafish larvae were exposed to 0.1% DMSO, [1 ng/μL] voriconazole, [2.5 ng/μL] posaconazole, [1 ng/μL] itraconazole, and [0.5 ng/μL] isavuconazole at 2 dpf and total RNA from 200 pooled larvae were extracted at 1- and 2-days post treatment. 2000 ng of RNA was used for cDNA synthesis. qPCR reaction mixtures were made containing: 5.0 μL SYBR Green Supermix (Bio-Rad), 0.3 μL of the forward and reverse primer for the respective gene, 0.4 μL water, and 4 μL of the 10X cDNA for the respective condition. The *rps11* housekeeping gene was used a comparison in this experiment. For each gene (*rps11, cyp1a, and cyp3c1*), triplicates of each cDNA sample were loaded into a 96 well plate for a total of 45 samples for each day. A negative control with 4 μL water instead of cDNA was also plated for each gene. Once all samples were loaded, a cover was added to both plates to ensure no evaporation occurred. The plates were then centrifuged for 3 minutes. Following centrifugation, the qPCR was run. Gene expression was normalized to *rps11* and DMSO vehicle control using ΔΔCq method.

To ensure no non-specific amplification occurred, gel electrophoresis for both 1- and 2-days post treatment samples were run. For each of the genes, one sample from each condition was run on the 1% gel at 80 mV for 1 hour.

CRISPR-Cas9 gRNA design and Primers

Using CHOPCHOP, a web tool used to select target sites for CRISPR/Cas9-directed mutagenesis, gRNAs were created for *cyp3c1*. The gRNA was picked based on results from CHOPCHOP ensuring that the exons were not located too close to the ends of the gene. The two

gRNAs generated as well as the constant oligo are listed in Table 3. To ensure the gRNA sufficiently induces mutations in the gene, primers for both gRNA were designed. These primers are listed in Table 4. gRNA and primers were synthesized and purchased from Integrated DNA Technologies (IDT). Primers were reconstituted at [10 μM] and gRNA at [100 μM], and were stored at 20 C.

Table 3. *cyp3c1* gRNA

***cyp3c1*_gRNA_1:**
5'- TAA TAC GAC TCA CTA TAG TTG GTT GGC CCT TTT GCT GAG TTT TAG AGC TAG AAA TAG CAA -3'
***cyp3c1*_gRNA_2:**
5'- TAA TAC GAC TCA CTA TAG GTC GAA GTC GAA GTC AAC GCT GAA GGG TTT TAG AGC TAG AAA TAG CAA -3'
Constant Oligo Sequence:
5'- AAA AGC ACC GAC TCG GTG CCA CTT TTT CAA GTT GAT AAC GGA CTA GCC TTA TTT TAA CTT GCT ATT TCT AGC TCT AAA AC-3'

Table 4. *cyp3c1* gRNA primers

***cyp3c1*_gRNA_1_Foward:**
5'- GCG GTC GGT CAT CAT TTG GA -3'
***cyp3c1*_gRNA_1_Reverse:**
5'- TGG AGA GAG TGA ACT TCG GA -3'
***cyp3c1*_gRNA_2_Foward:**
5'- CAG TGT TGT GGC TCC ATA CAG -3'
***cyp3c1*_gRNA_2_Reverse:**
5'- AGA GAG GAC TGA GTG GAT TGA -3'

gRNA Synthesis

For gRNA synthesis oligos were first annealed. For annealing reaction, each gRNA was first vortexed and spun down. Then 1 μL of the [100 μM] gene specific oligo, 1 μL of the [100 μM] constant oligo, and 8 μL of DI water were combined. The reaction mix was then run at 95 °C for 5 minutes, followed by 85 °C – 2 °C/second, followed by 25 °C -0.1 °C/second until it reaches 4°C. Once reaction is complete, T4 DNA polymerase (New England Biolabs) was then added

together with 2.5 µL [10 mM] dNTPs, 2 µL 10x NEB buffer 2.1, and 5 µL of water. These mixtures were then incubated at 12 °C for 20 minutes. The PCR was then cleaned up using the DNA Purification Kit. Following purification, each DNA sample was run on dsDNA nanodrop to obtain concentration of DNA present in the sample. *cyp3c1*_gRNA_1 contained 127.1 ng/µL and *cyp3c1*-_gRNA_2 contained 168.0 ng/µL. gRNAs were *in vitro* transcribed using 1 mg of template DNA. Then, RNA was cleaned up from this reaction using the Monarch RNA Cleanup Kit. Samples were nanodropped to obtain the amount of RNA present. *cyp3c1*_gRNA_1 had a concentration of 1877.5 ng/µL and *cyp3c1*_gRNA_2 had 3571.4 ng/uL present. Stock concentrations of 100 ng/µL and 125 ng/µL of each gRNA were made and stored at -80° C.

CRISPR-Cas9 gRNA injections.

On the day of injection, a gRNA mixture was made. This mixture contained 1 µg Cas9 protein (PNA Bio), 1 µL gRNA #1, 1µL gRNA #2, 0.5 µL phenol red, and 1.5 µL RNA free water. The [100 ng/µL] stock was used for the first survival experiment and the [125 ng/µL] was then used for the final two survival experiments. The needle was loaded with 1.5 µL of injection mix and the needle was calibrated. Once the needle was calibrated, zebrafish were spawned and embryos were collected approximately 30 minutes later. From each tank of embryos collected, half of the embryos were injected with gRNA and the other half were kept as uninjected controls.

