Nanomed Res J 5(1):90-100, Winter 2020

RESEARCH ARTICLE

An appraisal of antifungal impacts of nano-liposome containing voriconazole on voriconazole-resistant Aspergillus flavus isolates as a groundbreaking drug delivery system

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ARTICLE INFO

Article History:
Received 13 December 2019
Accepted 08 February 2020
Published 15 February 2020

Keywords:
Aspergillus flavus
voriconazole
nano-liposome containing voriconazole
cyp51A
MDR1

ABSTRACT

Objective(s): This study is an attempt to investigate the effect of nano-liposome containing voriconazole on voriconazole-resistant A. flavus strains on the one hand, and to consider the expression of cyp51A and MDR1genes, regarded as important genes involved in the development of resistance to triazoles before and after voriconazole and voriconazole-loaded nano-liposomes exert their effects, on the other hand.

Methods: Strains of A. flavus isolated from patients were investigated and their susceptibility to voriconazole was determined. Next, having applied a slight modification to the thin film hydration-sonication technique, the liposomal formulation of voriconazole was produced. After that, the voriconazole-loaded nano-liposome was subjected to in-vitro antifungal susceptibility testing to obtain minimum inhibitory concentration against fungal isolates. The qRT-PCR instrument was used to measure mRNA levels of Cyp51A and MDR1.

Results: The effect of nano-liposome containing voriconazole on the reduction of MIC in A. flavus isolates were considered to be significant. After using MIC50 concentration of VCZ, the cyp51A gene expression in voriconazole-susceptible A. flavus strains and voriconazole-resistant strains 10folds and 7folds depicted a downregulation, respectively, which was more pronounced in the expression of a liposomal formulation of VCZ (13folds and 15folds respectively). This procedure was applied exactly to MDR1, even though it induced 1, 2, 3, 4-fold reductions.

Conclusions: Considering the benefits of liposome-containing voriconazole formulation, such as the reduction of the side effects of the pure drug as well as minimizing the drug's toxicity coupled with the enhanced drug bioavailability and stability, the formulation can be used in drug-sensitive and drug-resistant species.

How to cite this article

Hassanpour P, Hamishehkar H, Baradaran B, Mohammadi M, Shomali N, Spotin A, Hazratian, Nami S. An appraisal of antifungal impacts of nano-liposome containing voriconazole on voriconazole-resistant Aspergillus flavus isolates as a groundbreaking drug delivery system. Nanomed Res J, 2020; 5(1): 90-100. DOI: 10.22034/nmrj.2020.01.010

INTRODUCTION

The most important clinical manifestations of *Aspergillus* as a saprophytic mold include invasive aspergillosis (IA), chronic pulmonary aspergillosis, *aspergillus* bronchitis, and allergic bronchopulmonary aspergillosis, with the emergence of clinical symptoms depending entirely on the immunological background of the infected host (1,2). IA in immunocompromised patients such as HIV-infected ones, hematologic malignancies who undergo intensive chemotherapy and bone marrow transplant recipients is considered as a life-

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threatening mycosis (3, 4). Today, *Aspergillus flavus* (*A. flavus*) is one of the most common causes of IA in some particular geographic areas such as the Middle East and Southeast Asia. *A. flavus* takes a back seat to *Aspergillus fumigatus* (*A. fumigatus*) since the latter accounts for 80% of invasive and noninvasive infections in Europe and the United States (4).

