Introduction

*Candida albicans* (C. albicans), the major opportunistic fungal pathogen of humans, causes various forms of candidiasis, ranging from superficial mucosal infections to life-threatening systemic disorders[1–3]. Extensive and repetitive use of antifungal azole derivatives such as fluconazole to treat refractory and recurrent candidosis infections has prompted *C. albicans* to develop multiple mechanisms of multidrug resistance (MDR)[4, 5]. Many genes have been confirmed to participate in the development of MDR, including *CDR1, CDR2, CaMDR1* and *ERG11*[6–8]. Recently, we have demonstrated that *RTA2* is also involved in calcineurin-mediated drug resistance in *C. albicans*[6]. Some transcription regulatory factors of these MDR genes have been identified, and many of them are zinc cluster proteins, such as Tac1p, Fcr1p and Upc2p[9]. Zinc cluster proteins form one of the largest families of transcriptional regulators in eukaryotes, displaying variable secondary structures and enormous functional diversity, and they can bind as homodimers to CGG triplets that are oriented in everted, inverted, or direct repeats[10]. These transcription factors, as well as the transcription processes, represent potential targets against MDR. Among the MDR genes regulated by zinc cluster transcription factors, the expression of *CDR1* and *CDR2* has received considerable attention, because their encoding ABC transporter proteins, Cdr1p and Cdr2p, could pump azoles out of cells to reduce azole accumulation as a self-defense mechanism[12–15]. Our previous studies have also demonstrated that elevated *CDR1* and *CDR2* levels are associated with the progression of MDR in antifungal treatment[16, 17].

Numerous attempts have been made to cope with clinical treatment failures resulting from drug resistance, such as developing novel antifungal compounds[18–21] and exploring combination therapies[22–29]. Small molecule approaches for gene regulation could bypass the need for delivery strategies. A number of natural and synthetic DNA binding molecules...
have been explored for their ability to regulate gene expression in vitro and in vivo. Polyamides, containing N-methylimidazole (Im) and N-methylpyrrole (Py), represent one approach to inhibit protein-DNA interactions, and they comprise a class of programmable DNA-binding ligands that can bind to a broad repertoire of DNA sequences with affinities and specificities comparable to those of natural DNA-binding proteins. They are cell permeable, have no cytotoxicity, bind to chromatin and have been shown to inhibit a broad range of transcription factors resulting in the down-regulation of endogenous gene expression in cell culture\textsuperscript{30, 31}. Sequence specificity is programmed by side-by-side pairings of the heterocyclic amino acids in the minor groove of DNA: Im/Py distinguishes GC and TA\textsuperscript{32, 33}, Py/Py binds both AT and TA\textsuperscript{34}. NMR spectrometer. Compounds 1-3 were synthesized as described by Dervan\textsuperscript{34}.

Figure 1. \textsuperscript{1}H NMR (DMSO-d$_6$, 300 MHz): $\delta$ 9.66 (s, 1 H), 7.78 (s, 1 H), 7.52 (s, 1 H), 4.75 (d, 1 H, $J$=4.8 Hz), 3.90 (s, 3 H).

**Materials and methods**

**Synthesis of polyamide**

Synthesis of SL-A92 was carried out using a solution-phase approach according to the synthesis route (Figure 1). Thin-layer chromatography was performed on silica gel HSGF254 plates, and column chromatography was performed using silica gel (300-400 mesh). Proton nuclear magnetic resonance ($^1$H NMR) spectra were recorded on a BRUKER AVANCE II 300 NMR spectrometer. Compounds 1-3 were synthesized as described by Dervan\textsuperscript{34}.

NO$_2$PyCOOH (4)

NO$_2$PyCOOMe (compound 1, 2 g, 7.37 mmol) was dissolved in methanol (50 mL), followed by the addition of 1 mol/L NaOH (30 mL). The reaction mixture was stirred at room temperature for 2 h. The methanol was removed, and the solution was washed with ethyl ether (2×50 mL). The pH of the aqueous layer was reduced to approximately 3 with 10% (v/v) HCl, and the mixture was extracted with ethyl acetate (3×50 mL). The combined ethyl acetate extracts were dried (sodium sulfate) and concentrated in vacuo to provide 1.2 g of NO$_2$PyCOOH as a brown powder (91% yield). $^1$H NMR (DMSO-d$_6$, 300 MHz): $\delta$ 9.13 (s, 1 H), 8.22 (q, 1 H, $J$=1.5 Hz), 7.25 (d, 1 H, $J$=4.8 Hz), 3.90 (s, 3 H).