Both gRNA injected and uninjected control larvae were dechorionated and treated with [1 ng/µL] voriconazole, [2.5 ng/µL] posaconazole, [1 ng/µL] itraconazole, [0.5 ng/µL] isavuconazole, [1.0 ng/µL] isavuconazole or [10 ng/µL] isavuconazole at 2 dpf as described above. To monitor survival, larvae were transferred to 96-well plates, 1 larva/well in 200 µL of drug. For 7 days, larvae are viewed under microscope and death is tracked.

Genomic DNA extraction

4 uninjected and 6 injected larvae were set aside for genomic DNA extraction during each experiment to check for gRNA efficiency. The larvae were anesthetized using tricaine and put in individual microcentrifuge tubes. All liquid was removed and 50 µL 50 mM NaOH is added. Samples are then placed on a heat block at 95° C for 10-15 minutes. Once removed, they are quickly spun down and 5 µL of Tris-HCl (pH 8) was added to neutralize. The samples are then quickly vortexed again. At max speed, the samples were then spun down for 10 minutes. The supernatant is then used for PCR.

PCR samples contained 2.4 µL water, 5 µL GoTaq (Promega), and 0.8 µL of both the forward and reverse primer. 1 µL of DNA from the respective fish was added to the samples as well. Based on the annealing temperatures for each of the primers generated, the PCR reaction was run following the manufacturer's protocol. 5 µL of PCR reaction samples were run on a 2.5% agarose gel as described above.

RESULTS

Identification of CYP450 genes important for triazole drug metabolism

Human CYP450 genes are known to metabolize drugs in the human body. I wanted to determine which CYP genes are modulated by triazole drug treatment. The four triazole antifungal drugs utilized in this experiment include: voriconazole, posaconazole, itraconazole, and isavuconazole. Therefore, through extensive literature research, the primary CYP450 enzymes involved in the metabolism of each of these drugs were first identified (Table 5). Since the *CYP3A4* and *CYP1A* enzymes account for 90% of drug metabolism in the human body, these genes are focused on in this thesis.

Table 5: CYP450 enzymes involved in triazole drug metabolism.

Voriconazole[13]	CYP2C9, CYP2C19, CYP2D6, **CYP3A4**, CYP3A5, and CYP3A7
Posaconazole[14,15]	**CYP3A4** and CYP3A7
Itraconazole[16]	**CYP1A1**, **CYP3A4**, CYP3A5, CYP3A7, CYP2D6, and CYP2E1
Isavuconazole[17]	**CYP3A4** and CYP3A5

cyp3c1 *and* cyp1a *are expressed in zebrafish larvae*.

Zebrafish orthologs to the two human CYP genes focused on in this thesis, *CYP3A4* and *CYP1A*, were first identified using ZFIN. These genes include *cyp3c1* and *cyp1a* respectively [18]. Therefore, I first tested if these genes are actively transcribed in zebrafish larvae. To do so, zebrafish were spawned in breeding tanks and embryos were collected. RNA was extracted from the larvae at 2-, 3-, and 5- days post fertilization and cDNA was synthesized. RT-PCR was run, and products were loaded into gel to determine gene expression (Figure 1A). The housekeeping gene, *rps11* was used as the control. Both *cyp3c1* and *cyp1a*, are actively transcribed in our

zebrafish model as indicated by the bands present for each gene at 2-, 3-, and 5- days post fertilization (Figure 1B).