IA treatment depends on several factors thus, the choice and administration of medication cannot be sufficient, for instance, understanding the underlying disease and duration of immunosuppression are key factors in the treatment of immunocompromised patients in particular. Voriconazole (VCZ) is a first-line treatment drug in IA and even in some transplant centers, it is utilized as a mold-prophylactic agent, it is also found to be more effective than amphotericin B (5-7). In recent years, selecting the right medicine has been complicated due to the observation of differing antifungal resistance patterns in *Aspergillus* isolates in different geographic regions. Even today, infection-related mortality rates are high despite the early diagnosis and use of VCZ in IA thanks to the emergence of acquired resistance to VCZ which has failed treatment in some patients (4, 8). Comprehensive molecular studies have been carried out on azole-resistant *A. fumigatus* strains, revealing mutations in the coding regions alongside the overexpression of the cyp51A (14α-demethylase) gene and non-cyp51-mediated resistance to be the main causes of resistance to VCZ (9, 10). Cyp51 genes (cyp51A, cyp51B, and cyp51C) play a crucial role in ergosterol biosynthesis; that is, VCZ disturbs the pathway by inhibiting 14-α-demethylases and finally destroying sterol metabolism in the cells (11, 12). As a result of recent studies on some VCZ-resistant *A.flavus* strains, it has been found that in these filamentous fungi, amino acid residue substitution or overexpression of the cyp51A gene is also one of the mechanisms of resistance to VCZ (13). But in this regard, some VCZ-R isolates (40-70%) lacked cyp51A alternations, indicating the involvement of other mechanisms for resistance in this triazole drug. Overexpression of multidrug resistance efflux pumps (MDR-EPs) is another important mechanism involved in *A. flavus* strains that well indicate acquired resistance to this antifungal (5, 14). The two main classes of MDR-EPs involved in antifungal resistance include ATP-binding cassette (ABC) and major facilitator superfamily (MFS) (5, 9). During studies conducted on EPs in *A. flavus* strains that are VCZ-resistant, overexpression of genes encoding MDR 1, 2, 4, AtrF, and/or MFS Eps has been observed. In some resistance isolates, there is neither a change in cyp51 genes nor a change in MDR-EPs (5, 13), which illustrates the need for further studies in this regard.

Although VCZ is a newly developed antifungal agent, the side effects of this drug as well as the observation of drug resistance in some isolates have led to solutions for optimizing this drug. One such solution is the use of a drug delivery system that has overcome some problems associated with the use of this drug (15, 16). In studies conducted on diverse drug delivery systems, the use of nanoparticles (NPs), as one of these systems, has shown an enhancement in therapeutic efficacy in addition to reducing side effects (17-19). Also, these NPs have been the Food and drug administration (FDA) and European medicines agency (EMA) approved (20). Some of the advantages of using NPs entail enhanced drug stability and prolonged drug residence time in the blood, moreover, NPs can protect encapsulated drugs from in vivo enzymatic degradation (18, 21, 22). One of the NPs is liposomes; in addition to reducing side effects, it can minimize drug toxicity and also increase drug bioavailability and stability; what is more, the small size of these lipid NPs enhances the rate of penetration to tissues (23, 24). Today, due to the increased mortality rates caused by aspergillosis, resulting from VCZ-resistant *A. flavus* strains, the need for the use of NPs to optimize the drug is urgently felt. Therefore, this study is an attempt to investigate the effect of nano-liposome containing voriconazole (VCZ-loaded nano-liposome) on VCZ-resistant *A. flavus* strains on the one hand, and to consider the expression of cyp51A and MDR1genes, regarded as important genes involved in the development of resistance to triazoles before and after VCZ and VCZ-loaded nano-liposomes exert their effects, on the other hand.

MATERIALS AND METHODS

Fungal isolates and In vitro antifungal susceptibility testing

In this study, 8 strains of *A. flavus* isolated from patients with different manifestations of aspergillosis were investigated. These strains were isolated and identified in previous studies (4, 14) from patients in the Serology Lab of the department of Mycology, Tehran University of Medical

Sciences, Tehran, Iran. Next, their susceptibility to VCZ (Sigma-Aldrich USA) was determined using the microdilution method, according to the Clinical and Laboratory Standard Institute M38 3rd edition (25) and were kept in fungi collections of the laboratory. Initially, to verify the authenticity of strains, *in vitro* antifungal susceptibility testing was performed for the second time to determine the minimum inhibitory concentration (MIC) of VCZ against fungal isolates. Standard strains of *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC22019) were also studied as qualitative controls.

Preparation of liposome containing voriconazole and Characterization of nanoparticles

Having applied a slight modification to the thin-film hydration-sonication technique, the liposomal formulation of VCZ was produced (26). Next, lecithin (Lipoid Company, Germany) and cholesterol (Sigma-Aldrich, Germany) (45:5 w/w) were mixed in 15 ml ethanol to act as the solvent. Then VCZ (350µg/ml) was added and the solvent was evaporated in a rotary evaporator at 50°C, resulting in dry lipid sediment on the wall of the flask. The obtained thin layer was hydrated by adding 5 ml of distilled water, culminating in multilamellar vesicles. The sample was subjected to a probing sonication (Vibra Cell - Sonics & Material, 130 W, 20 kHz, USA) at 80% sonication strength in ice bath for 10 min (10 cycles of 1 min sonication and 1 min rest intermittently to allow cooling of the sample) to let the sample shrink in size.

