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Repurposing of Ribavirin as an Adjunct Therapy against Invasive *Candida* Strains in an *In Vitro* Study

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ABSTRACT The use of antifungal agents in clinical settings is limited by the appearance of drug resistance and adverse side effects. There is, therefore, an urgent need to develop new drugs to strengthen the treatment of invasive fungal diseases. The aim of this study is to describe the potential repurposing of ribavirin as an adjunct therapy against *Candida* spp. Primary screening of a Prestwick Chemical library against *Candida albicans* ATCC 90028 and fluconazole-resistant *Candida albicans* strains was performed. Subsequently, we evaluated the responses of 100 *Candida* sp. strains to ribavirin, an antiviral agent, using the broth microdilution method as recommended by CLSI. We checked the involvement of efflux pump activity in the development of ribavirin resistance. We studied time-kill curves and performed a checkerboard assay for a ribavirin-antifungal combination study. Twenty-one non-standard antifungal compounds were identified, including ribavirin. Ribavirin had antifungal activity *in vitro* against 63 *Candida* strains, including strains of *C. albicans*, *C. parapsilosis*, and *C. tropicalis*, with MICs ranging from 0.37 to 3.02 $\mu\text{g/ml}$, while MICs for *C. krusei*, *C. glabrata*, *C. lusitanae*, and some *C. albicans* strains remained high ($\geq 24.16 \mu\text{g/ml}$). No relation was observed between efflux pump activity and ribavirin resistance. Ribavirin exhibited fungistatic activity against multidrug-resistant (MDR) *C. albicans* and fungicidal activity against a *C. parapsilosis* strain. In addition, ribavirin acted synergistically with azoles against *Candida* strains for which ribavirin MICs were $< 24.4 \mu\text{g/ml}$. This study highlights the potential clinical application of ribavirin, alone or in association with other antifungal agents, as an adjunct anti-*Candida* drug.

KEYWORDS *Candida* species, antifungals, *in vitro* activity, ribavirin

Fungi are considered to be among the main causes of human infections, particularly for immunocompromised individuals and hospitalized patients with serious immunosuppressive conditions, such as HIV and organ transplantation (1). Infections due to *Candida* spp. are among the most common invasive fungal diseases (2). In a recent study, *Candida* spp. were identified as the most frequent cause of bloodstream infection in hospitalized patients (3). Only three classes of antifungal agents are currently used to treat invasive *Candida* species infections: azoles, polyenes, and echinocandins (4). Although these classes of antifungal agents are usually active against many fungal infections, their applicability and efficacy might be compromised due to their high toxicity, their narrow spectrum of activity, and the development of drug resistance (5). Hence, there is a constant need for other compounds that possess antifungal properties. An interesting approach in this field is drug repurposing, whereby FDA-approved drugs may be tested and used in another therapeutic class (6). Applying this approach by screening 1,920 compounds belonging to three FDA-approved drug libraries against *Candida albicans* strains, Tournu et al. recently identified ribavirin, a purine nucleoside

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TABLE 1 Hits obtained by the primary screening of 1,280 FDA-approved drugs of the Prestwick Chemical library at 10 μ M against both *C. albicans* ATCC 90028 and fluconazole-resistant *C. albicans* strain Q181103513

Drug	Therapeutic class	Fungal growth inhibition (%)	Reference
Auranofin	Analgesic	99	25
Disulfiram	Antabuse effect	98	26
Pinaverium bromide	Antispastic	98	Unknown
Avermectin B1	Anthelmintic	98	Unknown
Pyrvinium pamoate	Anthelmintic	97	27
Triclabendazole	Anthelmintic	96	Unknown
Pentamidine isethionate	Antiprotozoal	96	28
Tetraethylenepentamine pentahydrochloride	Antilipemic	94	Unknown
Ribavirin	Antiviral	96	7
Pentetic acid	Chelating/radioprotectant	98	28
Thioguanine	Antineoplastic	96	Unknown
Anthralin	Antipsoriatic	96	Unknown
Thonzonium bromide	Antiseptic	98	29
Clioquinol	Antiamebic/antiseptic	98	7
Clotrimazole	Antibacterial	98	30
Chloroxine	Antibacterial	98	31
Chlorhexidine	Antibacterial/antiseptic	98	31
Dequalinium dichloride	Antibacterial/antiseptic	98	32
Methylbenzethonium chloride	Antibacterial	98	33
Benzethonium chloride	Antibacterial/antiseptic	98	33
Ciclopirox ethanolamine	Antibacterial	97	34