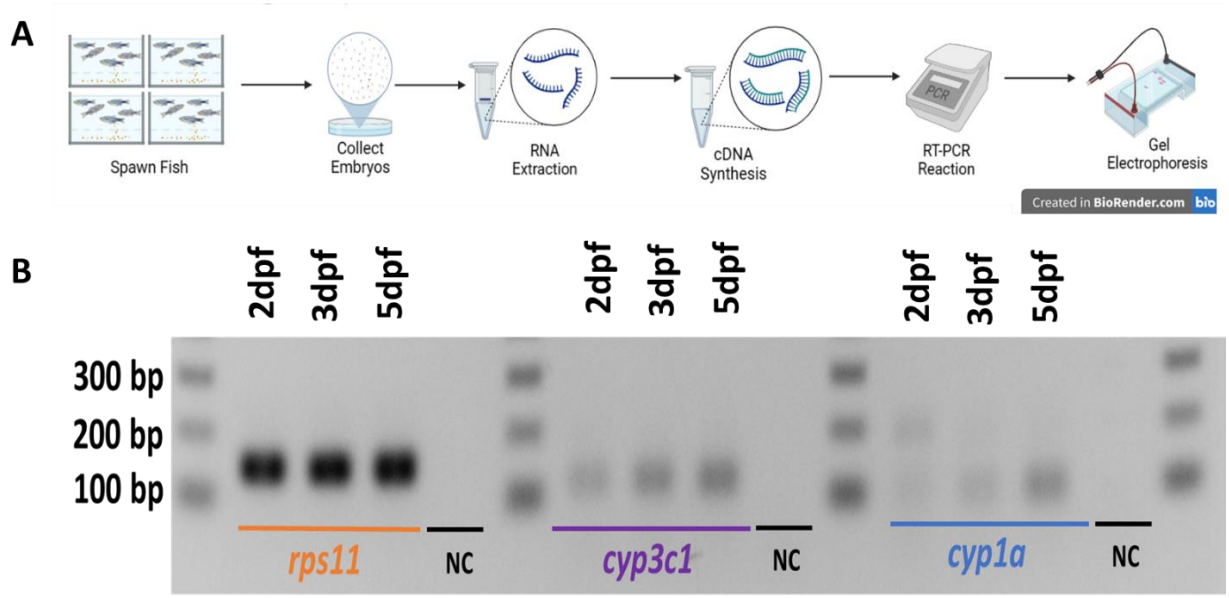

Figure 1. *cyp3c1* and *cyp1a* are expressed in zebrafish larvae. (A) Zebrafish are spawned, and embryos are collected and incubated. RNA is extracted from larvae at 2-, 3-, and 5-days post fertilization. cDNA synthesis is performed followed by RT-PCR to determine if these CYP genes are actively transcribed in the zebrafish larvae. Gel electrophoresis is run to examine gene expression. (B) Gene expression for both *cyp3c1* and *cyp1a* is monitored and housekeeping gene, *rps11,* is used as a control.

cyp3c1 *is upregulated by triazole drug treatment* in vivo.

cyp3c1 and *cyp1a* are important for the metabolism of antifungal drugs. Therefore, I hypothesized that exposure to triazole drugs would affect the expression of these genes. To test this, larvae were treated with four antifungal drugs: [1 ng/µL] voriconazole, [2.5 ng/µL] posaconazole, [1 ng/µL] itraconazole, [0.5 ng/µL] isavuconazole, and 0.1% DMSO vehicle control at 2 days post fertilization. RNA was extracted at 1- and 2- days post treatment and real-time quantitative PCR (RT-qPCR) was run to examine levels of gene expression (Figure 2A). RT-qPCR samples for each of the conditions tested were run on a gel to ensure that the expected 150bp amplicon was present in each of the samples (Figure 2B). *cyp1a* expression following treatment

with the four antifungal drugs remained relatively unchanged from 1- to 2-days post treatment for posaconazole, voriconazole, and itraconazole. Expression increased slightly in larvae treated with isavuconazole. *cyp3c1* expression remained relatively the same from 1- to 2-days post treatment in larvae treated with posaconazole, and voriconazole. The expression of this gene increased significantly from 1- to 2-days post treatment when larvae were treated with isavuconazole and increased significantly from 1- to 2-days post treatment with itraconazole (Figure 2C).

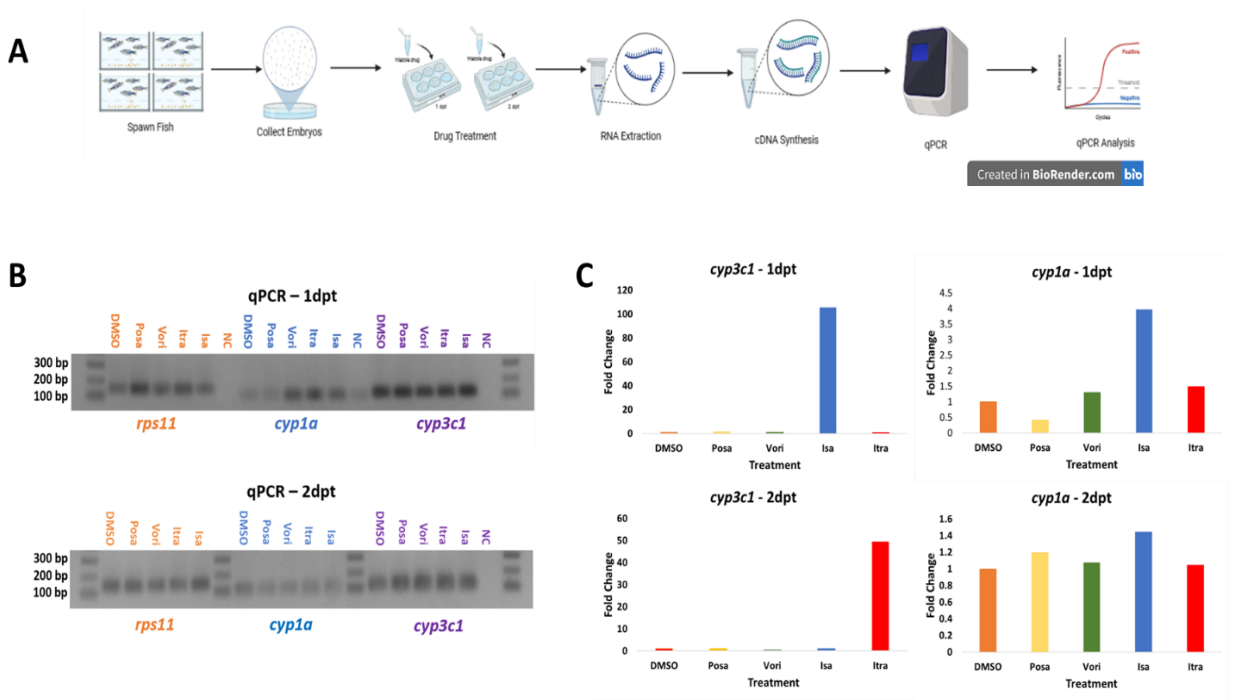

Figure 2. *cyp3c1* gene expression is upregulated after triazole drug treatment *in vivo*. (A) Schematic of experimental setup for qPCR reaction. Zebrafish larvae are treated with [1 ng/µL] voriconazole, [2.5 ng/µL] posaconazole, [1 ng/µL] itraconazole, and [0.5 ng/µL] isavuconazole at 1- and 2- days post fertilization. DMSO is used as the vehicle control and results were normalized to the *rps11* house keeping gene. (B) qPCR samples of wild-type larvae treated with antifungal drugs are run on a gel to ensure that expected amplicon is present at 1dpt and 2dpt for *rps11, cyp3c1*, and *cyp1a* at 150bp. (C) qPCR analysis is conducted on 1- and 2-day post treatment larvae.

cyp3c1 *may play a role in mediating triazole antifungal drug toxicity* in vivo.

