Atazanavir Resensitizes *Candida auris* to Azoles

Yehia Elgammal[a,b], Ehab A. Salama[a,b], Mohamed N. Seleem[a,b#]

[a]*Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA.*

[b]*Center for One Health Research, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 24061, USA.*

#Corresponding Author:

Mohamed N. Seleem
Department of Biomedical Sciences and Pathobiology
Virginia-Maryland College of Veterinary Medicine
Virginia Polytechnic Institute and State University
1410 Prices Fork Rd, Blacksburg, VA, 24061, USA
Phone: 540-231-2703
Email: seleem@vt.edu

Abstract

Candida auris represents an urgent health threat. Here, we identified atazanavir as a potent drug capable of resensitizing *C. auris* clinical isolates to the activity of azole antifungals. Atazanavir was able to significantly inhibit the efflux pumps, glucose transport, and ATP synthesis of all tested isolates of *C. auris*. In addition, the combination of itraconazole with atazanavir-ritonavir significantly reduced the burden of azole-resistant *C. auris* in murine kidneys by 1.3 log10 (95%), compared to itraconazole alone.

KEYWORDS

HIV protease inhibitors, *Candida auris*, azole resistance, efflux pumps, *in vivo* disseminated candidiasis mouse model.

Introduction

Candida auris is an emerging multidrug-resistant fungus associated with high mortality rates worldwide (1). Azole antifungals have been the standard-of-care for the treatment of most fungal infections (2). However, the overuse of azoles has led to an upsurge of azole resistance among *Candida* species (3). Most *C. auris* strains have developed high-level of azoles resistance (4). Due to the increasing resistance and prevalence of infections, the CDC has classified drug-resistant *C. auris* as an urgent health threat which critically needs development of new antifungals (5).

A promising strategy to thwart azole resistance and restore antimicrobial efficacy is using co-drugs that can potentiate the activity of azoles (3, 6-8). Repurposing FDA-approved drugs is a valuable approach for identifying new co-drugs that reduces the cost and the time required for drug development (9-12). Utilizing both approaches, we identified the HIV protease inhibitor, atazanavir as a co-drug for azole antifungals against *C. auris*. The objective of this study was to assess the effects of atazanavir on the activity of azole antifungal drugs against *C. auris in vitro* and *in vivo*.

The activity of atazanavir in combination with fluconazole and itraconazole was evaluated against a panel of pathogenic *C. auris* strains. The atazanavir/itraconazole combination was also evaluated in a time-kill assay. Additionally, mechanistic studies were performed to determine the possible mechanism of action by which atazanavir restores the activity of azoles against *C. auris*. Finally, we evaluated the *in vivo* efficacy of atazanavir/itraconazole combination, in presence of ritonavir as a pharmacokinetic enhancer (13), in a *C. auris* disseminated infection murine model.

Nineteen clinical isolates of *C. auris* (Table 1) were obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands), the CDC, and the BEI Resources. Drugs used in the study were purchased commercially: itraconazole, and ritonavir (TCI America, Portland, OR), atazanavir (Ambeed, Arlington Heights, IL), fluconazole (Acros Organics, Morris Plains, NJ). Media and reagents were obtained from commercial vendors.

Minimum inhibitory concentrations (MICs) of the drugs were identified following the CLSI guidelines (14). We also evaluated the interactions between atazanavir and two azole drugs (fluconazole and itraconazole) against *C. auris* isolates using the checkerboard method, as described elsewhere (15-17). Atazanavir alone did not exhibit any antifungal activity (MICs > 128 µg/ml) against all tested *C. auris* isolates. When tested in combination with fluconazole, atazanavir

interacted synergistically against 36.8% (7/19) of *C. auris* isolates with a fractional inhibitory concentration index (ΣFICI) ranging from 0.19 to 0.38. Interestingly, atazanavir displayed the most potent synergistic interaction with itraconazole against 100% (19/19) of *C. auris* isolates tested (ΣFICI= 0.09 - 0.38). These findings align with our earlier findings, where lopinavir displayed the most effective synergistic interaction with itraconazole (against 100% of *C. auris* isolates tested) (6). Because atazanavir interacted synergistically with itraconazole against all *C. auris* isolates tested, the focus for the rest of the work was to further investigate atazanavir/itraconazole combination using several experiments including killing kinetics, mechanistic study, and the *in vivo* efficacy against *C. auris*.