analog, as a potential *C. albicans* vacuole-disrupting agent (7). Ribavirin is a guanosine analog that produces broad-spectrum activity against many RNA and DNA viruses. However, it is used mainly to treat hepatitis C virus (HCV) in combination with alpha interferon (8). In this study, we investigated the *in vitro* activities of ribavirin against different clinical *Candida* species, including emerging fluconazole-resistant *Candida albicans* strains. We also evaluated the synergy between ribavirin and common antifungal agents.

RESULTS AND DISCUSSION

By testing of an FDA-approved library of 1,280 drugs against *C. albicans* ATCC 90028 (quality control) and a *C. albicans* strain (Q181103513) that has high resistance to fluconazole and all echinocandin antifungal agents, 21 nonstandard antifungal hits showed fungal growth inhibition greater than 90%. These primary compounds include 7 antibacterial drugs, 3 anthelmintic agents, and 11 compounds belonging to different therapeutic classes (Table 1).

The principal aim of our study is to identify new molecules whose antifungal action is not known and/or well investigated and which can be used in clinical practice without any constraints for patients. Among the 21 hits obtained, 14 have already been identified and well studied in previous work (Table 1), confirming the validity and reproducibility of our results and our screening technique. However, to our knowledge, the fungal growth inhibition induced by six of the remaining seven compounds—including pinaverium bromide, avermectin B1, triclabendazole, tetraethylenepentamine pentahydrochloride, thioguanine, and anthralin—has never been reported previously, and that induced by ribavirin has not been well studied (Table 1).

Among the aforementioned antifungal hits, we focused our study on the drug ribavirin for several reasons. (i) It presents a 96% reduction in fungal growth. (ii) It has been shown to disrupt vacuolar function in the pathogen *C. albicans* (7); however, no report describes its antifungal efficacy directly (i.e., MICs, fungicidal or fungistatic activity, synergy with antifungals, etc.). (iii) Most of these seven hits have been designed for a specific noninfectious indication (antispastic, antilipemic, antineoplastic, antipsoriatic) (Table 1), whereas ribavirin was approved to treat infectious disease of the liver (9), so its repurposing as an antifungal agent could be admissible. (iv) Reversible

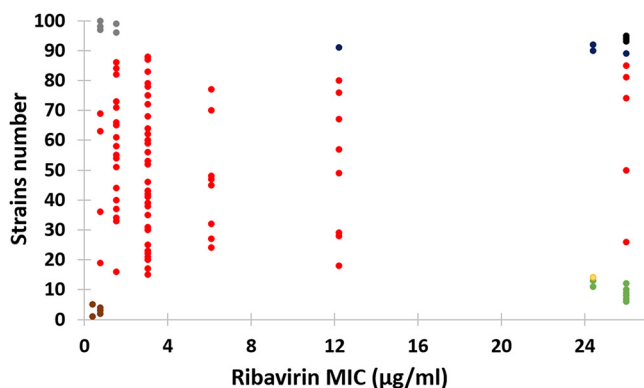


FIG 1 Distribution of ribavirin MICs for all clinical *Candida* sp. strains tested in this study. Data are color coded by strain as follows: brown, *C. parapsilosis*; green, *C. glabrata*; yellow, *C. auris*; red, *C. albicans*; blue, *C. krusei*; black, *C. lusitanae*; gray, *C. tropicalis*.

hemolytic anemia is the unique adverse effect observed with ribavirin treatment, in contrast to the cytotoxic effects that other molecules, such as thioguanine and avermectin B1, may have on human cells due to their nonselective activities (10, 11). (v) Ribavirin can be administered orally (as a capsule or tablet), intravenously, or through inhalation (9), while only topical application is possible for some molecules, such as anthralin (12).