Synthesized NPs were assessed for their particle size, polydispersity index (PDI) and zeta potential using dynamic light scattering (DLS) (Zetasizer-ZS, Malvern, UK). In the same vein, the NPs structure was probed by a scanning electron microscope (SEM) (TEscan, VEGA II XMU, Czech Republic). Briefly, the samples were fixed with 3% glutaraldehyde for 12 h at room temperature, then washed in cacodylate buffer. The specimens were dehydrated by ethanol and dipped in hexamethyldisilazane. However, a direct current sputter technique aided in coating the samples with a thin layer of gold previous to the scanning stage (EMITECH K450X, England) (27). The blank liposomal formulation was prepared in the same manner, without adding VCZ, though. After that, the VCZ-loaded nano-liposome was subjected to *in vitro* antifungal susceptibility testing to obtain MIC against fungal isolates. For comparison, the same concentration of VCZ-loaded nano-liposome was deployed.

Encapsulation efficiency determination

The aqueous solution was centrifuged and subsequently, the free drug and lipids were eliminated. Adding 2ml of chloroform to 1ml of sample leads to the extraction of VCZ from the liposomes as well as the degraded carrier. Then, the absorbance of drug dissolved in chloroform was read at 256 nm and the amount of encapsulated drug was calculated according to the calibration curve of free VCZ. The VCZ concentration in nanoliposomes was calculated using the following equation (28):

$$\text{Encapsulation on efficiency} = \frac{\text{Amount of VCZ incorporated in nanoliposomes}}{\textit{Amount of total VCZ}} \times 100$$

Isolation of total RNA and synthesis of complementary DNA (cDNA)

Total RNA was extracted from conidia which were previously grown in liquid culture (Merck, Germany) and incubated at 37°C, receiving constant shaking at 120 rpm for 48 - 72 hours. The mycelia were ground in liquid nitrogen and TRIzol lyzing reagent (Ambion life technologies) (1ml) was added and incubated at RT for 5min. After adding 250µl of chloroform, the microtube was incubated on ice for 10min. Then, it was centrifuged at 12500×g for 15min at 4°C and transferred the upper, aqueous phase to a new RNase free tube. The ice-cold isopropanol (500µl) was added (it was incubated on ice for 10min) and centrifuged at 12500×g for 10min at 4°C. The sediment was mixed with cold 75% ethanol and centrifuged at 7800×g for 8min at 4°C. The precipitant was dried at room temperature for 20min. Finally, the RNA was dissolved in 30µl diethyl pyrocarbonate (DEPC)-treated water and incubated for 10min at 55-60°C. RNA purity was evaluated by Nanodrop2000c spectrophotometer (Thermo fisher scientific). Thereafter, the RNA molecules were converted into their cDNA sequences by reverse transcriptase and used as the template for PCR amplification using a Sensiscript Reverse Transcription kit (QIAGEN, Germany). The comprehensive methodology of this protocol has been presented in the previously published article (4).

Table1. Sequences of primers

Primers Name	Sequence (5'-3')	PCR Product Size (bp)
cyp51A-F	TGA GCC TGC AGT CAT GGA AG	211
cyp51A-R	GAC GTA AGG TGT GCC AGG AA	
MDR1-F	TTC CGC TTC TTC GTC TGC TT	166
MDR1-R	TCT TGC CAT CTT CCG ACC AC	
tubulin-F	AAC GCT TTG CAA CTC CTG AC	162
tubulin-R	AGT TGT TAC CAG CAC CGG AC	