Because the *cyp3c1* gene expression was significantly altered when larvae were treated with triazole antifungal drugs: posaconazole, voriconazole, itraconazole, and isavuconazole, I hypothesized that the *cyp3c1* gene plays a role in mediating triazole drug toxicity in zebrafish larvae. To determine the level of toxicity that these drugs may have on larval zebrafish, a CRISPR-Cas9 system was used to mutate the *cyp3c1* gene. Two gRNAs were generated to target the gene as well as forward and reverse primers check that the gRNAs induce mutations following double strand breaks at the targeted regions of the gene (Figure 3).

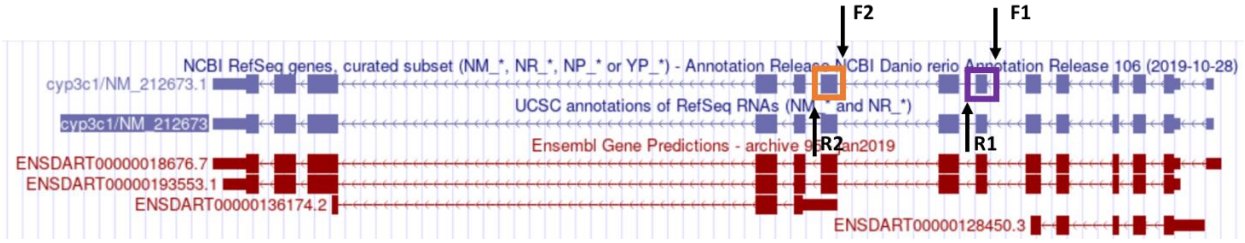

Figure 3. *cyp3c1* gene structure with labeled gRNA and primers. *cyp3c1* gRNAs induce double strand breaks at the targeted regions of the gene. Forward and reverse primers were created for each of the gRNA to flank the target sites as depicted.

Zebrafish embryos were injected with the two gRNAs and Cas9 protein at 1-cell stage. These larvae at 2 days post fertilization were treated with DMSO, [1 ng/µL] voriconazole, [2.5 ng/µL] posaconazole, [1 ng/µL] itraconazole, and [0.5 ng/µL] isavuconazole at 2 days post fertilization. The larvae were then transferred to 96-well plate, one larva/well, to examine survival (Figure 4A). To determine if the gRNA concentration of [100 ng/uL] is sufficient for cutting, gel electrophoresis of genomic DNA extracted from individual larvae was run. If the gRNA successfully induced double strand breaks causing mutations to occur in the *cyp3c1* gene, band smearing would be expected in the lanes with injected larvae in the F1R1, F2R2, and F1R1 gels. While there is small amounts of smearing in the F1R1 and F2R2 gels, there are no clear bands in

the injected larvae in the F1R2 gel. This indicates the [100 ng/uL] of each gRNA in the mixture did not induce mutations at the target region of the gene (Figure 4B). Survival was tracked and graphed for uninjected (Figure 4C) and gRNA injected (Figure 4D) larvae treated with the four antifungal drugs. Overall, more than 70% of injected larvae survived the whole treatment period, except for those treated with isavuconazole. In both conditions, uninjected and injected larvae, there is a baseline mortality with only ~70% DMSO-treated larvae surviving the treatment period. In the uninjected larvae, isavuconazole increases mortality three times the amount as compared to larvae in DMSO. Other antifungal drugs produced similar or less toxic effects on the larvae as compared to larvae in DMSO (Figure 4C). In the injected larvae, voriconazole, posaconazole, and itraconazole had less toxic side effects than larvae in DMSO. However, isavuconazole increased mortality in the larvae compared to DMSO (Figure 4D).

Because the [100 ng/uL] gRNA mixture did not effectively induce sufficient mutations in the target region of the *cyp3c1* gene, the injection mixture was increased to [125 ng/uL] of each gRNA. Gel electrophoresis of the F1R2 revealed that [125 ng/uL] gRNA mixture does induce double strand breaks in the *cyp3c1* gene of the injected larvae (Figure 5B). Survival was tracked and graphed for uninjected (Figure 5C) and injected (Figure 5D) larvae treated with the four antifungal drugs. Overall, more than 70% of both uninjected and injected larvae survived the whole treatment period. In both conditions, uninjected and injected larvae, there is a baseline mortality with only ~85% DMSO-treated larvae surviving the treatment period. None of the antifungal drugs produced significant toxic effects as compared to the larvae in DMSO for the uninjected or injected larvae (Figure 5C and D). The lack of death of treated larvae in both the uninjected and injected larvae could indicate that the gRNA concentration was too low to effectively cause mutations in the *cyp3c1* gene sequence.