That is why, in this study, a large panel of 100 *Candida* sp. strains was tested for susceptibility to ribavirin, and ribavirin MICs were determined. Consequently, we report here the efficacy of this compound against a collection of 60 *Candida* sp. strains, including 6 *Candida parapsilosis*, 5 *C. tropicalis*, and 49 *C. albicans* strains, with MICs ranging from 1.56 to 12.5 μM (i.e., 0.37 to 3.02 $\mu\text{g}/\text{ml}$). Among these 60 clinical strains tested, *C. parapsilosis* and *C. tropicalis* strains showed the greatest susceptibility to ribavirin; MICs were mainly $\leq 3.02 \mu\text{M}$ (i.e., $\leq 0.75 \mu\text{g}/\text{ml}$). Interestingly, ribavirin was effective against the clinical strain that was resistant to both fluconazole and echinocandin agents (*C. albicans* Q181103513), with a low MIC of 6.25 μM (i.e., 1.51 $\mu\text{g}/\text{ml}$). On the other hand, the ribavirin MICs obtained for *C. krusei*, *C. lusitanae*, *C. auris*, and *C. glabrata* were mainly $\geq 100 \mu\text{M}$ (i.e., $\geq 24.16 \mu\text{g}/\text{ml}$) (Fig. 1).

In order to study the fungicidal and fungistatic activities of the ribavirin compound, time-kill curves were performed with *C. albicans* and *C. parapsilosis*. Ribavirin exhibited fungistatic activity against the multidrug-resistant (MDR) *C. albicans* strain tested and *C. albicans* ATCC 90028. At ribavirin concentrations equal to $0.5 \times \text{MIC}$, no inhibitory effect was observed, and the curve was nearly identical to those for the control. At concentrations of $\geq 1 \times \text{MIC}$, a concentration-independent fungistatic effect was observed. In contrast, fungicidal activity of ribavirin against *C. parapsilosis* was noted, with a minimal fungicidal concentration (MFC) of ≥ 2 -fold the ribavirin MIC (MFC, 3.12 μM [i.e., 1.5 $\mu\text{g}/\text{ml}$]) (Fig. 2).

Ribavirin is on the WHO Model List of Essential Medicines as an effective and safe drug used in a health system. Ribavirin is administered orally at a dose of 1,200 mg (600 mg twice a day) over 4 weeks to achieve a stable concentration of 2.2 $\mu\text{g}/\text{ml}$ in plasma (9). A retrospective study performed by J. F. Jen et al. on patients infected with hepatitis C virus (HCV) showed that 49% of the sustained virological response was achieved at week 4 via plasma ribavirin concentrations ranging from 3.5 to 4 $\mu\text{g}/\text{ml}$. In addition, the authors achieved higher plasma ribavirin concentrations in the treatment of a patient infected with HCV genotype 1 (13). Moreover, a prospective open-label study of patients infected with HCV genotype 1 demonstrated that an increase in the ribavirin treatment dose (a maximum daily dose of 3,600 mg) resulted in plasma ribavirin concentrations of 5.37 $\mu\text{g}/\text{ml}$. In this case, HCV RNA was rendered undetectable in 9/10 patients during the fourth week, but this result was associated with more-frequent side effects, such as anemia (14). Thus, concentrations in plasma equal