A time-kill assay was used to investigate the killing kinetics of the atazanavir/itraconazole combination against *C. auris* AR0390, as described before (3, 6, 18). As shown in Fig. 1A and 1B, atazanavir/itraconazole (16/0.5 µg/ml) exerted a fungistatic activity against the tested isolate, lowering the fungal burden by 4.5 and 4.9-$\log_{10}$ after 24 and 48 hours, respectively as compared to the negative control.

One of the key methods by which *C. auris* withstands the activity of azole antifungals, resulting in treatment failure, is the overexpression of efflux pumps (3). Therefore, using an efflux pump inhibitor could be a potential strategy to overcome the microorganism's resistance to antifungals. Encouraged by our previous work with lopinavir (6), we investigated the effect of atazanavir on the efflux activity, glucose utilization and ATP synthesis in *C. auris*. Glucose-induced Nile red efflux assays were performed using five isolates of *C. auris* as reported previously (19-21). As shown in Fig. 2A, atazanavir (8 µg/ml) hindered the efflux of Nile red in all tested *C. auris* isolates, leading to an increase in Nile red fluorescence intensity by 40 to 50%.

The glucose-induced acidification assay was performed to assess the impact of atazanavir on glucose utilization (22). As expected, atazanavir (32 µg/ml) significantly obstructed *C. auris*' ability to utilize glucose, and less reduction in OD_{590} values was detected (Fig. 2B).

Finally, the impact of atazanavir on the cellular ATP content of *C. auris* was evaluated as reported (23). Atazanavir (8 µg/ml) decreased the cellular ATP content in *C. auris* AR0390 by 20% (Fig. 2C). These results are consistent with our published data (6).

The efficacy of itraconazole/atazanavir combination in the presence of the bioavailability enhancer, ritonavir (13), was evaluated in an *in vivo* mouse model of disseminated candidiasis, as outlined in previous reports (24, 25). The animal experiment was conducted following the

guidelines from the Virginia Tech Animal Care and Use Committee. Briefly, female immunocompromised CD-1 mice were infected with 3.33×10^7 CFU/ml *C. auris* AR0390 intraperitoneally. Treatments were administered orally two hours after infection and continued for 48 hours which included itraconazole (5 mg/kg), atazanavir-ritonavir (90-30mg/kg), and itraconazole/atazanavir-ritonavir combination. Mice were then euthanized, and fungal count in kidneys were determined by plating onto YPD agar containing chloramphenicol (100 µg/ml). The itraconazole/atazanavir-ritonavir combination had a significantly lower fungal burden of *C. auris* in murine kidneys by 1.15-$\log_{10}$ (93%) and 1.3-$\log_{10}$ (95%) as compared to the vehicle control, and itraconazole alone, respectively (Fig. 3).

Our findings highlight that HIV protease inhibitors are a promising class of drugs that can be combined with azole antifungals to potentially treat fungal infections caused by challenging pathogens such as *C. auris*. It is noteworthy that patients with HIV are at higher risk for serious fungal infections, and protease inhibitors, such as atazanavir and ritonavir, are part of the routine therapy for them (33). Therefore, the combination therapy of azoles and HIV protease inhibitors could be the best fit for such scenarios (34).

In conclusion, our results show that atazanavir is a promising drug that enhances the activity of azole antifungals against *C. auris* both *in vitro* and *in vivo*.

Acknowledgments

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Conflict of interest

No conflict of interest.

