



Reversal of Azole Resistance in *Candida albicans* by Sulfa Antibacterial Drugs

Hassan E. Eldesouky,^a Abdelrahman Mayhoub,^{b,c} Tony R. Hazbun,^{d,e} Mohamed N. Seleem^{a,f}

^aDepartment of Comparative Pathobiology, College of Veterinary Medicine, Purdue University, West Lafayette, Indiana, USA

^bDepartment of Pharmaceutical Organic Chemistry, College of Pharmacy, Al-Azhar University, Cairo, Egypt

^cBiomedical Sciences, University of Science and Technology, Zewail City of Science and Technology, Giza, Egypt

^dDepartment of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, Purdue University, West Lafayette, Indiana, USA

^eBindley Bioscience Center, Purdue University, West Lafayette, Indiana, USA

^fPurdue Institute for Inflammation, Immunology, and Infectious Disease, Purdue University, West Lafayette, Indiana, USA

ABSTRACT Invasive candidiasis presents an emerging global public health challenge due to the emergence of resistance to the frontline treatment options, such as fluconazole. Hence, the identification of other compounds capable of pairing with fluconazole and averting azole resistance would potentially prolong the clinical utility of this important group. In an effort to repurpose drugs in the field of antifungal drug discovery, we explored sulfa antibacterial drugs for the purpose of reversing azole resistance in *Candida*. In this study, we assembled and investigated a library of 21 sulfa antibacterial drugs for their ability to restore fluconazole sensitivity in *Candida albicans*. Surprisingly, the majority of assayed sulfa drugs (15 of 21) were found to exhibit synergistic relationships with fluconazole by checkerboard assay with fractional inhibitory concentration index (Σ FIC) values ranging from <0.0312 to 0.25. Remarkably, five sulfa drugs were able to reverse azole resistance in a clinically achievable range. The structure-activity relationships (SARs) of the amino benzene sulfonamide scaffold as antifungal agents were studied. We also identified the possible mechanism of the synergistic interaction of sulfa antibacterial drugs with azole antifungal drugs. Furthermore, the ability of sulfa antibacterial drugs to inhibit *Candida* biofilm by 40% *in vitro* was confirmed. In addition, the effects of sulfa-fluconazole combinations on *Candida* growth kinetics and efflux machinery were explored. Finally, using a *Caenorhabditis elegans* infection model, we demonstrated that the sulfa-fluconazole combination does possess potent antifungal activity *in vivo*, reducing *Candida* in infected worms by $\sim 50\%$ compared to the control.

KEYWORDS *Candida albicans*, antifungal, azole resistance, fluconazole resistance, sulfa antibacterial, biofilm, *Caenorhabditis elegans*

Invasive candidiasis remains the single most important cause of fungal bloodstream infections and the fourth leading cause of nosocomial bloodstream infections in the United States (1). The mortality rate of invasive candidiasis can reach more than 40%, even with the introduction of new antifungal therapies (2). Moreover, the development of new antifungal drugs is currently unable to keep pace with the urgent demand for safe and effective treatment options. Collectively, this points to an urgent need for different strategies to develop antifungal drugs to deal with this emerging scourge. The Infectious Diseases Society of America has adopted fluconazole as a primary

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Address correspondence to Mohamed N. Seleem, mseleem@purdue.edu.

drug of choice for controlling and treating invasive candidiasis (3). Fluconazole has several advantages over other antifungal drugs in terms of the cost, safety, oral bio-availability, and ability to cross the blood-brain barrier (4, 5). However, the extensive use of fluconazole has increased the incidence of resistance to the drug among different fungal strains, especially *Candida albicans* (6–8). Unfortunately, fluconazole resistance has the potential to cross over to other azole drugs, such as itraconazole and voriconazole (9).

Several studies (10–13) have reported the ability of some drugs and compounds to reverse azole resistance in *C. albicans*. Unfortunately, the concentrations required for the majority of these drugs to suppress the azole resistance are generally above their clinically achievable concentration. In addition, some of these drugs can result in serious side effects, such as those caused by tacrolimus and cyclosporine (14).

Interestingly, a few reports (15–18) have superficially outlined the ability of some sulfa antibacterial drugs to act synergistically with different antifungals. For example, sulfamethoxazole was found to exhibit synergistic activity with different azole antifungal drugs, such as miconazole, ketoconazole, and clotrimazole, against *C. albicans*. Also, sulfamethoxazole showed a synergistic interaction with caspofungin against different *Aspergillus* species, such as *Aspergillus fumigatus* and *Aspergillus niger*.

Sulfa antibacterial drugs were the first systemic antimicrobial agents to be discovered and have been used extensively for several decades in human and veterinary medicine to treat bacterial infections (19). Sulfa drugs exert their antibacterial action by inhibiting the folate pathway through a competitive antagonism to the dihydropteroate synthase enzyme (DHPS), which is required for the conversion of para-amino benzoic acid (PABA) into dihydrofolate (20). In fungi, sulfa drugs and dihydrofolate reductase (DHFR) inhibitors, such as methotrexate and pyrimethamine, have been reported to inhibit the folate pathway by the same mechanism, albeit very high concentrations are required to inhibit the growth (21). For instance, sulfamethoxazole and trimethoprim have been shown to have antimycotic activity against *A. fumigatus* through inhibition of the folate pathway (22). Furthermore, interruption of the folate pathway in *C. albicans* was found to inhibit ergosterol biosynthesis, which could explain the synergistic activity of folate inhibitors and azole antifungal agents (23, 24).

In an effort to repurpose drugs and explore new leads in the field of antifungal drug discovery, we explored sulfa antibacterial drugs for the purpose of reversing azole resistance in *Candida*. In this study, we investigated a library of 21 sulfa antibacterial drugs for their ability to restore fluconazole sensitivity in *C. albicans*.