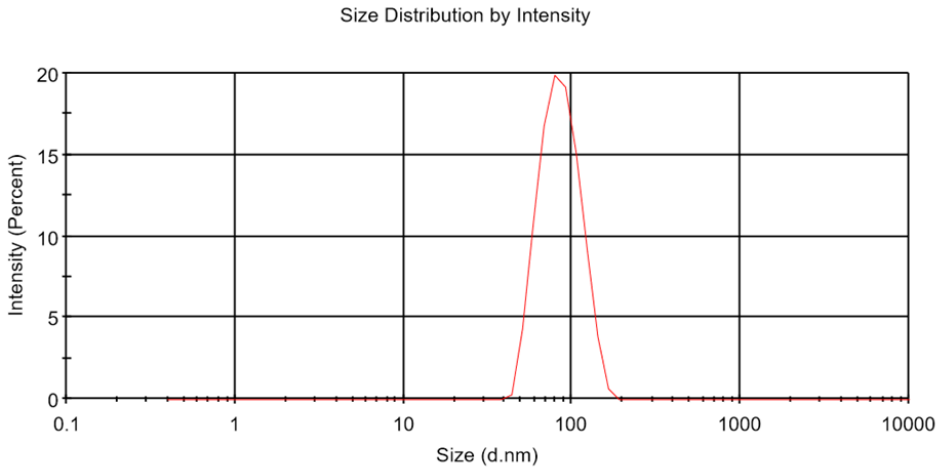

Fig.1. Particle-size distribution spectrum of VCZ-loaded nano-liposome

Quantitative real-time polymerase chain reaction (qRT-PCR)

The published gene sequence of *A. flavus* NRRL3357 (http://www.ncbi.nlm.nih.gov/pubmed/) was the place of origin for nucleotide sequences of cyp51A (NCBI accession numbers: XM_002375082) and MDR1 (NCBI accession numbers: XM_002382940). After that, primers (Table 1) were designed for cyp51A and MDR1with tubulin acting as a housekeeping gene. The qRT-PCR instrument was used to measure mRNA levels of Cyp51A and MDR1 using SYBR Green Master Mix (AMPLIQON, Denmark). The real-time was carried out in a 10 µl reaction volume, containing: 0.5µl of cDNA template, 0.25µl of each primer, 5µl of master mix and 4.25µl of nuclease-free distilled water. The program was 95°C for 5min, 45cycles at 95°C for 10sec, 58°C for 35sec, and 70°C for 20sec. The relative gene expressions were analyzed by $2^{-\Delta CT}$ method. Finally, a *P*-value < 0.05 was considered as statistically significant and data were represented as mean ± standard error of mean (SEM). GraphPad PRISM 6 (GraphPad Software, La Jolla California USA, www.graphpad.com) was used to draw graphs.

RESULTS

Physicochemical characterization

Zeta Potential, PDI value and Particle Size of VCZ-Loaded Nano-Liposomes

After the preparation of the liposomal formulation of VCZ by the technique of thin-film hydration-sonication, particle size, PDI value, and zeta potential of synthesized NPs were measured by DLS, with the results being 80.91 ± 2.78 nm, 0.076 ± 0.012 and -2.33 ± 0.54 mV respectively. Comparing the results of Blank liposomal formulation, the findings were as follows: 53.46 ± 2.11 nm, 0.112 ± 0.01 and -11.8 ± 0.71 mV, respectively (Fig. 1). It should be noted that all tests were repeated in 3 rounds.

The SEM micrograph

The SEM analysis divulged the spherical shape of vesicles with a relatively uniform size distribution, with a majority being smaller than 100 nm (Fig. 2).

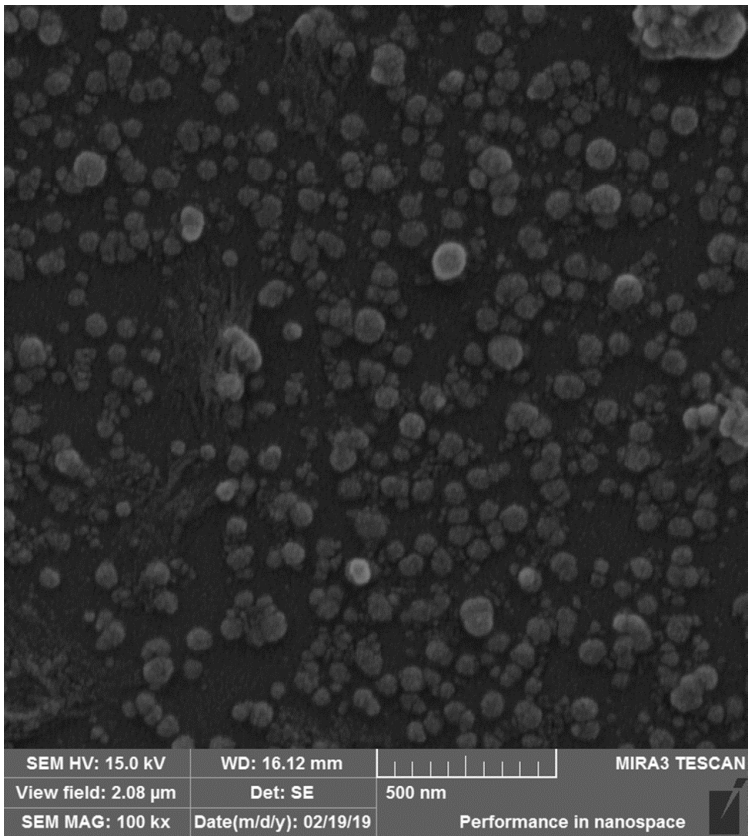

Fig. 2. Morphology of VCZ- loaded nano-liposome determined by SEM

Table 2. MIC50 and MIC90 values for VCZ and liposomal VCZ in the isolates of A. flavus. (Mean range) 0.32 -16 (µg/mL