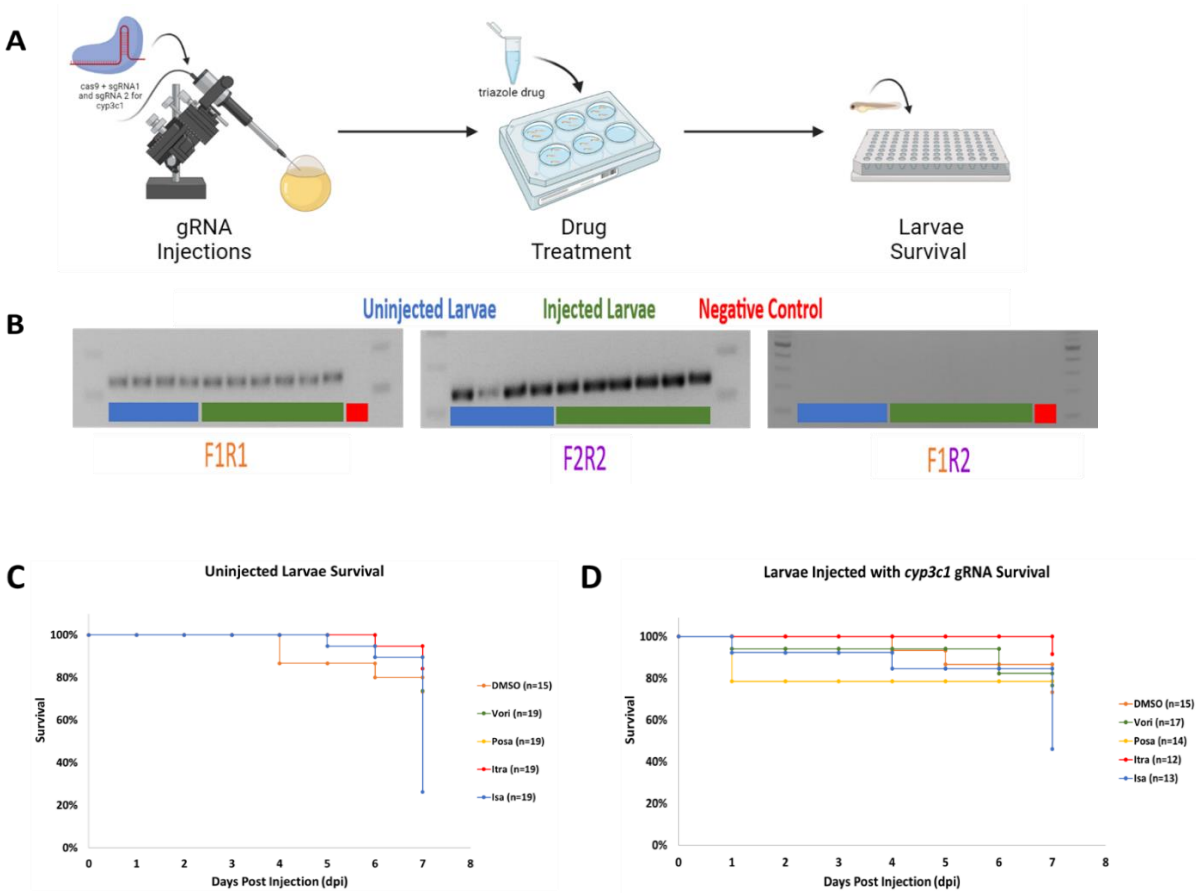

Figure 4. *cyp3c1* may play a role in mediating triazole antifungal drug toxicity *in vivo.* (A) Schematic showing experimental setup for testing drug toxicity *in vivo*. Wild-type larvae are injected with various concentrations of *cyp3c1* gRNA mixture or kept uninjected as a control at the one-cell stage. Larvae are then treated with 0.1% DMSO, [1 ng/µL] voriconazole, [2.5 ng/µL] posaconazole, [1 ng/µL] itraconazole, and [0.5 ng/µL] isavuconazole at 2 days post fertilization. Individual larvae are then transferred to 96-well plate and survival is monitored. (B) To determine if the gRNA concentration of [100 ng/uL] is sufficient for cutting, gel electrophoresis of DNA extracted from the larvae was run. Survival was tracked and graphed for uninjected (C) and injected (D) larvae treated with the four antifungal drugs.