RESULTS

Antifungal susceptibility testing and identification of azole-resistant *Candida* strains. The MICs of fluconazole, itraconazole, voriconazole, amphotericin B, and 5-fluorocytosine were examined against a panel of 53 *C. albicans* strains. We identified 10 strains as azole resistant (i.e., resistant to fluconazole, itraconazole, and voriconazole) (Table 1). These 10 azole-resistant strains were selected for further studies.

Assembly of the sulfa antibacterial drugs and testing of their antifungal activity. The library of 21 sulfa antibacterial drugs were initially screened at a single concentration of 1,024 $\mu\text{g/ml}$ against *C. albicans* strains to identify any possible antifungal activity. Eight sulfa antibacterials showed weak antifungal activity at the high concentration of 512 $\mu\text{g/ml}$ (Table 1). The rest of the drugs did not show any antifungal activity up to 1,024 $\mu\text{g/ml}$.

Sulfa drugs exert potent synergistic activity with fluconazole (checkerboard assay). We assessed the activity of sulfa drugs in combination with fluconazole against azole-resistant *Candida* strains using the checkerboard assay (25–28). As shown in Table 1, 15 sulfa drugs were found to exhibit synergistic relationships with fluconazole against 7 of the 10 *Candida* strains tested with fractional inhibitory concentration (ΣFIC) values ranging from <0.0312 to <0.25 . Sulfamethoxazole and sulfamonomethoxine were found to have the most potent synergistic activities. At $1/32 \times \text{MIC}$ for sulfa, we observed a >128 -fold reduction in the MIC for fluconazole (when combined with

TABLE 1 Synergistic activity of combinations of fluconazole and sulfonamide antibacterial drugs against different resistant strains of *Candida albicans* isolates

Sulfa drug	MIC ₅₀ (μg/ml) ^a				FIC index	Interpretation ^c
	FLC		Sulfa drug			
	Alone	In combination (MIC _c) ^b	Alone	In combination (MIC _c)		
4-Amino phenyl sulfone (dapson)	512	1	512	32	0.0625	SYN
Sulfadiazine	512	0.5	512	32	0.0625	SYN
Sulfamethazine	1,024	1	1024	128	0.125	SYN
Sulfamethoxazole	512	1	512	16	0.0312	SYN
Sulfanilamide	>1,024	>128	>1,024	>1,024	2	IND
Sulfacetamide	>1,024	>128	>1,024	>1,024	2	IND
Sulfabenzimide	>1,024	>128	>1,024	>1,024	2	IND
Sulfadoxine	512	1	512	64	0.125	SYN
Sulfantran	>1,024	>128	>1,024	>1,024	2	IND
Sulfamerazine	1,024	0.5	1,024	128	0.125	SYN
Sulfamonomethoxine	512	0.5	512	16	0.0312	SYN
Sulfadimethoxine	1,024	0.5	1,024	64	0.0625	SYN
Succinylsulfathiazole	>1,024	>128	>1,024	>1,024	2	IND
Sulfathiazole	1,024	0.5	1,024	128	0.125	SYN
Sulfaguanidine	>1,024	>128	>1,024	>1,024	2	IND
Sulfapyridine	512	1	512	64	0.125	SYN
Sulfametopyrazine (sulfalene)	1,024	0.5	1,024	64	0.0625	SYN
Sulfametoxydiazine (sulfameter)	512	0.5	512	64	0.125	SYN
Sulfisomidine	1,024	0.5	1,024	128	0.125	SYN
Sulfisoxazole (sulfafurazole)	1,024	1	1,024	128	0.125	SYN
Sulfamethoxyipyridazine	512	1	512	32	0.0625	SYN

^aMIC₅₀ is the lowest concentration of the drug that showed at least 80% reduction of the growth of 50% of the tested *Candida* strains.

^bMIC_c is the MIC of the drug when used in combination with fluconazole.

^cFIC index interpretations: ≤0.5, synergism (SYN); >0.5 to 4, indifferent (IND); >4, antagonism.

sulfamethoxazole or sulfamonomethoxine) for all seven clinical *Candida* strains in which synergy was detected (data not presented). Six drugs showed an additive effect (indifference) with an FIC index (FICI) of >0.5 to 4. Interestingly, five drugs, i.e., sulfamethoxazole (SMX), sulfadiazine (SDZ), sulfadoxine (SDX), sulfadimethoxine (SDM), and sulfamethoxyipyridazine (SMP), reversed azole resistance in a clinically achievable range (MIC when combined with fluconazole [MIC_c] values were below their achievable therapeutic concentrations in plasma).

Growth kinetics of sulfa antibacterial drugs. After confirming that the sulfa antibacterial drugs showed synergistic relationship with fluconazole against azole-resistant *Candida*, we next assessed the growth kinetics of *C. albicans* strain NR 29448 when exposed to the sulfa drugs that showed synergistic activity with fluconazole. As shown in Fig. 1, the combination of sulfamethoxazole (16 μg/ml) and fluconazole (1 μg/ml) significantly inhibited the growth of *C. albicans* NR 29448. However, neither

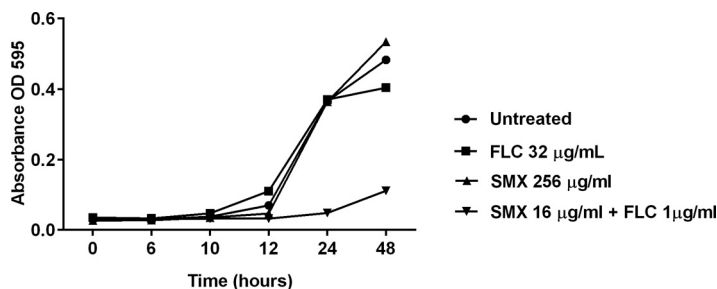


FIG 1 Growth kinetics of *C. albicans* (NR 29448) when exposed to sulfamethoxazole-fluconazole combination. An overnight culture of the yeast cells was diluted to 1×10^3 CFU/ml in RPMI 1640 medium. Cells were treated with 256 μg/ml sulfamethoxazole (SMX), 32 μg/ml fluconazole (FLC), or a combination of the two drugs at their MIC (16 μg/ml SMX and 1 μg/ml FLC). Cells were grown in the assay medium for 48 h, and OD₅₉₅ values were measured at different time points (0, 6, 10, 12, 24, and 48 h) and plotted against the values for control (untreated) cells.