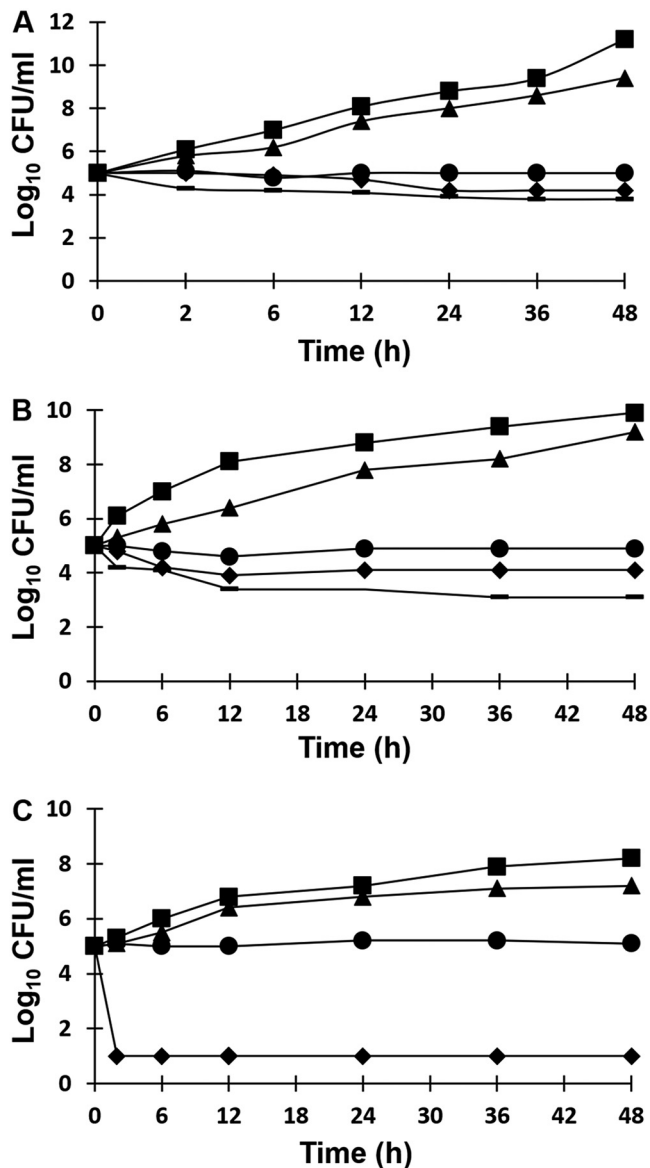


FIG 2 CFU counts (expressed as \log_{10} CFU per milliliter) versus time for *Candida* strains tested against ribavirin at the following concentrations: control (squares), $0.5 \times$ MIC (triangles), $1 \times$ MIC (circles), $2 \times$ MIC (diamonds), $4 \times$ MIC (heavy lines). (A) *C. albicans* Q181103513; (B) *C. albicans* ATCC 90028; (C) *C. parapsilosis* Q181208447.

to the MICs obtained from most of the *Candida* sp. strains tested in this study can be easily achieved. The main side effect of ribavirin treatment is reversible hemolytic anemia, which may be observed in patients with chronic hepatitis C (15). The use of this drug for short-term therapy, along with the use of hematopoietic growth factors, can manage the risk of severe anemia by maintaining an effective concentration.

Nonetheless, some *Candida* strains were resistant to ribavirin, including some *C. albicans* strains and all the *C. glabrata*, *C. krusei*, and *C. lusitanae* strains tested. Among the many drug resistance mechanisms that have been described in yeasts, one specific or nonspecific mechanism is the overexpression of membrane-associated carriers acting as drug efflux pumps (16). Thus, we supposed that reduced permeability of the membrane to the ribavirin drug and the potential role of efflux pumps in the extrusion of the ribavirin molecule may be the mechanisms that explain ribavirin resistance. That is why we checked the efflux pump activity using the nonspecific efflux pump inhibitor (EPI) carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and the specific calcium channel blocker verapamil for

ribavirin-resistant strains. The activity of ribavirin did not change with the addition of CCCP or verapamil. No changes were reported in the ribavirin MICs for any of the strains tested after EPI treatment, which could indicate the potentially normal transport of ribavirin in the isolates tested, although we did not quantify the intracellular level of ribavirin. This result leads us to the possibility of the existence of a specific pathway that could induce the development of ribavirin resistance in yeasts.