Tables and Figures

Table 1. Effect of atazanavir (ATV) on the antifungal activity of fluconazole (FLC) and itraconazole (ITC) against *C. auris*

C. auris **Isolate**	**FLC/ATV combination**				**ITC/ATV combination**			
	MIC (µg/ml)		**ΣFICI**	**Mode**	**MIC (µg/ml)**		**ΣFICI**	**Mode**
	Alone	**Combined**			**Alone**	**Combined**		
CBS 10913	0.5/>128	0.5/1	1.00	IND	0.25/>128	0.0015/32	0.13	SYN
CBS 12372	64/>128	32/16	0.56	IND	2/>128	0.5/32	0.38	SYN
CBS 12373	128/>128	32/16	0.31	SYN	1/>128	0.25/32	0.38	SYN
CBS 12766	128/>128	64/8	0.53	IND	2/>128	0.5/8	0.28	SYN
CBS 12768	>128/>128	128/8	0.53	IND	2/>128	0.5/16	0.31	SYN
CBS 12770	>128/>128	128/8	0.53	IND	2/>128	0.5/4	0.27	SYN
CBS 12771	>128/>128	128/16	0.56	IND	2/>128	0.25/16	0.19	SYN
CBS 12772	>128/>128	128/8	0.53	IND	2/>128	0.25/16	0.19	SYN
CBS 12773	>128/>128	128/2	0.51	IND	2/>128	0.5/16	0.31	SYN
CBS 12774	>128/>128	64/16	0.31	SYN	2/>128	0.5/16	0.31	SYN
AR0931	>128/>128	>128/>128	2.00	IND	1/>128	0.25/16	0.31	SYN
AR0390	>128/>32	64/16	0.31	SYN	1/>128	0.125/16	0.19	SYN
AR1097	32/>128	8/32	0.38	SYN	2/>128	0.5/16	0.31	SYN
AR1099	2/>128	1/32	0.63	IND	0.5/>128	0.125/16	0.31	SYN
NR-52713	64/>128	16/32	0.38	SYN	1/>128	0.125/16	0.19	SYN
NR-52714	64/>128	16/32	0.38	SYN	1/>128	0.125/16	0.19	SYN
NR-52715	1/>128	0.5/4	0.52	IND	0.25/>128	0.06/32	0.37	SYN
NR-52716	8/>128	8/>128	2.00	IND	1/>128	0.25/4	0.27	SYN
NR-52717	>128/>128	32/16	0.19	SYN	2/>128	0.06/16	0.09	SYN