NO$_2$ImmCOOEt (5)

NH$_2$ImCOOEt (compound 3) was collected from 100 mg of NO$_2$ImmCOOEt as described by Dervan\textsuperscript{34} and then dissolved in ethyl acetate (30 mL). NO$_2$ImmCOOH (88.5 mg, 0.52 mmol) was added, followed by HOBT/DCC (80/120 mg). The mixture was stirred for 4 h. DCU was removed by filtration. The filtrate was concentrated in vacuo and then purified by column chromatography using methanol and chloroform as an eluent (gradient eluate) to provide NO$_2$ImmCOOEt as a light yellow powder (72.8 mg, 45% yield). $^1$H NMR (CDCl$_3$, 300 MHz): $\delta$ 7.96 (s, 1 H), 7.46 (s, 1 H), 7.24 (s, 1 H), 4.43 (d, 2 H, $J$=7.2 Hz), 4.18 (s, 3 H), 4.03 (s, 3 H), 1.44 (tt, 3 H, $J$=7.2 Hz).

NO$_2$PyImmCOOEt (6)

Pd/C catalyst (10%, 10 mg) was added to a solution of NO$_2$ImImCOOEt (100 mg, 0.31 mmol) in 15 mL of methanol, and the mixture was stirred under a slight positive pressure of H$_2$ for 4 h. The catalyst was removed by filtration through Celite and washed with 50 mL of ethyl acetate. The filtration was concentrated in vacuo and then purified by column chromatography using methanol and chloroform as an eluent (gradient eluate) to provide NO$_2$PyImmCOOEt as a light yellow powder (72.8 mg, 45% yield). $^1$H NMR (CDCl$_3$, 300 MHz): $\delta$ 7.46 (s, 1 H), 7.56 (s, 1 H), 4.43 (d, 2 H, $J$=7.2 Hz), 4.18 (s, 3 H), 4.03 (s, 3 H), 1.44 (tt, 3 H, $J$=7.2 Hz).

**Drugs, strains and media**

Fluconazole (2 mg/mL) was purchased from Pfizer Inc (New York, NY). SL-A92 was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mg/mL. The C. albicans collection strain SC5314 was kindly provided by William A FONZI (Department of Microbiology and Immunology, Georgetown University, Washington, DC). Clinically isolated C. albicans Y0109, 102 and Y012 strains were obtained from the Department of Dermatology, Changhai Hospital (Shanghai, China). The strains were cultivated at 30°C under constant shaking (200 r/min) in a liquid complete YPD medium consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose. RPMI 1640 medium was buffered to pH 7.0
with 3-(N-morpholino)-propane-sulfonic acid (MOPS) to a final concentration of 0.165 mol/L.

**Antifungal susceptibility testing**

The *in vitro* minimal inhibitory concentrations (MIC) of SL-A92 were determined using the micro-broth dilution method as defined by the National Committee for Clinical Laboratory Standards (NCCLS)\[36\]. SC5314, Y0109 and 102 were cultured in RPMI-1640 medium, with an inoculum concentration of 10^5 cells/mL. The final concentrations of SL-A92 ranged from 0.39 to 200 μg/mL. The microdilution plates inoculated with SL-A92 were incubated at 30°C. MIC endpoints for SC5314 were determined after incubation for 24 h. The drug MIC_{80} was defined as the first well with an approximate 80% reduction in growth compared to the growth of the drug-free well.

**Growth curves**

SC5314 in YPD medium was prepared at the starting inoculum of OD_{600}=0.003. The concentrations of SL-A92 that were added were 5, 20 and 200 μg/mL and the concentration of fluconazole was 2 μg/mL. The growth was monitored by measuring the optical density at 600 nm (OD_{600}) of the cultures throughout the subsequent 48 h at predetermined time points (0, 2, 4, 6, 8, 12, 16, 24, 36, and 48 h).

**Antifungal drug resistance assay**

A randomly selected colony of Y012 was inoculated in 1 mL of YPD medium and cultivated for 24 h. When the culture reached a density of 10^8 cells/mL, each aliquot of this culture containing 10^6 cells was then transferred to 1 mL×4 fresh YPD broth. SL-A92 was then added to three strains reaching the final concentrations of 5, 20 and 200 μg/mL, respectively. The fourth strain was supplemented with an equal amount of DMSO to broth and used as a blank control. Each day, 10 μL from each overnight culture was serially transferred into 1 mL of fresh medium, and the drug concentrations in the experiment were never reduced. At intervals of 4 days over the following 20 days of incubation, the susceptibilities of the induced Y012 strains were tested in each aliquot by the NCCLS microdilution method.