The mutation frequencies of *C. albicans* ATCC 90028 on RPMI medium containing 1.52, 3.04, and 6.08 $\mu\text{g/ml}$ of ribavirin were 1.5×10^{-4} , 1.1×10^{-4} , and 9.2×10^{-4} , respectively. The mutation frequencies of *C. albicans* Q181103513 on RPMI agar plates with 3.04, 6.08, and 12.16 $\mu\text{g/ml}$ of ribavirin were 1.6×10^{-4} , 1.1×10^{-4} , and 9.8×10^{-4} , respectively. Thus, the overall mutation frequency was in the range of 10^{-3} to 10^{-4} per strain, relatively higher than that reported for classical antifungals (17). This result indicates that it is preferable to use ribavirin for a short time or in association with other antimicrobial drugs. Therefore, in addition to the discovery of new agents effective against fungi, a pragmatic approach would be to improve the activities of old antifungal agents by using a combination of drugs. Thus, testing ribavirin-antifungal combinations *in vitro* could be very useful for increasing the antimicrobial spectra of the usual antifungals used and for minimizing the development of drug resistance and side effects by reducing the drug concentration.

Investigations for potential *in vitro* synergy between ribavirin and antifungal agents were carried out with *Candida* strains resistant to an azole (fluconazole [6 strains], itraconazole [9 strains], posaconazole [19 strains], and/or flucytosine [11 strains]) (Table 2). The combination of ribavirin and fluconazole was evaluated against six fluconazole-resistant *Candida* sp. strains (five *C. glabrata* strains and one MDR *C. albicans* strain) (Table 2). Interestingly, significant synergism (fractional inhibitory concentration index [FICI], 0.12) was demonstrated against the MDR *C. albicans* strain (Q181103513), since this combination lowered the MICs of fluconazole and ribavirin from 256 $\mu\text{g/ml}$ and 1.5 $\mu\text{g/ml}$ to 1 $\mu\text{g/ml}$ and 0.18 $\mu\text{g/ml}$, respectively. However, this combination (i.e., ribavirin with fluconazole) was synergistic for only one *C. glabrata* strain and was indifferent for the remaining four *C. glabrata* strains tested. It is noteworthy that the latter strains had initially high ribavirin MICs ($\geq 24.4 \mu\text{g/ml}$) (Table 2). When ribavirin was combined with itraconazole and tested against nine itraconazole-resistant strains, synergistic activities were observed for the two *C. albicans* strains tested, while indifferent activities were noted for most *C. glabrata* strains (5/7) analyzed in this study (Table 2). Furthermore, in ribavirin-posaconazole combinations, the highest rates of synergy were observed for *C. albicans* (7/9 [78%]) and *C. tropicalis* (2/2 [100%]) strains (Table 2) but not for *C. glabrata* (1/7) and *C. krusei* (0/1) strains. It is worth mentioning again that the ribavirin-azole association was synergistic mainly against *Candida* strains for which ribavirin MICs were $< 24.4 \mu\text{g/ml}$. Synergy was not seen with ribavirin in combination with flucytosine against any of the *Candida* sp. strains tested in this study (11 strains) (Table 2). This could be explained by the fact that both molecules (i.e., ribavirin and flucytosine) belong to the same therapeutic class of nucleoside analogs, which can disturb the DNA synthesis of a given microorganism. Finally, none of the combinations tested was found to be antagonistic against *Candida* strains.

The mechanisms of action of ribavirin on HCV have been correctly elucidated, and four pathways have been described: (i) inhibition of HCV replication (RNA polymerase), (ii) inhibition of the IMP dehydrogenase (IMPDH) enzyme, (iii) induction of RNA mutagenesis, and (iv) adaptation of the immune response to HCV (9). In contrast, the mechanism of ribavirin action on *Candida* species remains unclear. Thus, the most important aim of further studies will be the characterization of the mode of action of ribavirin against *Candida* species.