	Antifungal Drugs	VCZ (MIC range) 0.32 -16 (µg/mL)		liposomal VCZ (MIC range) 0.32 -16 (µg/mL)	
Species (NO)	Site of isolation	MIC50	MIC90	MIC50	MIC90
489	fingernail	16	32	8	16
66041	nose	8	16	4	8
65822	sinus	8	16	2	4
65817	sinus	8	16	1	2
65811	sinus	8	16	2	4
65836	sinus	1	2	0.5	1
66246	nose	2	4	1	2
65770	sinus	1	2	0.5	1

Drug-encapsulation efficiency

In this study, liposomes utterly encapsulated VCZ; occupying roughly 97% of the formulation.

Antifungal activity

At this stage, in vitro susceptibility testing was carried out on clinical species of *A. flavus* according to the CLSI M38 3rd edition protocol in two separate sections. In the first stage, pure VCZ was used to determine MIC, and in the second stage, after preparing the liposome containing VCZ, the above test was repeated at the same concentration and once again, the MIC for all isolates was determined. It is worth noting that all the steps were done in duplicate and the results are presented in Table 2.

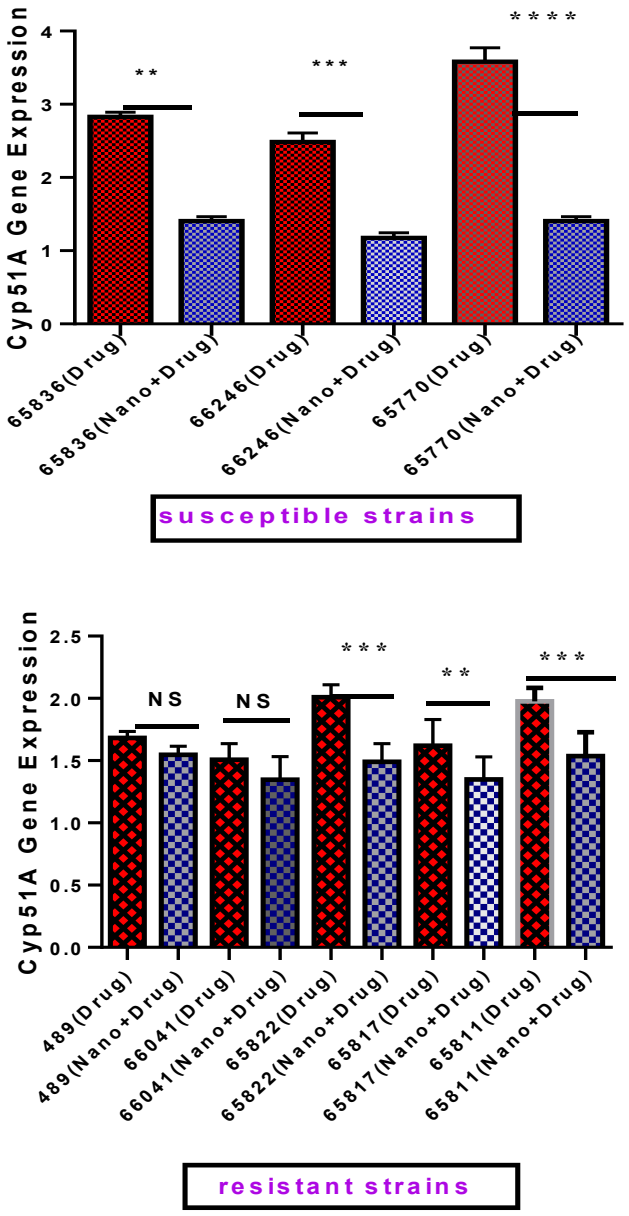

Fig. 3. Cyp51A gene expression in VCZ-resistant *A.flavus* isolates and VCZ-susceptible *A.flavus* isolates

Quantitative real-time polymerase chain reaction (qRT-PCR)