**Induction of azole resistance and the identification**

The incubation of SC5314 with SL-A92 at different concentrations were used. As shown in Figure 2, fluconazole at 2 μg/mL showed obvious inhibition on the growth of SC5314. However, the curves of the strains incubated with SL-A92 at 5, 20 and 200 μg/mL were in accordance with that of the only DMSO incubated strain within 48 h (for the OD_{600} data, Table S4). These curves indicate that SL-A92 did not affect the growth of the strains incubated at those concentrations.

**Real time RT-PCR**

**RNA isolation**

Total RNA isolation was carried out using the Fungal RNA out kit (TIANDZ, Sichuan, China) as described in the manufacturer’s manual. After quantifying the isolated total RNA, the yields were 20-50 μg per well, and genomic DNA was digested by treatment with DNase I (TaKaRa, Dalian, China). Purification of the RNA was carried out using the Column RNA clean kit (TIANDZ) to provide RNA for synthesis of the first strand cDNA.

**Results**

**Antifungal activity of SL-A92**

The MIC_{80} against a laboratory collection strain of SC5314, and the clinically isolated drug-susceptible Y0109 and clinical isolated drug-resistant 102 strains were measured to evaluate the antifungal activity of SL-A92. As shown in Table S3, all the MIC_{80} of SL-A92 to the above three strains were >200 μg/mL, which indicates that there was no obvious antifungal activity of SL-A92.

**Growth curve**

Growth curves were plotted to evaluate the effects of SL-A92 on the growth of the strains. The OD_{600} was used to monitor the growth of the cultured strains. As illustrated in Figure 2, fluconazole at 2 μg/mL showed obvious inhibition on the growth of SC5314. However, the curves of the strains incubated with SL-A92 at 5, 20 and 200 μg/mL were in accordance with that of the only DMSO incubated strain within 48 h (for the OD_{600} data, Table S4). These curves indicate that SL-A92 did not affect the growth of the strains incubated at those concentrations.

**Evaluation of azole resistance inhibition**

The susceptibilities to fluconazole of the Y012 strains induced with SL-A92 at different concentrations were used. As shown...
in Table S5, the susceptibility did not change during any part of the induction process at each concentration, which suggests that SL-A92 had no significant inhibition on the drug resistance that had already developed.

Blocking the development of drug resistance during fluconazole induction

The susceptibilities of different induced SC5314 strains to fluconazole are shown in Table 2. On day 21, the positive control strain had already developed resistance, as indicated by an MIC$_{80}$ > 64 µg/mL, and the resistance was stable in the following induction process. However, drug resistance did not appear in the strain with 200 µg/mL of SL-A92. The resistance of the strain with 20 µg/mL of SL-A92 was unstable, with MIC$_{80}$s of 4 µg/mL and 2 µg/mL on day 21 and day 30, respectively. As 5 µg/mL of SL-A92 showed no effect on the drug resistance in S3, we speculate that the inhibition to drug resistance could affect the MIC$_{80}$s when the concentration of SL-A92 in the co-incubation mixture reached 20 µg/mL. The results described here indicated that 200 µg/mL of SL-A92 could block the development of drug resistance during fluconazole induction.

Table 2. The susceptibility of the induced SC5314 strains to fluconazole.

| Strain Code* | MIC$_{80}$ (µg/mL) |
|--------------|-------------------|
| SC5314       | 12 d | 15 d | 18 d | 21 d | 24 d | 27 d | 30 d | 33 d |
| S1           | 0.5  | 2    | 0.5  | 0.5  | 2    | 0.5  | 0.5  | 0.5  |
| S2           | 0.25 | 0.25 | 0.5  | >64  | >64  | >64  | >64  | >64  |
| S3           | 0.5  | 0.125| >64  | >64  | >64  | >64  | >64  | >64  |
| S4           | 1    | 1    | >64  | 4    | >64  | >64  | 2    | >64  |
| S5           | 0.25 | 0.125| 0.125| 0.125| 0.125| 0.125| 0.125| 0.125| 0.125| 0.125| 0.125| 0.125| 0.125| 0.5  |

*SC5314 strains induced by DMSO and fluconazole were coded as S1 and S2, respectively, and those induced by fluconazole combined with SL-A92 5, 20, 200 µg/mL were as S3, S4, and S5, respectively.