Our findings demonstrate that ribavirin exhibits antifungal activities against *Candida* species, especially *C. albicans*, *C. parapsilosis*, and *C. tropicalis*. Its efficacy against MDR *C. albicans* could offer a promising alternative strategy for the treatment of invasive fungal infections. Nonetheless, the ribavirin MICs for some *Candida* strains (especially strains of *C. glabrata*, *C. krusei*, and *C. lusitanae*) remain higher than the concentration

TABLE 2 MICs and fractional inhibitory concentration indexes for the combinations of ribavirin with antifungals^a

Candida species and strain	MIC					FICI			
	FLU	ITRA	POSA	5-FC	RBV	RBV-FLU	RBV-ITRA	RBV-POSA	RBV-5-FC
<i>C. glabrata</i>									
Q181201614	8	8	1	<0.06	>24.4	ND	0.7 (I)	ND	ND
Q181198565	64	1	8	<0.06	>24.4	0.7 (I)	1 (I)	0.7 (I)	ND
Q181203672	128	1	>8	<0.06	>24.4	0.6 (I)	0.6 (I)	0.4 (S)	ND
Q181202903	16	4	8	0.06	>24.4	ND	0.7 (I)	1 (I)	ND
Q181255715	64	16	4	<0.06	>24.4	0.6 (I)	0.6 (I)	0.8 (I)	ND
Q181280604	4	0.25	0.5	<0.06	24.4	ND	ND	0.7 (I)	ND
8070855959	128	16	8	<0.06	>24.4	0.6 (I)	0.1 (S)	2 (I)	ND
Q181208658	128	16	8	<0.06	24.4	0.5 (S)	0.5 (S)	1 (I)	ND
<i>C. albicans</i>									
Q181103513	256	0.25	0.25	<0.06	1.52	0.12 (S)	ND	0.1 (S)	ND
Q181198103	4	0.25	1	<0.06	3.05	ND	ND	0.2 (S)	ND
Q181201380	0.5	0.06	0.03	>64	1.52	ND	ND	ND	1.5 (I)
Q181203339	1	0.06	0.12	<0.06	>24.4	ND	ND	0.7 (I)	ND
490890568	1	0.5	0.25	0.5	3.05	ND	ND	0.4 (S)	ND
Q181264539	0.25	0.12	0.06	>64	0.76	ND	ND	ND	1.2 (I)
Q181252599	1	0.12	0.12	0.12	6.1	ND	ND	0.3 (S)	ND
Q181253570	<0.012	0.03	0.15	0.12	>24.4	ND	ND	0.5 (S)	ND
Q181211559	2	1	0.5	1	12.2	ND	0.4 (S)	0.6 (I)	ND
Q181226987	0.25	0.06	0.03	64	6.1	ND	ND	ND	2 (I)
Q181260978	2	1	0.5	0.5	12.2	ND	0.5 (S)	0.5 (S)	ND
Q181108884	16	0.5	0.5	1	1.52	ND	ND	0.3 (S)	ND
<i>C. krusei</i>									
ATCC 6258	32	0.12	0.25	8	>24.4	ND	ND	0.6 (I)	ND
Q181262141	32	0.12	0.12	4	24.4	ND	ND	ND	0.8 (I)
Q181263662	16	0.12	0.12	2	12.2	ND	ND	ND	0.6 (I)
Q181208438	32	0.25	0.25	8	24.4	ND	ND	ND	0.6 (I)
<i>C. lusitanae</i> Q181206420	0.5	0.12	0.03	32	>24.4	ND	ND	ND	0.7 (I)
<i>C. tropicalis</i>									
Q181257439	1	0.12	0.12	64	0.76	ND	ND	0.4 (S)	0.7 (I)
Q181250041	1	0.12	0.06	64	1.52	ND	ND	ND	1 (I)
8070845333	2	0.12	0.12	>64	1.52	ND	ND	0.4 (S)	1.1 (I)
Q181203338	1	0.12	0.06	64	0.76	ND	ND	ND	1 (I)