The MIC50 concentration of VCZ and Liposomal formulation of VCZ were used to evaluate Cyp51A and MDR1 mRNA levels by qRT-PCR in voriconazole-resistant *A.flavus* strains and voriconazole-susceptible strains (Fig. 3 and 4). After using MIC50 concentration of VCZ, the cyp51A gene expression in VCZ-susceptible *A. flavus* strains and VCZ-resistant strains 10folds and 7folds depicted a downregulation, respectively, which was more pronounced in the expression of a liposomal formulation of VCZ (13folds and 15folds respectively). This procedure was applied exactly to MDR1, even though it induced 1, 2, 3, 4-fold reductions (Fig. 5).

DISCUSSION

Today, due to the increasing importance of aspergillosis, an opportunistic infection especially in immunocompromised individuals whose mortality is due to resistance to drugs, the

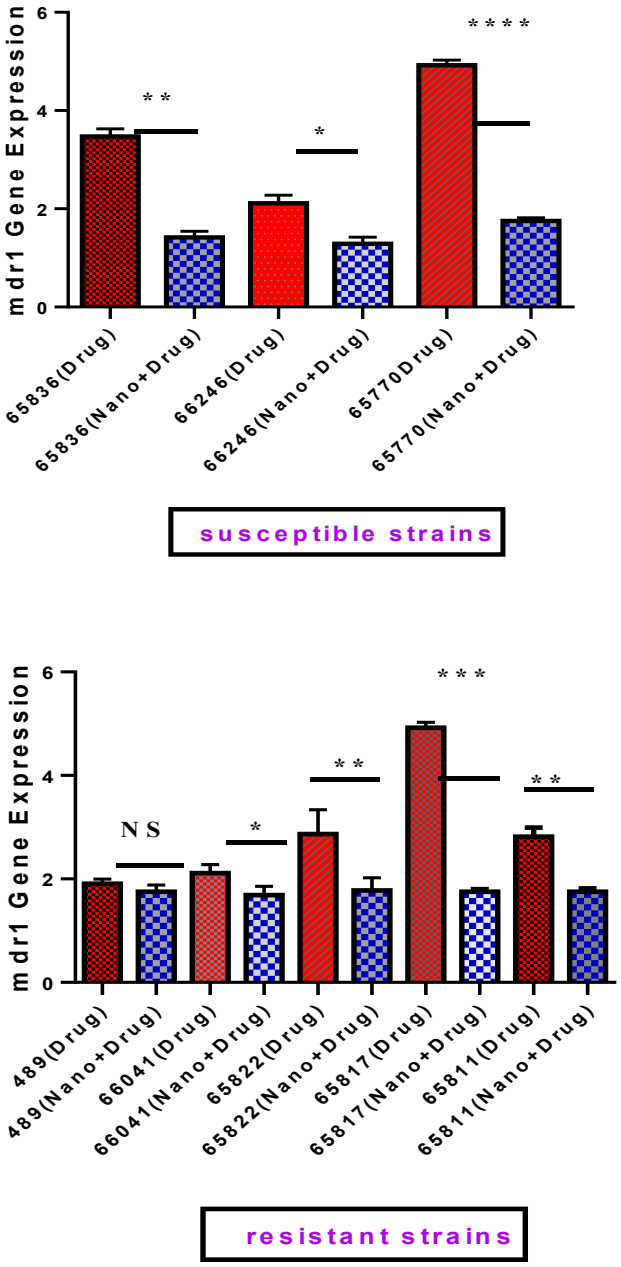

Fig. 4. MDR1 gene expression in VCZ-resistant *A.flavus* isolates and VCZ-susceptible *A.flavus* isolates

identification of *Aspergillus* species and deploying an appropriate effective drug are of immense significance. Therefore, in this study, those *A. flavus* strains which were isolated from Iranian patients with different manifestations of aspergillosis were studied, revealing a better efficacy of VCZ-loaded nano-liposome in the removal of VCZ-resistant *A.flavus* isolates when compared to pure VCZ. In other words, the main objective of this study was to attain the best formulation for both particle size and VCZ encapsulation to better investigate the antifungal properties of the formulations compared with pure VCZ on isolates, what is more, cyp51A and MDR1gene, known as two salient drug resistance genes in resistance to VCZ in *A.flavus* isolates, were studied in terms of their expressions. Fortunately, we were able to gain positive results in this regard.