Determination of the CDR1 and CDR2 mRNA levels in the induced SC5314

Real time RT-PCR was performed to determine the CDR1 and CDR2 mRNA levels of the induced strains. Strains were incubated with DMSO, fluconazole, and fluconazole combined with 200 µg/mL of SL-A92 for 24 days; the strains were coded as S1-24, S2-24 and S5-24, respectively. At the same concentrations, the strains incubated for 30 days were coded as S1-30, S2-30 and S5-30, respectively. The CDR1 and CDR2 mRNA levels of SC5314 were normalized to 1. As shown in Figure 3, both CDR1 and CDR2 in S1-24 and S1-30 were at almost the same levels as those of SC5314. However, the CDR1 level of S2-24 was up-regulated to 2.14-fold compared to that of SC5314, while the CDR2 level showed no significant change. The CDR1 and CDR2 levels of S5-24 were reduced to 26% and 24% compared to those of SC5314, respectively. Similarly, on day 30, the CDR1 level of S2-30 was up-regulated to 2.87-fold compared to that of SC5314, and the CDR2 level showed no significant change. The CDR1 and CDR2 levels of S5-30 were reduced to 43% and 31% of those of SC5314, respectively (for the relative fold change data, see supplementary Table 6). In other words, participation of SL-A92 in the induction process resulted in a decrease in CDR1 mRNA production as well as 88% and 85% the transcripts that were induced by fluconazole only in cultured SC5314 cells on days 24 and 30, respectively.

Figure 2. Time-growth curves were taken according to the OD$_{600}$ to evaluate the effects of SLA92 on the growth of strains. Strains incubated only with DMSO and fluconazole (FCZ) 2 µg/mL was used as blank and positive controls, respectively.

Figure 3. Relative CDR1 and CDR2 mRNA levels of the induced strains compared to SC5314.

Discussion

Widespread and long-term use of azole derivatives to treat _C. albicans_ infection has promoted the development of MDR, which has become a general and severe problem in clinical therapy. In the present study, we designed and synthesized a cell-permeable, sequence-specific, DNA-binding polyamide SL-A92 to target the sequence CGG, which is the binding site of zinc cluster transcription factors, with the goal of interrupting some MDR gene expression and thus further inhibiting drug resistance.

Just as reported previously[30], we also found that SL-A92 had no cytotoxicity, as revealed by its antifungal activity and effects on strain growth. Based on the theory[31] of how polyamide exerts its function of gene regulation, a clinically isolated drug-resistant strain Y012 was incubated with SL-A92 for 20 days. Unfortunately, inhibition of drug resistance in
induced Y012 strains was not found. On the one hand, this may be due to unknown resistance mechanisms in Y012; resistance to drugs can be conceived of as a gradually evolving process wherein various mechanisms may appear during the course of chemotherapy. On the other hand, we speculate that SL-A92 could not exhibit its function when some MDR genes have already been up-regulated; therefore, we produced a model in which SL-A92 exerts its function during the development of drug resistance. Interestingly, we succeeded in promoting SC5314 in developing drug resistance using fluconazole induction, and the concomitance of SL-A92 induction blocked this induced drug resistance successfully at 200 µg/mL. These results indicate that SL-A92 could block the development of drug resistance during fluconazole induction, but not the resistance that had already developed.

The reduced intracellular accumulation of drugs is a common mechanism of resistance, which is correlated with the increased expression of the genes CDR1 and CDR2 (members of the ABC efflux pump family) and of the gene CaMDR1 (a member of the MFS efflux pump family)[37–39]. Four kinds of CDR genes in C. albicans have been cloned[13, 40–42], but only over-expression of CDR1 and CDR2 resulted in azole resistance[43]. Tac1p is the major transcription factor needed for the regulation of CDR1 and CDR2, and is characterized by a highly conserved Zn(II)$_2$Cys$_6$ zinc finger motif formed by six cysteines that coordinate two zinc atoms within the DNA-binding domain[31, 44, 45]. Previous studies have demonstrated that the binding site of Tac1p lies in the drug-responsive element (DRE) and consists of a direct CGG repeat with four intervening nucleotides (CGGAA/TATCGG)[44]. In this study, we showed that fluconazole could up-regulate the level of CDR1 after induction for 33 days in vitro, but there was no up-regulation of CDR2. However, the participation of SL-A92 in the induction resulted in significant decreases of both CDR1 and CDR2 mRNA production, and both were down-regulated below normal levels.

In conclusion, we demonstrate here that SL-A92 can block the development of drug resistance during fluconazole induction, but cannot block the resistance that had already developed. This effect could be partially due to the down-regulation of CDR1 and CDR2, although the detailed mechanisms need further study. Future work is also needed to explore the molecular target of SL-A92 in C. albicans and to optimize the cellular and nuclear uptake of such functionalized compounds. This would provide a new method of antidrug resistance in C. albicans. The results presented here are a promising proof of principle for further studies in this area. Our findings may open a new doorway for the development and design of new effective agents for the regulation of MDR genes.

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Author contribution

Shao-long ZHU contributed new reagents, performed the research, and wrote the paper; Zhi-hui JIANG assisted the synthesis; Ping-hui GAO designed the bioactivity research; Yue QIU and Liang WANG analyzed the data; Yuan-ying JIANG and Da-zhi ZHANG revised the manuscript.

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