^aAbbreviations: FLU, fluconazole; ITRA, itraconazole; POSA, posaconazole; 5-FC, flucytosine; RBV, ribavirin; S, synergy; I, indifference; ND, not determined.

achievable in human plasma. This phenomenon of high MICs is not associated with the overexpression of efflux pump activity. Finally, ribavirin has synergistic activities with fluconazole, itraconazole, or posaconazole, but not with flucytosine, particularly against *Candida* strains for which ribavirin MICs are <24.4 µg/ml. Consequently, the *in vivo* activities of ribavirin need further investigation in order to introduce ribavirin as an antifungal agent, alone or in association with other antifungals, in clinical mycology practice.

MATERIALS AND METHODS

Screening of 1,280 FDA-approved drugs against *C. albicans*. Using the concept of drug repurposing, an initial screen of an FDA-approved library of 1,280 drugs (Prestwick, Illkirch-Graffenstaden, France) (18) at a fixed concentration of 10 µM was performed against *Candida albicans* ATCC 90028 (quality control) and a clinical *Candida albicans* strain that is resistant to fluconazole and all echinocandin agents. This concentration was chosen according to previous screening studies in order to select "hits" that present antifungal activities at low concentrations (thus avoiding the toxicity and adverse effects of drugs tested at high concentrations). The fungal inoculum was prepared using RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) according to the Clinical and Laboratory Standards Institute protocol (19). Sixteen 96-well plates were used. Each plate contained 80 compounds; the first column served for the positive control with untreated fungi, and the last column contained the medium as a negative control. The plates were incubated for 24 h at 37°C. Then the optical density (OD) was determined using a spectrophotometer, and the percentages of fungal growth inhibition were calculated in relation to the growth in the untreated wells.

Fungal strains. A collection of 96 *Candida* sp. strains was tested, including 72 *C. albicans*, 5 *Candida parapsilosis*, 8 *Candida glabrata*, 5 *Candida tropicalis*, 3 *Candida krusei*, and 3 *Candida lusitanae* strains with different antifungal susceptibilities (see Table S1 in the supplemental material). The species of all

isolates were identified using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (20). All strains were recovered from La Timone University Hospital in Marseille, France, and were isolated from different clinical samples, mainly from blood cultures, urine, and vaginal swabs (Table S1). *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. auris* DSMZ 21092, and *C. krusei* ATCC 6258 were used as quality controls (Table S1).

Antifungal susceptibility testing. Antifungal susceptibility testing was performed using commercial broth microdilution plates (Sensititre YeastOne; Thermo Fisher Scientific, Schwerte, Germany) containing nine antifungals belonging to the four therapeutic classes: polyenes (amphotericin B), azoles (fluconazole, posaconazole, voriconazole, and itraconazole), echinocandins (anidulafungin, caspofungin, and micafungin), and nucleoside analogs (5-flucytosine). The MICs obtained for the *Candida* sp. strains tested (Table S1 in the supplemental material) were compared to the breakpoints provided by the manufacturers, or to the CLSI cutoffs (19), so as to assess the susceptibilities of each strain to the different antifungal agents tested.

Ribavirin susceptibility testing. Using the broth microdilution method as outlined by the CLSI (19), ribavirin MICs were determined. First, ribavirin powder (Sigma-Aldrich, St. Louis, MO) was dissolved in water to obtain a stock solution at a concentration of 10 mg/ml. Then the ribavirin stock solution was diluted in RPMI 1640 broth medium (Sigma-Aldrich, St. Louis, MO) to yield a concentration of 200 μ M (i.e., 48.32 μ g/ml), 2-fold more concentrated than the starting concentration. Fungal suspensions at a 0.5 McFarland standard were prepared, and working yeast suspensions were obtained by a 1:100 dilution followed by a 1:20 dilution of the initial suspension in RPMI broth medium. Further, serial ribavirin dilutions (2-fold dilutions) ranging from 100 to 0.2 μ M (i.e., 24.16 to 0.047 μ g/ml) were tested by inoculating a well containing 100 μ l of ribavirin at the appropriate concentration with 100 μ l of the final fungal suspension. The ribavirin concentration achievable in human plasma without toxic effects (\approx 6 μ g/ml) was considered in order to establish the concentration ranges used in the 2-fold dilution testing. The MIC of ribavirin was defined as the concentration that resulted in complete growth inhibition relative to the growth in untreated control wells. This MIC was later determined visually after 24 h of incubation at 37°C by using the OD obtained by spectrophotometric measurement.