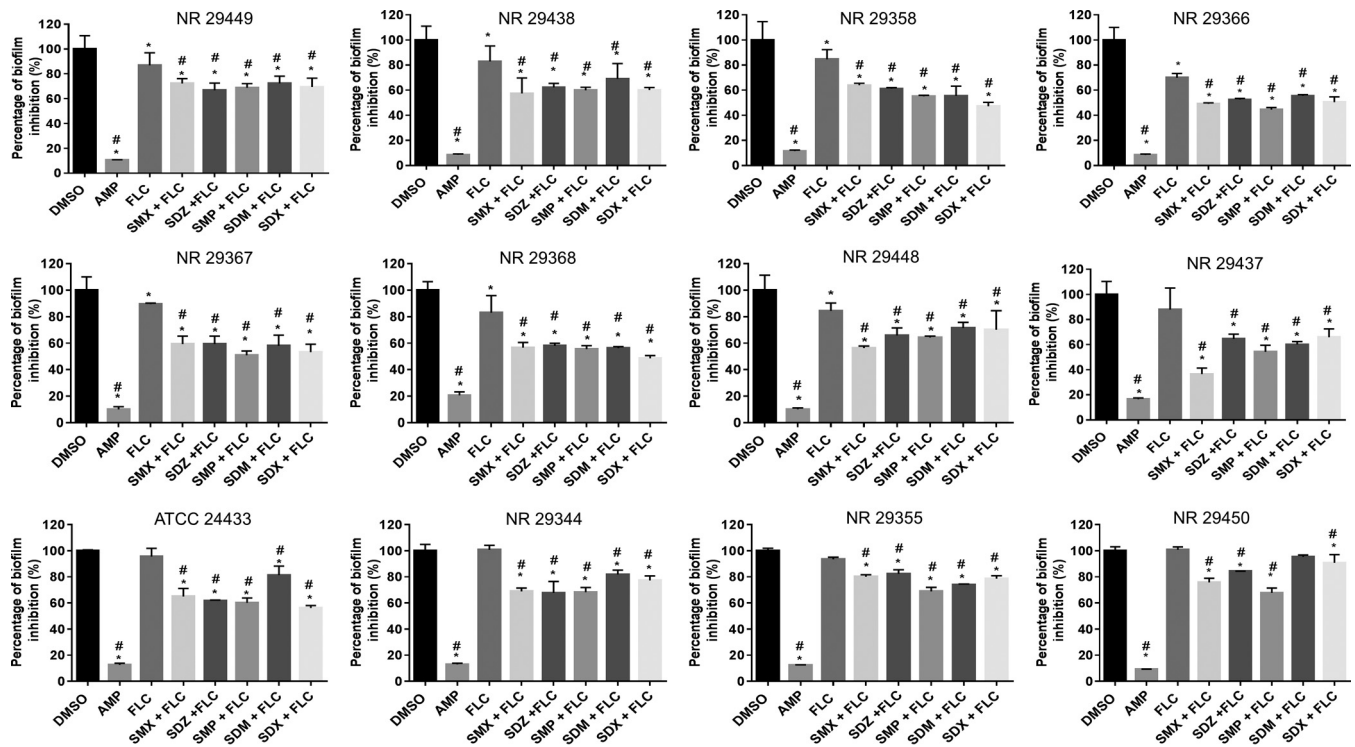


FIG 2 Effects of sulfa drug-fluconazole combinations on the biofilm-forming ability of 12 *C. albicans* clinical isolates. Fresh overnight cultures of *C. albicans* strains were back diluted 1:100 in RPMI 1640 medium, treated with different combinations of fluconazole (0.0625 $\mu\text{g/ml}$) and the indicated sulfa drugs (0.125 \times MIC), and then incubated for 24 h. Amphotericin B, fluconazole, and DMSO treatments served as controls. After 24 h, biofilms were stained by crystal violet and then destained with absolute ethanol. The absorbance of crystal violet at OD₅₉₅ was measured and compared to that of the controls. *, significant difference from untreated; #, significant difference from fluconazole.

sulfamethoxazole (256 $\mu\text{g/ml}$) nor fluconazole (32 $\mu\text{g/ml}$) was solely able to inhibit the fungal growth at such high concentrations. Similar growth kinetics were observed for all other active sulfa drugs (data not shown).

Sulfa drug-fluconazole combinations do inhibit biofilm formation. We examined whether the potential therapeutic application of sulfa drugs could be expanded beyond just inhibition of planktonic *Candida*. As presented in Fig. 2, sulfa drugs at subinhibitory concentrations (0.125 \times MIC) resulted in a significant reduction (\sim 40%) of the biofilm-forming ability of 12 *C. albicans* strains when combined with fluconazole (0.0625 $\mu\text{g/ml}$).

Sulfa drug-fluconazole combinations reduced the fungal burden of infected *Caenorhabditis elegans*. In order to validate the *in vitro* results, the temperature-sensitive sterile mutant *C. elegans* strain AU37, genotype [glp-4(bn2) I; sek-1(km4) X] was utilized as a whole-animal model to assess the ability of the sulfa drugs to reverse azole resistance in a living system. In comparison to fluconazole alone, sulfa drug-fluconazole combinations produced a significant reduction (\sim 50%) in the mean fungal burden (Fig. 3). This result was comparable to the effect of the antifungal drug 5-fluorocytosine.