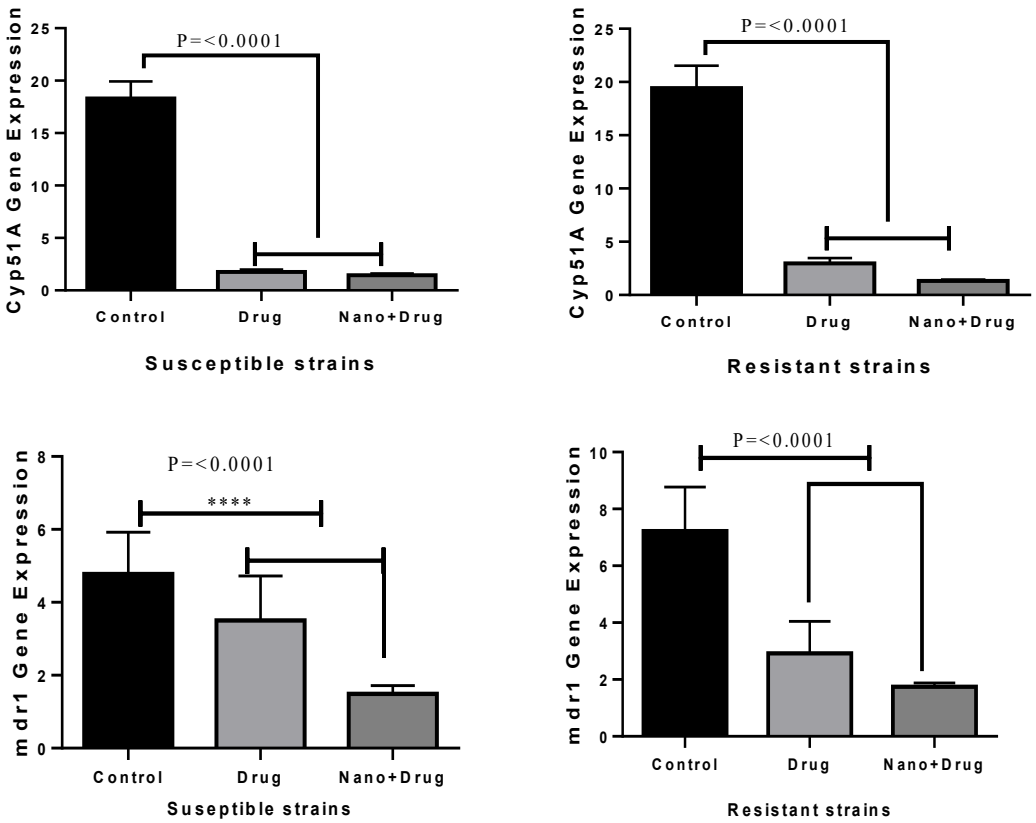

Fig. 5. After using MIC50 concentration of VCZ, the cyp51A and MDR1 gene expressions in VCZ-susceptible *A. flavus* strains and VCZ-resistant strains depicted a downregulation which was more pronounced in the expression of a liposomal formulation of VCZ.

In this study, after the preparation of the liposomal formulation of VCZ by the technique of thin-film hydration-sonication, the physicochemical characterization of synthesized NPs was measured by DLS. The adhesive and interactive properties of liposomal systems such as nanocarriers in the face of biological cells are predominantly determined by their particle size, thus, adequate and decent drug accumulation in fungal cells is conceivable due to the nanometer range of NPs (29). Plus, the antifungal activity directly regulates the NP cell-uptake efficiency (16). The stability of the colloidal system and the status of superficial electrical dispersions are decided by zeta potentials indicating a negative surface charge (30). The zeta potential of the colloidal system is magnified when cholesterol is added; and this, in turn, boosts the stability of the particles, in the same vein, bilayer phospholipid gains rigidity and rigor when cholesterol builds up (23). Zeta potential edges towards negativity when the lipid composition of liposomal formulations houses both cholesterol and lecithin (31). Absolute zeta potential >30 mV generally is an indicator of acceptable stability (16). A PDI of 0.3 and below is reckoned acceptable, representing a homogenous population of phospholipid vesicles, when liposome and nanoliposome formulations, known as lipid-based carriers, are utilized in drug delivery applications. Generally, mono dispersion of the suspension arises when PDI fluctuates between 0 and 0.5, however, poly dispersion emerges if PDI exceeds 0.5 (32), hence, the good stability of these NPs is further validated. Then the scanning electron microscope was used to probe the shape of the particles. In this study, SEM analysis divulged the spherical shape of vesicles with a relatively uniform size distribution, with a majority being smaller than 100 nm. After that, the encapsulation efficiency (EE%) of prepared nanoliposome showed a significant entrapment of VCZ in the nanoliposomes. In a study conducted by Tian et al. in 2015, after preparing VCZ loaded nanostructured lipid carriers by melt, high-pressure homogenization, they also reported that VCZ was successfully entrapped into loaded nanostructured lipid carriers (NLC) NPs, with EE% of VCZ-NLCs being 75.37% (16).