SYN: synergy, IND: indifference

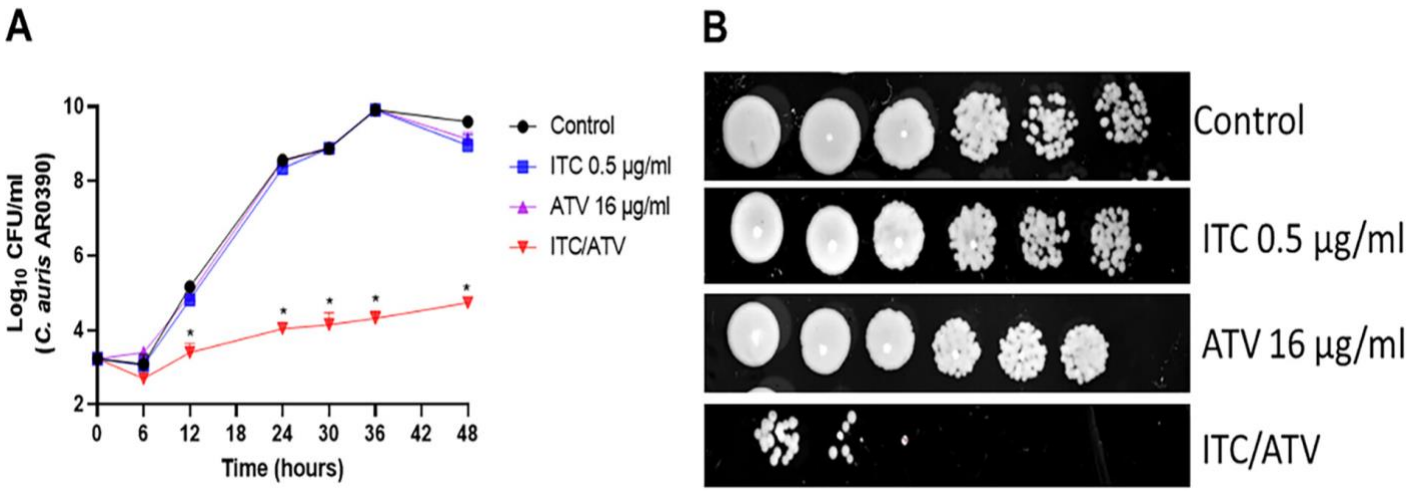

Figure 1. Time-kill curve of itraconazole (ITC) at 0.5 µg/ml, atazanavir (ATV) at 16 µg/ml, or a combination of both (ITC/ATV). A) The drugs were assessed against *C. auris* AR0390 for 48 hours at 35°C. B) Scanning images of *C. auris* AR0390 from the 24-hour time point plated onto YPD agar plates and incubated for 24 hours.