Fungal efflux machinery is not inhibited by sulfa drugs. To test whether the sulfa drugs would have any effect on efflux of drugs in *Candida*, we assessed the effect of sulfa on rhodamine 123 (Rh123) efflux. Under our experimental conditions, sulfa drugs did not inhibit the efflux pump machinery expressed by *C. albicans* NR 29448 as indicated by intracellular accumulation of rhodamine dye (Fig. 4). On the other hand, cyclosporine significantly increased the accumulation of intracellular rhodamine dye, in accordance with previous reports (11, 29, 30).

PABA supplementation inhibits the synergistic activity of sulfa drugs. We asked whether the ability of sulfa antibacterial drugs to reverse azole resistance is due to their

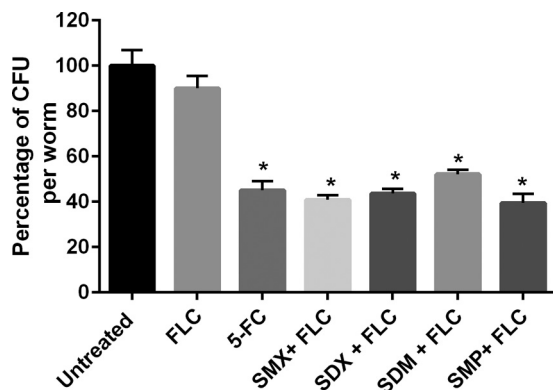


FIG 3 *In vivo* activity of sulfa drug-fluconazole combinations on *C. elegans* infected with *C. albicans* (NR 29448). Synchronized *C. elegans* worms, strain AU37 genotype [glp-4(bn2) I; sek-1(km4) X], were infected with *C. albicans* strain NR 29448 for 3 h and then treated with the indicated drugs. Sulfa drugs were used at 10× MICc in combination with fluconazole (10 µg/ml). 5-Fluorocytosine (5-FC) was used as a positive control. After 24 h of treatment, the worms were washed out and disrupted using silica carbide beads, and then the CFU/worm were counted on YPD plates containing streptomycin-penicillin.

ability to disrupt the folate pathway and the related ergosterol biosynthesis in *Candida* (23, 24). This was tested via supplementation of different concentrations of PABA and calculation of FIC indices. Supplementation of PABA restored *Candida* growth and reversed the inhibition caused by sulfa drug-fluconazole combinations in a concentration-dependent manner (Table 2). The FIC indices increased up to 64-fold with all sulfa drugs, losing their synergistic activity with fluconazole (except for sulfamonomethoxine).

DISCUSSION

Fifteen sulfa drugs were found to exhibit a synergistic relationship with fluconazole against azole-resistant *Candida* strains, with ΣFIC values ranging from <0.0312 to 0.25. Interestingly, of the 15 drugs with synergistic activity, 5 sulfa drugs (sulfamethoxazole, sulfadiazine, sulfadoxine, sulfadimethoxine, and sulfamethoxyypyridazine) showed great promise and were able to reverse azole resistance in *Candida in vitro* at a clinically applied concentration. The significance of this finding cannot be overstated because of its potential in clinical applications.

We were curious to determine if the synergistic relationship observed between sulfa drugs and fluconazole was limited to fluconazole only or if it could be applied to other

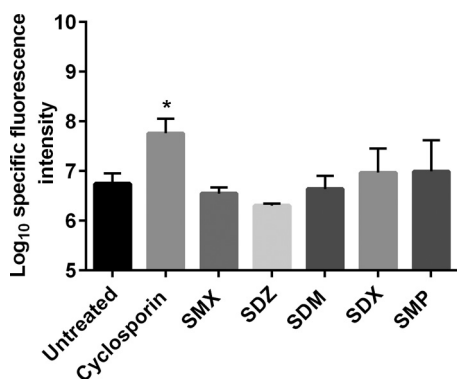


FIG 4 Effects of different sulfa antibacterial drugs on intracellular accumulation of rhodamine 123 by *C. albicans* (NR 29448). Deenergized pretreated *Candida* cells with subinhibitory concentrations of the indicated sulfa drugs (128 µg/ml) were loaded with 10 µM rhodamine for 30 min at 35°C. Then, the cells were washed thrice to remove extracellular dye. Pellets were resuspended in PBS, and fluorescence at excitation and emission wavelengths of 485 and 538 nm, respectively, was recorded. Cyclosporine was used as a positive control.

TABLE 2 Effects of PABA on the FIC indices of the sulfa drug-fluconazole combinations

Sulfa drug-fluconazole combination	FIC index under PABA concn ($\mu\text{g/ml}$) of:							
	0	1	4	8	16	32	64	128
4-Amino phenyl sulfone (dapsona)	0.0625	0.125	0.25	0.5	0.5	2	2	>2
Sulfadiazine	0.0625	0.125	0.5	0.5	0.5	>2	>2	>2
Sulfamethazine	0.125	0.5	1	1	2	2	>2	>2
Sulfamethoxazole	0.0312	0.0312	0.0625	0.0625	0.125	0.25	2	2
Sulfadoxine	0.125	0.125	0.25	0.25	0.5	2	2	>2
Sulfamerazine	0.125	0.125	0.25	0.25	0.5	2	>2	>2
Sulfamonomethoxine	0.0312	0.0625	0.125	0.125	0.125	0.25	0.5	0.5
Sulfadimethoxine	0.0625	0.125	0.25	0.25	0.25	>2	>2	>2
Sulfathiazole	0.125	0.25	0.5	0.5	0.5	2	>2	>2
Sulfapyridine	0.25	0.5	2	2	2	2	2	>2
Sulfametopyrazine (sulfalene)	0.0625	0.0625	0.125	0.125	0.125	0.5	0.5	2
Sulfametoxydiazine (sulfameter)	0.125	0.125	0.25	0.25	0.5	2	>2	>2
Sulfisomidine	0.125	0.5	2	2	2	>2	>2	>2
Sulfisoxazole (sulfafurazole)	0.125	0.25	0.25	0.5	0.5	2	>2	>2
Sulfamethoxy pyridazine	0.0625	0.125	0.25	0.25	0.5	2	>2	>2

azole antifungals drugs too, such as itraconazole and voriconazole. Itraconazole and voriconazole were used to further explore the partnership between sulfa drugs and azole antifungal agents. As expected, sulfamethoxazole at 16 $\mu\text{g/ml}$ was able to reverse resistance to both itraconazole and voriconazole in *Candida* (data not shown).