According to the literature review, this was the first study on nano-liposome containing VCZ on VCZ-resistant *A.flavus* isolates in Iran. According to the findings of this study and our hypothesis, the effect of nano-liposome containing VCZ on the reduction of MIC in VCZ-resistant *A.flavus* isolates was considered to be significant. In a study by Tian et al. who investigated the effect of VCZ nanostructured lipid carriers against *Candida albicans*, it was reported that VCZ-NLCs perpetuate the antifungal activity of VCZ and boost the efficiency of antifungal drug delivery to *C. albicans* (16). In another study by Asadi et al. which was conducted on the antifungal effects of nanoliposomal fluconazole on resistant *Candida* species, the same results on fluconazole were obtained and the positive effect of nanoliposomal fluconazole formulations in reducing MIC compared to pure fluconazole was reported (23). A further study by Sarrafha on nanoliposomal fluconazole in *A. flavus* and *A. fumigatus* isolates yielded positive results of nanoliposomal formulation as opposed to pure drug regarding antifungal effects (24).

Based on our results and in line with the previously performed experiments on *Aspergillus* spp., unlike the susceptible strains, the VCZ-resistant strains manifested a striking up-regulation in cyp51A gene (5folds) ($P<0.0001$) as well as MDR1 gene (7folds) ($P<0.0001$) (4). As noted, in several studies, point mutations and overexpression of cyp51A and MDR1 genes are responsible for the resistance of some *Aspergillus* spp. to azoles such as VCZ (5, 33, 34). In a study conducted by Sharma et al. on VCZ-resistant *A. flavus* clinical isolates, the upregulated levels of MDR1 and cyp51A gene expressions were reported (35) which confirms our study. In this study, after observing the elevated expression of genes in resistant isolates as compared to sensitive isolates, to evaluate the effect of pure VCZ as well as the liposomal formulation of VCZ, the level of gene expression was re-evaluated in two stages. The results of this phase overlapped with the results of *in vitro* antifungal susceptibility testing. That is, after using MIC50 concentration of VCZ, the cyp51A gene expression in VCZ-susceptible *A. flavus* strains and VCZ-resistant strains 10folds and 7folds depicted a downregulation, respectively, which was more pronounced in the expression of a liposomal formulation of VCZ (13folds and 15folds respectively). This procedure was applied exactly to MDR1, even though it induced 1, 2, 3, 4-fold reductions (Fig. 5). According to the available literature, the positive effect of liposomal VCZ formulation versus pure VCZ can be observed in the number of gene expressions in drug-resistant species, in particular.

CONCLUSIONS

The results of this study and the subsequent complementary *in vivo* studies promise the utilization of VCZ-resistant *A.flavus* isolates to formulate liposome-containing soon. On the other hand, considering the benefits of this drug formulation, such as the reduction of the side effects of the pure drug as well as minimizing the drug's toxicity coupled with the enhanced drug bioavailability and stability, the formulation can also be used in drug-sensitive species. The yielded results emanating from the application of liposome containing VCZ give good grounds for an alternative therapeutic platform and fruitful treatment of invasive aspergillosis.

ACKNOWLEDGMENTS

This study was financially supported by Tabriz University of Medical Sciences, Tabriz, Iran and the study was approved by the ethics committee of Tabriz University of Medical Science (Grant no. IR.TBZMED.VCR.REC.1398.103).

ETHICAL ISSUES

None to be declared

CONFLICT OF INTEREST

The authors declare no conflict of interest in this study.

AUTHORS' CONTRIBUTIONS

S.N., P.H.; participated in the design of the study, contributed to data collection and wrote the manuscript. H.H., B.B., M.M., N.S., A.S., T.H.; carried out the experiments and performed the statistical analysis and have been involved in critically revising the manuscript for important intellectual content. All authors read and approved the final manuscript.

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