Time-kill assay. In order to assess the fungicidal or fungistatic activity of the ribavirin compound against three fungal strains (2 *C. albicans* strains and 1 *C. parapsilosis* strain), ribavirin time-kill analysis was performed as described previously (21); *C. albicans* ATCC 90028 was used as the quality control. The action of ribavirin was investigated at four different concentrations (0.5 \times MIC, 1 \times MIC, 2 \times MIC, 4 \times MIC), and fungal growth was determined by the CFU count (expressed as CFU per milliliter) after 2, 6, 12, 24, 36, or 48 h of incubation at 37°C. The results were analyzed using GraphPad Prism software (version 5.3; GraphPad Inc., San Diego, CA, USA).

Testing of efflux pump activity. The involvement of efflux pumps in the ribavirin resistance mechanism in some ribavirin-resistant *Candida* sp. strains (two *C. glabrata*, one *C. krusei*, one *C. lusitanae*, and one *C. albicans* strain) was verified using the inhibitors CCCP (carbonyl cyanide 3-chlorophenylhydrazone) and verapamil, which decrease the MICs when resistance is correlated to this mechanism (22). The CCCP and verapamil inhibitors were tested at two final concentrations (0.5 μ g/ml and 10 μ g/ml). To ensure that these concentrations did not affect yeast growth, control growth wells containing efflux pump inhibitors (EPIs) alone in RPMI medium were added. We determined ribavirin MICs for all strains before and after adding EPIs to the RPMI medium.

Mutation frequency. The frequencies of spontaneous mutations were determined on *C. albicans* ATCC 90028 and the multidrug-resistant (MDR) *C. albicans* strain Q181103513 by plating 100 μ l of the growing yeast at an appropriate dilution (10^3 CFU) on ribavirin-free plates and without dilution (10^5 CFU) on ribavirin-containing plates (17). RPMI 1640 agar plates with different ribavirin concentrations (1 \times MIC, 2 \times MIC, 4 \times MIC) were prepared as described previously (23). Spontaneous mutation frequencies were calculated, after 24 h of incubation at 37°C, by dividing the number of resistant colonies grown on a given plate by the initial inoculum pipetted. Mutant resistance phenotypes were confirmed by determining the ribavirin MICs for mutant colonies.

In vitro interaction studies of combinations of ribavirin with antifungal drugs. In order to exploit the potential for improved efficacy, reduced toxicity, and reduced risk of drug resistance development (24), ribavirin–antifungal agent combinations were tested against 29 *Candida* sp. strains, including 12 *C. albicans* strains, 8 *C. glabrata* strains, 4 *C. tropicalis* strains, 1 *C. lusitanae* strain, and 4 *C. krusei* strains. Based on the checkerboard association assay, combinations of ribavirin with each of the following antifungals were tested: fluconazole, itraconazole, posaconazole, and flucytosine (Sigma-Aldrich, St. Louis, MO). The effects of different combinations on different strains were analyzed using the fractional inhibitory concentration index (FICI), calculated as described previously (24), and interpreted as follows: an FICI of \leq 0.5 indicates synergy between the antimicrobials tested; an FICI of $>$ 0.5 but \leq 4 indicates indifference or additivity; and an FICI of $>$ 4 indicates antagonism between the antimicrobials tested.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00263-19>.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

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