We then evaluated the structure-activity relationships (SARs) of the amino benzene sulfonamide scaffold as antifungal agents. In some respects, the SAR was similar to the antibacterial activity of sulfa drugs, whereby all sulfa prodrugs (derivatives with substitutions at the aniline amino group), such as succinylsulfathiazole, are completely inactive in the *in vitro* assay (31). In contrast, unlike the case with antibacterial activity, the fluconazole-antifungal synergistic activity is highly correlated with the N1 substitution. The simplest unsubstituted derivative, sulfanilamide, does not work synergistically with fluconazole and has to have an aromatic system substituted to synergize the antifungal activity of fluconazole. Furthermore, drugs with N1 aliphatic derivatives, for example, N1-imidiny and acetyl derivatives (sulfaguanidine and sulfacetamide), lose their synergistic antifungal activity with fluconazole.

On the other hand, aromatic substitution at the N1 position appears to be a requirement for sulfa drugs to exert synergistic activity with fluconazole. We noticed that the 2-pyrynyl and 2-oxazolyl derivatives (sulfapyridine and sulfathiazole) demonstrated potent synergistic activity with fluconazole (FIC indices of 0.25 and 0.125, respectively). In addition, isoxazole and 4-pyrimidine were among the most-active drugs (sulfamethoxazole and sulfamonomethoxine), with an FIC index of 0.0312. Thus, we concluded that the aromatic ring is critical to the synergistic activity of sulfa drugs with fluconazole. In addition, the electron properties of substituents on the N1 ring play a pivotal role in this synergistic activity. Using the example of pyrimidine-containing sulfa drugs, it was observed that a methoxy group at pyrimidine position 6 (sulfamonomethoxine) demonstrated the best synergistic antifungal activity. Adding a second methoxy group at position 5 (sulfadoxine) led to a less potent compound. In the same vein, the replacement of 5,6-dimethoxy groups with more-lipophilic 2,6-dimethyl moieties resulted in sulfisomidine with remarkably decreased antifungal synergistic activity. The same observation was made with sulfisoxazole, where two methyl groups are at isoxazole positions 3 and 4. Sulfisoxazole has a weaker antifungal synergistic activity than that of its 6-methylisoxazole analogue (sulfamethoxazole), which demonstrated the best synergistic antifungal potency. The SAR information presented here will prove critical to the medicinal chemistry community in relation to the development of new sulfa antifungal analogues.

Fungal cells present within biofilms are more resistant to antifungal agents than planktonic cells (32). Thus, there is an unmet need to identify and develop new agents to attack fungal biofilm and circumvent increasing public health concerns about

antifungal resistance. To determine whether or not the potential therapeutic application of sulfa drugs could be expanded beyond merely inhibiting planktonic *Candida*, the ability of a sulfa drug-fluconazole combination to inhibit biofilm was evaluated. This combination proved to be far superior to fluconazole alone, significantly inhibiting biofilm formation in *C. albicans* (Fig. 2). The biofilm-forming ability of six strains was significantly reduced (~15%) by fluconazole alone (Fig. 2). The ability of fluconazole to inhibit biofilm formation in fluconazole-resistant *Candida* is in agreement with previous reports (33). In addition, there was a significant difference in biofilm-forming ability in these six strains between the sulfa drug-fluconazole combination group and fluconazole alone (Fig. 2).

The finding that the sulfa drug-fluconazole combinations have synergistic activity *in vitro* against numerous resistant *Candida* strains and inhibit biofilm formation prompted us to investigate the efficacy of this combination *in vivo* in a *C. elegans* animal model. Utilizing the *C. elegans* sensitive strain AU37, which is more sensitive to the effects of pathogens than other strains (34), we confirmed that the sulfa drug-fluconazole combination does possess potent antifungal activity *in vivo*. As shown in Fig. 3, the sulfa drug-fluconazole combination reduced the presence of *Candida* in infected worms by ~50%.

The activity of most azole resensitizing drugs has been attributed mainly to their ability to inhibit the overexpression of efflux pumps in *Candida* (35–37). Hence, we turned our attention to studying the effects of sulfa drugs on the efflux pump in *Candida* using a rhodamine 123 accumulation assay (27). The sulfa drugs did not affect the accumulation of rhodamine dye inside the *Candida* cells and therefore did not result in efflux pump inhibition (Fig. 4). This is in agreement with previously reported findings whereby sulfa drugs, such as sulfamethoxazole, had no effect on multiple transport-related genes (38). The results of our accumulation assay were confirmed using additional dyes, namely, Nile red and calcein AM (data not shown).

After confirming that sulfa drugs did not result in the inhibition of the *Candida* efflux pump, we were curious to explore the potential mechanism for the synergistic relationship between sulfa drugs and fluconazole. We hypothesized that the inhibition of the fungal dihydropteroate synthase (DHPS) enzyme by sulfa drugs would lead to restriction of the *Candida* ergosterol biosynthesis pathway, resulting in synergy with the azole drugs (23, 24). To test this hypothesis, we relied on the premise that the mode of antibacterial action by sulfa drugs depends on structural similarity between the sulfa drugs and para-amino benzoic acid (PABA). Hence, the sulfa drugs act as competitive inhibitors of the DHPS enzyme, preventing folic acid synthesis (39). PABA supplementation restored *Candida* growth and reversed the inhibition caused by sulfa drugs in a concentration-dependent manner (Table 2). Interestingly, the resensitization activity of sulfa drugs is medium dependent. Strong synergetic activity with fluconazole was observed in chemically defined media, such as RPMI 1640 medium, and weaker synergetic activity was observed in complex media, such as yeast extract-peptone-dextrose (YPD) medium. This difference can be attributed to the high content of PABA and folic acid precursors in the complex media. A similar observation about the effect of the medium was reported in a similar study in which the activity of sulfamethoxazole against *A. fumigatus* was evaluated (22). The results of this study provide critical information that will facilitate development and testing of novel sulfa drugs with potential antifungal activity.