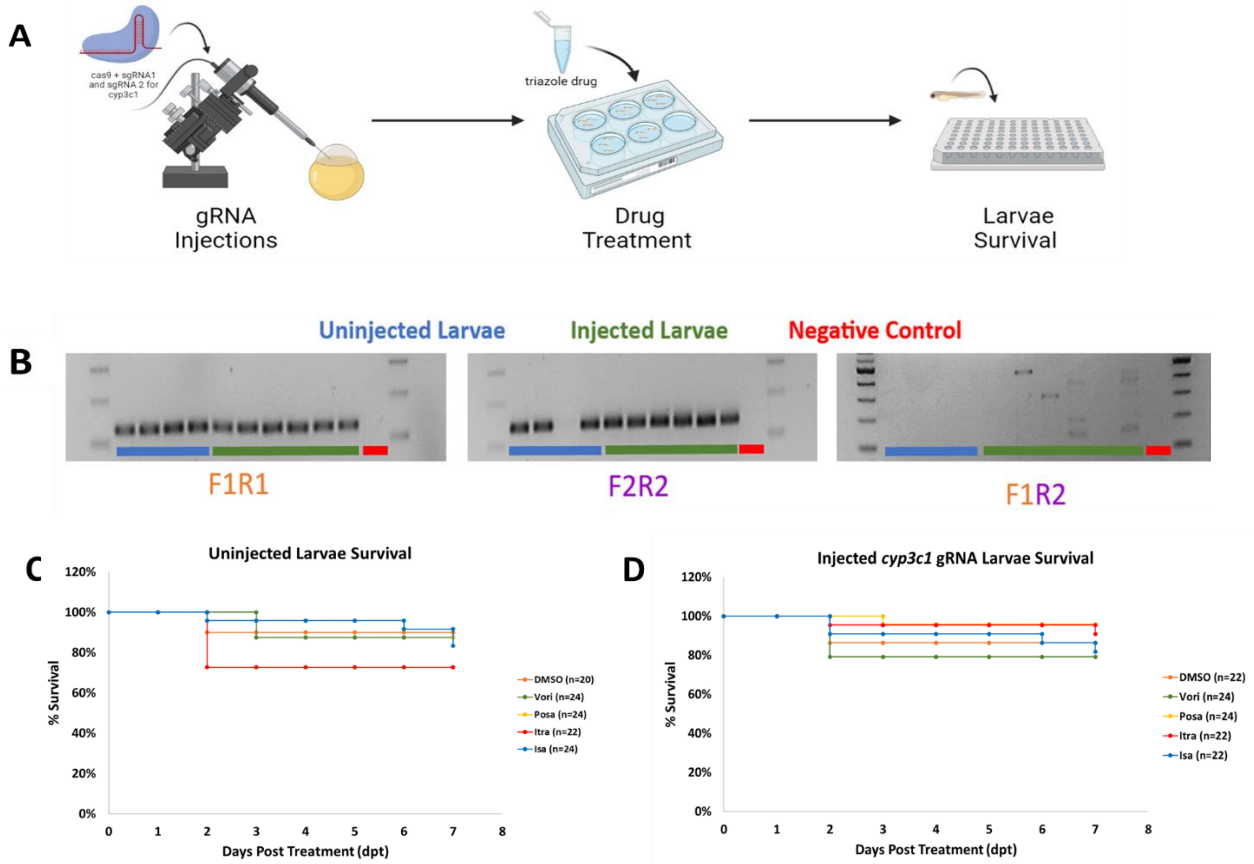

Figure 5. *cyp3c1* may play a role in mediating triazole antifungal drug toxicity *in vivo.* (A) Schematic showing experimental setup for testing drug toxicity *in vivo*. Wild-type larvae are injected with various concentrations of *cyp3c1* gRNA mixture or kept uninjected as a control at the one-cell stage. Larvae are then treated with 0.1% DMSO, [1 ng/µL] voriconazole, [2.5 ng/µL] posaconazole, [1 ng/µL] itraconazole, and [0.5 ng/µL] isavuconazole at 2 days post fertilization. Individual larvae are then transferred to 96-well plate and survival is monitored. (B) To determine if the gRNA concentration of [125 ng/uL] is sufficient for cutting, gel electrophoresis of DNA extracted from the larvae was run. Survival was tracked and graphed for uninjected (C) and injected (D) larvae treated with the four antifungal drugs.

cyp3c1 *mediates isavuconazole toxicity.*

Because *cyp3c1* gene expression increased when larvae were treated with isavuconazole (Figure 2C), I hypothesized that this gene is likely involved in mediating isavuconazole toxicity. To better understand the effects of isavuconazole when the *cyp3c1* gene is mutated, wild-type larvae were injected with [125 ng/µL] of each gRNA at the one-cell stage and then treated larvae

with various concentrations of isavuconazole. Drug toxicity was then examined by moving individual larvae to 96-well plate (Figure 6A). Gel electrophoresis of the F1R2 revealed that [125 ng/uL] gRNA mixture did induce mutations in the *cyp3c1* gene of the injected larvae (Figure 6B). Survival was tracked and graphed for uninjected and injected larvae treated with DMSO, [0.5 ng/μL] isavuconazole, [1 ng/μL] isavuconazole, and [10 ng/μL] isavuconazole at 2 days post fertilization. Overall, in the uninjected larvae there is a baseline mortality in DMSO-treated larvae with only ~80% surviving the treatment period. [0.5 ng/μL] isavuconazole proved to be less toxic in these larvae as compared to the DMSO larvae. However, [1 ng/μL] and [10 ng/μL] isavuconazole was completely toxic to the larvae as they were all dead by day 4 (Figure 6C). Of the injected larvae, there was significant death in larvae in DMSO as only ~30 survived the duration of the treatment. There was increased survival in larvae treated with [0.5 ng/μL] isavuconazole as compared to DMSO. However, [1 ng/μL] and [10 ng/μL] isavuconazole proved to be completely toxic as all larvae were dead by 4 days post treatment (Figure 6D).