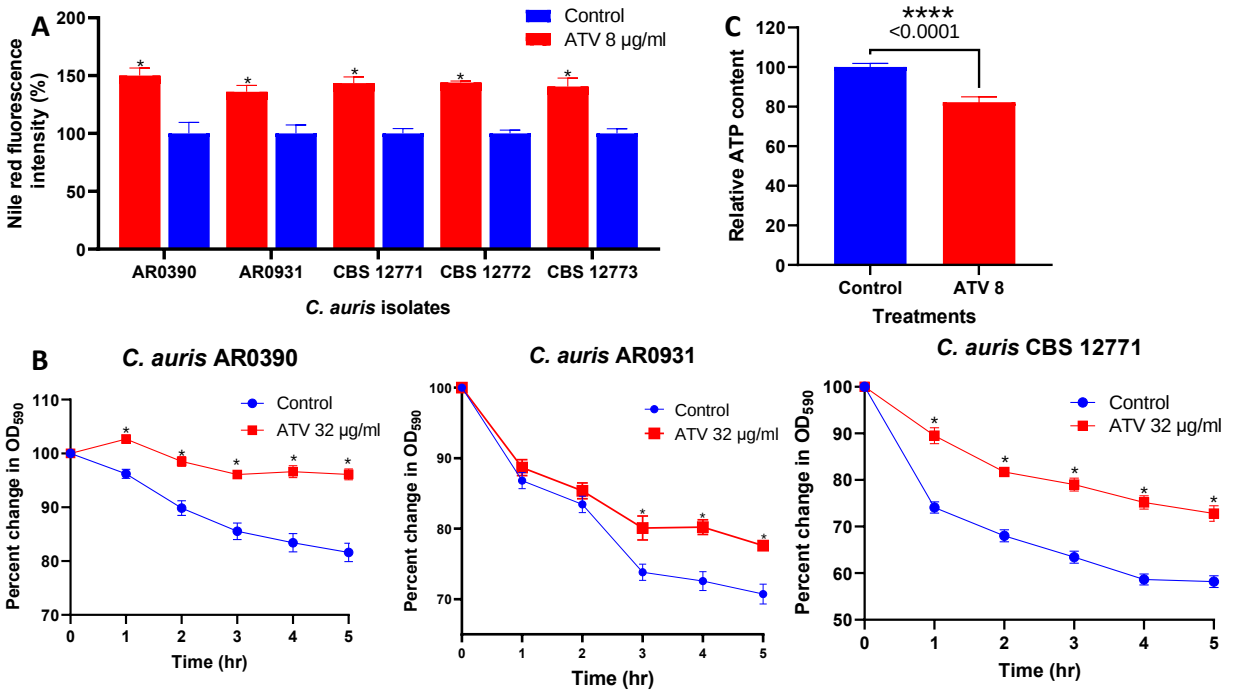

Figure 2. Effect of atazanavir (ATV) on Nile red efflux, glucose utilization, and ATP content against *C. auris* isolates. A) Effect of ATV on efflux of Nile red from five *C. auris* clinical isolates. Asterisks (*) denote a statistical difference (P<0.05) between the untreated and ATV-treated *C. auris* cells in the intensity of Nile red florescence. B) Effect of ATV on glucose utilization of *C. auris*. Asterisks (*) represent a statistical difference (P<0.0001) in percent change in OD_{590} between ATV-treated cells and the untreated control as determined by the unpaired *t* test. C) Effect of ATV against *C. auris* ATP content. Asterisks (*) represent a statistical difference (P<0.0001) in ATP levels between ATV-treated and untreated cells as determined by the unpaired *t* test.

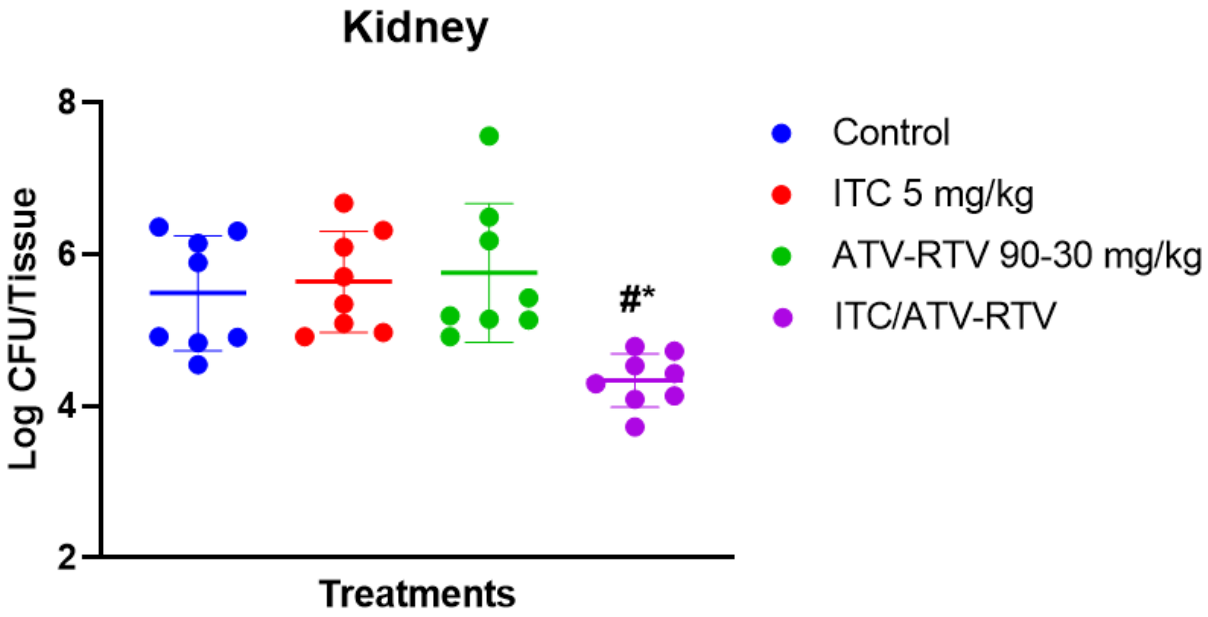

Figure 3. *In vivo* efficacy of itraconazole/atazanavir-ritonavir combination in a murine model of *C. auris* disseminated infection. Female CD-1 mice (n = 8) were infected with azole-resistant *C. auris* AR0390 and then treated with either vehicle control, itraconazole (ITC) (5 mg/kg), atazanavir/ritonavir (ATV-RTV) (90-30 mg/kg), or ITC/ATV-RTV (5/90-30 mg/kg). Statistical difference was measured via One-way analysis of variance (ANOVA) with post-hoc Dunnett's test for multiple comparisons. Asterisk (*) denotes a statistical significance of the combination treatment ($P < 0.01$) as compared to the untreated control. A pound (#) denotes a statistical significance ($P < 0.01$) in comparison to the ITC-treated group.

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