MATERIALS AND METHODS

Chemicals and reagents. RPMI 1640 broth powder with glutamine but without NaHCO₃ was purchased from Thermo Fisher Scientific (Waltham, MA), and yeast extract-peptone-dextrose (YPD) broth medium and agar were obtained from BD (Franklin Lakes, NJ). Fluconazole, rhodamine 123, sulfaguandine, and 4-aminophenyl sulfone (dapson) were obtained from Fisher Scientific (Pittsburgh, PA). Itraconazole, voriconazole, Nile red, sulfamonomethoxine, sulfathiazole, 5-fluorocytosine, sulfamethoxy-pyridazine, sulfisomidine, sulfametopyrazine (sulfalene), sulfapyridine, and succinylsulfathiazole were obtained from TCI America (Portland, OR). Sulfabenzamide, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfantran, sulfanilamide, sulfisoxazole (sulfafurazole), sulfametoxydiazine (sulfameter), and 3-(N-morpholino) propanesulfonic acid (MOPS) were obtained from Sigma-Aldrich (St. Louis, MO). Sulfacet-

TABLE 3 Strain data and susceptibility testing of *Candida albicans* isolates against standard antifungal agents 5-fluorocytosine (5-FC), fluconazole (FLC), itraconazole (ITC), voriconazole (VOC), and amphotericin B (AMP)

Strain ID	Designation	Isolation source	Geographical region	MIC ($\mu\text{g/ml}$)				
				5-FC	FLC	ITC	VOC	AMP
ATCC 14053	NIH 3172	Bloodstream isolate	Maryland, USA	0.062	0.25	0.062	0.031	1
ATCC 24433	Wasson	Nail infection	NA ^a	1	1	0.125	0.062	1
ATCC 26790	H-29	Human bronchomycosis	NA	0.062	>64	>16	>16	1
ATCC 10231	3147	Human bronchomycosis	NA	0.125	0.5	0.5	0.5	0.5
ATCC 64124	Darlington	Oral isolate	NA	0.5	>64	>16	8	0.5
ATCC 90028	NCCLS 11	Bloodstream isolate	Iowa, USA	1	1	0.031	0.25	1
ATCC 90029	NCCLS 67	Bloodstream isolate	Iowa, USA	>128	0.5	0.25	0.031	0.5
ATCC MYA573	M4	Bloodstream isolate	Germany	0.125	>64	0.5	0.25	0.5
NR 29339	23R	Human isolate	China	0.062	1	0.125	0.125	0.5
NR 29340	23B	Human isolate	China	0.125	0.25	0.062	0.031	1
NR 29341	23Q	Human isolate	China	0.125	0.25	0.062	0.031	1
NR 29342	23P	Human isolate	China	0.125	0.125	0.062	0.031	1
NR 29343	25N	Human isolate	China	0.062	0.25	0.062	0.031	1
NR 29344	24A	Human isolate	China	0.062	0.25	0.062	0.031	1
NR 29345	24C	Human isolate	China	0.062	0.5	0.25	0.125	1
NR 29346	11E	Human isolate	China	0.125	2	0.25	0.5	1
NR 29347	24F	Human isolate	China	0.062	1	0.125	0.5	0.5
NR 29348	23C	Human isolate	China	0.062	1	0.0312	0.0312	0.5
NR 29349	22F	Human isolate	China	0.062	2	0.25	0.125	1
NR 29350	23K	Human isolate	China	0.125	0.125	0.0312	0.0312	1
NR 29351	18 M	Human isolate	China	0.25	0.125	0.0312	0.0312	1
NR 29352	22C	Human isolate	China	0.062	0.5	0.25	0.25	0.5
NR 29353	18K	Human isolate	China	0.25	0.125	0.0312	0.0312	1
NR 29354	18E	Human isolate	China	0.25	0.125	0.0312	0.0312	1
NR 29355	24E	Human isolate	China	0.062	1	0.0625	0.0625	1
NR 29356	23G	Human isolate	China	0.125	0.25	0.0625	0.0312	1
NR 29357	25O	Human isolate	China	0.062	0.25	0.0625	0.0312	1
NR 29358	28H	Human isolate	China	0.125	64	>16	>16	1
NR 29359	25 M	Human isolate	China	0.062	0.25	0.0625	0.0312	1
NR 29360	22O	Human isolate	China	0.062	0.5	0.0625	0.0625	1
NR 29361	22K	Human isolate	China	0.062	0.5	0.0625	0.25	1
NR 29362	11C	Human isolate	China	0.25	0.25	0.0625	0.0312	1
NR 29363	18J	Human isolate	China	0.25	0.125	0.0312	0.0312	1
NR 29364	25P	Human isolate	China	0.125	0.25	0.0625	0.0312	1
NR 29365	23F	Human isolate	China	0.062	0.25	0.0625	0.0312	0.5
NR 29366	28I	Human isolate	China	0.062	>64	>16	>16	1
NR 29367	28A	Human isolate	China	0.062	>64	>16	>16	1
NR 29368	28C	Human isolate	China	0.062	>64	>16	>16	1
NR 29434	P78048	Bloodstream isolate	Manitoba, Canada	0.125	0.25	0.0625	0.0312	1
NR 29435	P57072	Bloodstream isolate	Iowa, USA	0.062	0.25	0.031	0.031	1
NR 29436	P34048	Bloodstream isolate	Istanbul, Turkey	0.062	0.25	0.031	0.031	1
NR 29437	P75010	Bloodstream isolate	Brussels, Belgium	0.062	>64	>16	>16	1
NR 29438	P75016	Bloodstream isolate	Tel-Hashomer, Israel	0.062	0.25	0.031	0.031	1
NR 29444	12C	Oral isolate	Michigan, USA	1	0.25	0.0625	0.0312	1
NR 29445	L26	Vaginal isolate	Iowa, USA	2	1	0.0625	0.0625	1
NR 29446	P94015	Bloodstream isolate	Utah, USA	0.5	>64	4	8	0.5
NR 29447	P37005	Oral isolate	Florida, USA	0.5	0.125	0.0625	0.0312	1
NR 29448	P60002	Bloodstream isolate	Arizona, USA	0.125	>64	>16	>16	2
NR 29449	19F	Vaginal isolate	Michigan, USA	>128	0.5	0.062	<0.031	1
NR 29450	P37037	Oral isolate	Wisconsin, USA	0.5	8	0.0625	0.0312	2
NR 29451	P37039	Oral isolate	New Jersey, USA	0.125	8	0.125	0.5	1
NR 29452	GC75	Oral isolate	Medunsa, South Africa	0.062	0.25	0.125	0.0312	1
NR 29453	P87	Oral isolate	Pretoria, South Africa	0.062	0.125	0.062	0.031	0.5