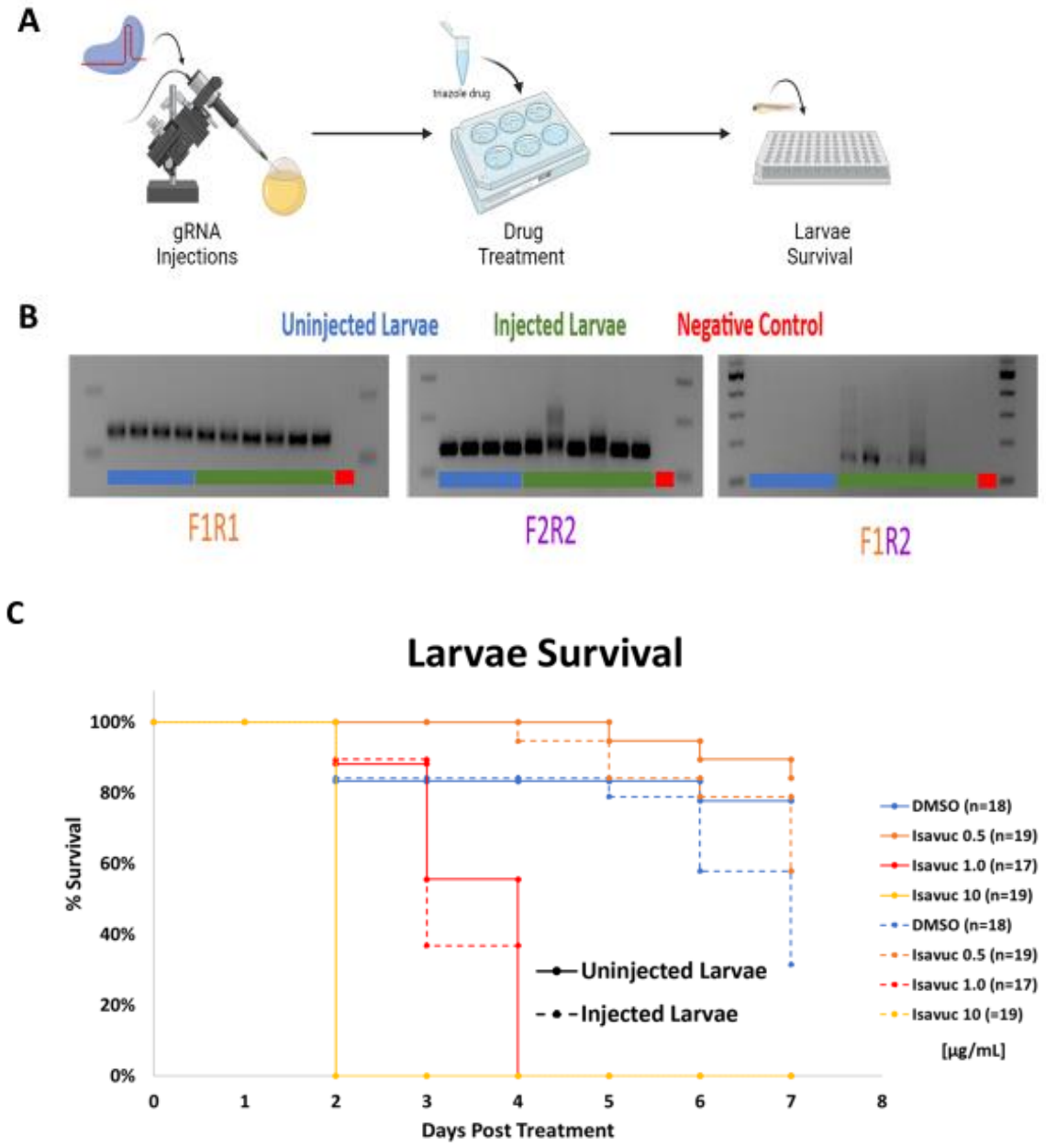

Figure 6. *cyp3c1* may play a role in mediating triazole antifungal drug toxicity *in vivo*. (A) Schematic showing experimental setup for testing drug toxicity *in vivo*. Wild-type larvae are injected with [125 ng/µL] of *cyp3c1* gRNA mixture or kept uninjected as a control at the one-cell stage. Larvae are then treated with 0.1% DMSO, [0.5 ng/µL] isavuconazole, [1 ng/µL] isavuconazole, and [10 ng/µL] isavuconazole at 2 days post fertilization. Individual larvae are then transferred to 96-well plate and survival is monitored. (B) To determine if the gRNA concentration of is sufficient for cutting, gel electrophoresis of DNA extracted from the larvae was run. (C) Survival was tracked and graphed for uninjected and injected larvae treated with the four antifungal drugs.

DISCUSSION

Given the rising incidence of opportunistic invasive fungal infections in the immunocompromised population, safe and effective antifungal drug treatments are needed [19]. While many different antifungal drugs exist, including polyenes, allylamines, and azoles, many of these drugs have toxic side effects and severe drug interactions with common medications that many individuals worldwide rely on. Azoles are currently considered the safest form of antifungal drug in regard to a reduction in drug-to-drug interactions as well as drug toxicity [20]. While studies have proven azole antifungal drug efficacy *in vitro*, much is still unknown about why they are less efficient in humans.

In this study, zebrafish larvae were utilized to test if CYP450 enzymes mediate triazole drug efficacy and toxicity *in vivo*. Zebrafish larvae are increasingly used as a whole organism model in assessing drug toxicity and safety due to the similarity in toxicity profiles between mammals and larvae [21]. I found that that *cyp3c1* and *cyp1a*, two genes important for triazole drug metabolism in mammalian models, are actively transcribed in zebrafish larvae, validating that they are a good whole organism model to study the effects of the CYP450 enzymes on triazole drugs.