^aNA, not available.

amide, sulfadoxine, and sulfamerazine were obtained from Alfa Aesar (Tewksbury, MA). FK 506 and cyclosporine were obtained from Biotang Inc., Lexington, MA. Sulfamethoxazole and amphotericin B were obtained from Chem-Impex International (Wood Dale, IL). Penicillin-streptomycin was obtained from Lonza (Walkersville, MD). Calcein AM was obtained from BD Biosciences (San Jose, CA).

Clinical isolates and antifungal susceptibility testing. A total of 53 *Candida albicans* clinical isolates (Table 3) were screened against fluconazole, itraconazole, voriconazole, 5-fluorocytosine, and amphotericin B in accordance with the Clinical and Laboratory Standards Institute (CLSI) M27-A3 guidelines for yeast (40) to determine their MICs. All experiments were carried out in triplicates and repeated at least twice.

TABLE 4 Name, indication, and therapeutic concentration in plasma of different sulfa antibacterial drugs^a

Name of sulfa antibacterial drug	Route of administration and indication	Therapeutic concn in plasma ^b
4,Amino phenyl sulfone (dapson)	Oral treatment for leprosy and <i>Pneumocystis</i> pneumonia	0.63–1.66
Sulfadiazine	Oral treatment of UTI	19–40
Sulfamethazine	Potential treatment of <i>Escherichia coli</i> enteritis in veterinary medicine	64.0–88.4
Sulfamethoxazole	Oral treatment for UTI and prostatitis	15–65
Sulfanilamide	Topical treatment of vaginal yeast infections	NA
Sulfacetamide	Topical treatment of acne and eye infections	NA
Sulfabenzamide	Topical intravaginal antibacterial preparation	NA
Sulfadoxine	Oral treatment used in combination with pyrimethamine to treat or prevent malaria	130–200
Sulfanitran	Veterinary use, as an aid in the prevention of coccidiosis	NA
Sulfamerazine	In some countries, this medicine may be approved only for veterinary use, for example, in control of acute fowl cholera	NA
Sulfamonomethoxine	Used in veterinary medicine for the treatment of toxoplasmosis	NA
Sulfadimethoxine	Oral treatment of respiratory, urinary tract, enteric, and soft tissue infections	120–180
Succinylsulfathiazole	Treatment of intestinal amebiasis	5% of a dose is absorbed after oral dose
Sulfathiazole	Oral and topical antimicrobial	NA
Sulfaguanidine	Treatment of bacillary dysentery	15–40
Sulfapyridine	Help in control of dermatitis herpetiformis (Duhring's disease)	35–60
Sulfametopyrazine (sulfalene)	Treatment of UTI and chronic bronchitis	25–33
Sulfametoxydiazine (sulfameter)	Long-acting sulfonamide antibacterial used as a leprostatic agent	62–90
Sulfisomidine	Treatment of pertussis	NA
Sulfisoxazole (sulfafurazole)	Treatment of bladder infections, ear infections, or meningitis	127–210
Sulfamethoxyypyridazine	Treatment of vaginal irritation, severe acute thrush, and dermatitis herpetiformis, where it is an alternative therapy to dapson	110–180

^aUTI, urinary tract infection; NA, not available.

^bValues are in micrograms per milliliter unless otherwise indicated. Data are from reference 55.

Identification of azole-resistant *Candida* strains. Resistance to fluconazole, itraconazole, and voriconazole was identified by following the guidelines of the Clinical and Laboratory Standards Institute (40). Strains with MIC values of $>32 \mu\text{g/ml}$ fluconazole, $\geq 1 \mu\text{g/ml}$ itraconazole, and $\geq 1 \mu\text{g/ml}$ voriconazole were considered azole resistant (R) (41, 42). *Candida albicans* ATCC 64124 was used as a reference strain for azole resistance, due to this strain's known genetic mutation in the azole target (*Erg11*) (43).

Assembly of the sulfa antibacterial drugs and testing of their antifungal activity. A library of 21 FDA-approved sulfa antibacterial drugs (Table 4) were purchased from commercial sources, and the drugs were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO). The MICs of the sulfa antibacterial drugs were determined against azole-resistant *C. albicans* strains according to the CLSI M27-A3 guidelines as described above (40).