If the drug is toxic to the larval zebrafish and a particular CYP enzyme is responsible for metabolizing it to a non-toxic product, the larvae will respond by upregulating the expression of that enzyme. Compared to the DMSO vehicle of control, *cyp1a* gene expression was not significantly altered by the triazole drugs, indicating that the *cyp1a* gene is likely not involved in triazole drug toxicity *in vivo.* CYP1A2 is one of the six main cytochrome-P450 enzymes known to metabolize 90% of drugs. Therefore, this result was surprising as this enzyme is known to be involved in itraconazole drug metabolism [22].

The *cyp3c1* gene expression however did significantly increase in larvae treated with isavuconazole and itraconazole. *cyp3c1* expression in larvae treated with isavuconazole was nearly 100-fold higher than that of larvae treated with DMSO. This change in expression could potentially indicate that isavuconazole is toxic to larval zebrafish. Itraconazole also impacted the *cyp3c1* gene expression in the treated larvae as the *cyp3c1* gene expression increased nearly 50-fold higher than the control larvae. This finding supports prior research as itraconazole is known to be primarily metabolized via CYP3A4 in humans. The delayed increase in gene expression could be due to the concentration of the itraconazole used in the study. Drugs that have a long half-life and persist in the body for long periods of time, require a higher initial dose to start metabolism [23]. This change in expression could potentially indicate that itraconazole may have some toxic effects in larval zebrafish. However, isavuconazole seems to produce more toxic side effects than itraconazole.

While these drugs are considered the safest antifungal compounds, they still produce unwanted side effects in human patients [20]. To examine if *cyp3c1* mediates triazole drug toxicity, the *cyp3c1* gene was knocked down using a CRISPR-Cas9 system and gRNA were injected into larvae. Because isavuconazole increased expression of the *cyp3c1* gene, I began to examine the concentration of this drug that would produce toxic effects in the mutated larvae. There was baseline death in larvae treated with DMSO. This was unexpected as DMSO is considered a safe solvent for developmental toxicity testing of pharmaceutical drugs [24]. In future experiments, a control group with E3 could be added to compare the DMSO larvae to. Adding this control group would serve as a comparison to determine if DMSO is truly the reason for larvae death or if the larvae are dying from other causes such as improper development due to toxicity from gRNAs. There was less death in both uninjected and injected larvae treated with [0.5 mg/mL] isavuconazole when compared to DMSO. However, there was significant death in a much shorter amount of time

for both uninjected and injected larvae treated with [1 mg/mL] as all larvae were dead by 4 days post treatment. This indicates that the threshold for toxicity may be between these two concentrations. The [10 mg/mL] proved to be incredibly toxic as all fish were dead by 2 days post treatment in both the uninjected and injected larvae. It can be concluded that *cyp3c1* may play a role in mediating triazole antifungal drug toxicity *in vivo*.

The major limitation to this research was time. Therefore, future investigations would need to be performed to replicate the experiments performed in regard to the impact of CYP450 genes in drug metabolism as well as drug toxicity. Next steps for this project first must include optimizing the experimental conditions. The concentrations of gRNA injected in embryos should first be increased as this would allow for better cutting by the gRNA and ensure that mutation are introduced in the *cyp3c1* gene. Due to the small amount of smearing seen on the gels for [125 ng/µL], the concertation of each gRNA will likely need to be increased to [300 ng/µL] or more. Once the concentration of gRNA is optimized, the antifungal drug concentrations will then need to be optimized to better determine the role of *cyp3c1* in antifungal drug toxicity and efficacy *in vivo*. For isavuconazole specifically, concentrations between 0.5 ng/µL and 1 ng/µL should be tested as the toxicity in larvae was significantly different between these two concentrations.

Following optimization of experimental conditions, it would then be important to determine the role of *cyp3c1* in drug efficacy against invasive fungal infection. To test this, *cyp3c1* mutant larvae would be infected with *Aspergillus fumigatus.* Once the larvae are infected, we will treat them with the four triazole antifungal drugs discussed throughout this paper. This would allow us to determine the role of *cyp3c1* in drug efficacy against an opportunistic fungal infection.

CONCLUSIONS

In the last 25 years, therapeutic challenges associated with invasive fungal infections have changed drastically. The new medical advancements that improve the lives of individuals with health conditions, cancer, liver failure, kidney failure, etc. render many individuals around the world immunocompromised. These immunocompromised individuals have an increasingly higher chance of developing life-threatening fungal infections. While treatment for these infections is known to be effective at preventing spore germination, most cases are not caught early enough for optimal efficacy of the drug. It has been estimated that nearly 50% of the population provided with antifungal drug treatment after diagnosis still succumb to disease [25]. It is therefore important to understand what is causing the difference between triazole drug efficacy *in vitro* as compared to *in vivo*. One main difference is the presence of presence of CYP450 enzymes in humans that are not present in media on a petri dish.

Therefore, the aim of this study was to determine how CYP450 genes affect the efficacy and toxicity of triazole antifungal drug treatment *in vivo* using a zebrafish animal model. In the current study, described here in this thesis, I identified zebrafish orthologs of two of the most common CYP450 enzymes involved in drug metabolism in humans, *cyp1a* and *cyp3c1*. Both these genes are expressed in zebrafish larvae, and *cyp3c1* expression is altered in response to triazole drug exposure. Through CRISPR/Cas9-mediated knockdown, I find that *cyp3c1* gene may play a role in mediating triazole antifungal drug toxicity *in vivo*, supporting the hypothesis that CYP450 genes affect triazole toxicity in a whole organism. Future studies focus on if CYP450 genes also affect triazole drug efficacy against fungal infections using larval zebrafish model.

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