Interaction of sulfa antibacterial drugs with fluconazole against azole-resistant *Candida*. The interaction between sulfa antibacterial drugs and azole antifungals against azole-resistant *C. albicans* clinical isolates was investigated using the checkerboard assay, and the fractional inhibitory concentration index (FICI) was calculated as described previously (25, 26, 44–46). An FIC index of ≤ 0.5 is considered synergism (SYN); an FIC index of >4 is considered antagonism; and a result of >0.5 to 4 is considered indifferent (IND).

Growth kinetics of sulfa antibacterial drugs. *C. albicans* strain NR 29448 was used in this experiment because it exhibited rapid resistance (~ 16 h) to all azole antifungal drugs used in this study. All sulfa drugs that showed synergetic activity with fluconazole were further studied in a growth kinetic curve to confirm their ability to reverse azole resistance (47). Briefly, an overnight culture of *C. albicans* strain NR 29448 was adjusted to 2.5×10^3 CFU/ml in RPMI 1640 medium. Then, each drug at its MIC_c (MIC when combined with fluconazole) was incubated with *C. albicans* at 35°C, either alone or in combination with 1 $\mu\text{g/ml}$ fluconazole. The growth kinetic was monitored at an optical density at 595 nm (OD₅₉₅) at 0, 6, 10, 12, 24, and 48 h after incubation.

Effects of sulfa drug-fluconazole combinations on biofilm-forming ability of *C. albicans*. Biofilm-forming *C. albicans* strains were used to study whether sulfa drug-fluconazole combinations can interfere with their abilities to form biofilms. Cells were prepared as previously described (48–50). Briefly, overnight cultures of 12 strains of *C. albicans* (Fig. 2) in YPD broth were diluted in RPMI 1640 medium to an inoculum size of 1×10^5 CFU/ml. Sulfa drugs, i.e., sulfamethoxazole, sulfadiazine, sulfadoxine, sulfadimethoxine, and sulfamethoxyypyridazine, were added to the yeast cell suspension of *C. albicans* at concentrations of 0.5 \times , 0.25 \times , and 0.125 \times MIC in the presence of a fixed concentration of fluconazole (0.0625 $\mu\text{g/ml}$). Amphotericin B, at concentrations of 0.5 \times , 0.25 \times , and 0.125 \times MIC, was used as a positive control. *Candida* cells were then transferred to the wells of microtiter plates, and the plates were incubated at 35°C for 24 h. The formed biofilms were rinsed twice with phosphate-buffered saline (PBS) and then left to air dry at room temperature. Air-dried biofilms were stained for 10 min with 200 μl crystal

violet (0.1%). Stained biofilms were rinsed three times with PBS and air dried for 1 h. The amount of crystal violet was quantitated by destaining the biofilms for 10 min with 200 μ l of absolute ethanol, and then the absorbance of the crystal violet solution at OD₅₉₅ was measured. All experiments were carried out in quadruplicates and repeated at least twice.

Caenorhabditis elegans infection study. To examine the efficacy of sulfa drugs in reversing azole resistance *in vivo*, we used the *C. elegans* animal model (51–54). The biofilm-forming strain NR 29448 was found to colonize the worms effectively, so we used it in this experiment. Briefly, L4 stage worms [strain AU37 genotype glp-4(bn2) I; sek-1(km4) X] were infected with *C. albicans* NR 29448 for 3 h at room temperature. After infection, worms were washed five times with M9 buffer and transferred into tubes (~20 worms per tube). Worms were treated for 24 h (in triplicate) with a combination of 10 \times MIC_c sulfa drugs and 10 μ g/ml fluconazole (SDZ was insoluble at 10 \times MIC_c and was excluded from this experiment). DMSO, 5-fluorocytosine at 10 \times MIC (0.625 μ g/ml), and fluconazole (10 μ g/ml) served as controls. Posttreatment, worms were examined microscopically to evaluate morphological changes and ensure viability, after which they were washed with M9 five times and then disrupted using silicon carbide particles, and the resulting suspensions were serially diluted and transferred to YPD agar plates containing penicillin (100 μ g/ml) and streptomycin (100 μ g/ml). Plates were incubated for 48 h at 35°C before the viable *Candida* CFU per worm was determined.

Effects of sulfa drugs on efflux of rhodamine. To test if sulfa drugs have inhibitory effects on the efflux pump in *Candida*, a rhodamine accumulation assay was performed (27). Briefly, *C. albicans* strain NR 29448 was grown overnight at 35°C in YPD broth and then transferred to a fresh YPD broth and incubated at 35°C for 4 h until the cells reached the mid-log phase. Cells were harvested and adjusted to 1 \times 10⁶ CFU/ml in YPD broth. *Candida* cells were then incubated with sulfa drugs at subinhibitory concentrations (0.25 \times MIC) for 1 h. Then, rhodamine 123 dye (Rh123) at a final concentration of 10 μ M was added to the cell suspension and incubated at 35°C in a reciprocating shaker for an additional 30 min. After incubation, 1-ml samples were taken and centrifuged at 5,000 \times g for 5 min. Supernatants were discarded, and the pellets were washed three times with PBS to remove extracellular Rh123. Fluorescence at excitation and emission wavelengths of 485 and 538 nm, respectively, was recorded for 6 replicates for each drug using a Spectramax-ix3 microplate reader. Rh123 accumulations were expressed as arbitrary fluorescence per OD₅₉₅ unit (specific fluorescence).

Effects of PABA supplementation on synergistic activity of sulfa antibacterial drugs and fluconazole. To study the effects of para-amino benzoic acid (PABA) on the synergistic relationship between sulfa drugs and fluconazole, a checkerboard assay was performed as described above, in the presence and absence of different concentrations of PABA (1, 4, 8, 16, 32, 64, and 128 μ g/ml). Then, FIC indices for each drug were calculated as described above.

Statistical analysis. Data are presented as means \pm standard deviations. Statistical analyses were performed using GraphPad Prism 6.0 (Graph Pad Software, La Jolla, CA, USA). *P* values were calculated using one-way analysis of variance (ANOVA). *P* values of <0.05 were considered significant.

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