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Glucose directly promotes antifungals resistance in the fungal pathogen, *Candida* spp.

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Running title: ***Effect of glucose on antifungal susceptibility***

Key words: Antifungal agents, Antifungal susceptibility, Diabetes mellitus, Glucose

Backgroud: Glucose level alters the susceptibility of antifungal agents during chemotherapy in diabetes patients.
Results: Glucose selectively interact to antifungal agents, strongly affects azole durgs and form complex by hydrogen bonding, subsequently reduced the susceptibility.
Conclusion: It is flagrant to researchers and pharmaceuticals for making new antibiogram of diabetes patients.
Significance: Selection of drugs is important to control fungal infections in diabetes patients

ABSTRACT

Effects of glucose on the susceptibility of antifungal agents are investigated against *Candida* spp. Increasing the concentration of glucose decreased the activity of antifungal agents, voriconazole was mostly affected drugs followed by amphotericin B. No significant change has been observed for anidulafungin. Biophysical interaction between antifungal agents with glucose molecules were investigated using ITC, FTIR and ^{1}HNMR. Glucose have higher affinity to bind with voriconazole by hydrogen bonding and decrease the susceptibility. In addition to confirm the results observed *in vitro*, theoretical docking studies demonstrated that voriconazole presented three important hydrogen bonds and amphotericin B presented two hydrogen bonds that stabilized the complex compound-glucose. *In vivo* results also suggest that the physiologically relevant higher glucose level in blood stream of diabetes mellitus (DM) mice might interact with the available selective agents during antifungal therapy, decreased the activity by complex formation. Thus, selection of drugs for DM patient is important to control the infectious diseases.

INTRODUCTION

Among several emerging diseases, DM is considered one of the largest emerging threats to public health in 21st century. From medical profession to general public are well accepted that greater frequency of metabolic disorders to morbimortality in diabetic patients is due to the hyperglycemic environment. Extra glucose in blood and urine provides an increased propensity to develop infections, especially prone to foot infections, yeast infections, urinary tract infections and surgical site infections [1,2]. The prevalence of yeast infection is more common with higher density of candidal growth in patients with DM [3,4]. *Candida* species are the part of our body's normal oral and intestinal flora, infection becomes sever in DM patients with high-minded inflammation [5-6]. Antifungal medications, such as clotrimazole, nystatin, fluconazole, and ketoconazole are very effective to control topical yeast infections. In case of Candidial infections in blood,

intravenous fluconazole or echinocandin or Amphotericin B (AmpB) is the choice of drugs [7-8]. No such specific indication for choice of drug for people suffering with DM. When a person suffering with DM and have a yeast infection, he/ she is more likely to get other infections because the combination of *Candida* and high blood sugar inhibits the body's natural defense mechanisms to fight against other bacteria and viruses. Thus, increased sugar level is seems to be the main offender for infection prevalence in body. This article aims to address the biophysical mechanism of glucose level to alter the susceptibility of antifungal agents during chemotherapy.

RESULTS

The antifungal activities of anidulafungin, amphotericin B and voriconazole were tested against two model fungal strains, *C. albicans* and *C. tropicalis* with variation of glucose concentration. The antifungal activities of tested drugs were ranged between 1.562 to 3.125 against *C. albicans* and 0.19 to 1.562 against *C. tropicalis* in absence of glucose. Interestingly, the MIC values are altered with increasing the glucose concentration only for voriconazole and amphotericin B but remain unchanged with anidulafungin (Table S1). Voriconazole is more sensitive to glucose and decreased four folds activity in the presence of 2% glucose in culture medium, whereas amphotericin B showed only two fold reduction.

Titrations between glucose and antifungal drugs were done using ITC and the binding constant (K), entropy (ΔS), and enthalpy (ΔH) of their reactions are described in Table S2. The binding theromgram and isotherms are clearly indicates that the interactions of antifungals with glucose are exothermic in nature because ΔH was negative. The binding affinity was very strong to voriconazole, followed by AmpB whereas interaction of anidulafungin with glucose molecules shows very low binding constant (Fig. 1, upper panel).

FTIR analysis of voriconazole (Table S3) revealed the presence of C=N, C-N, C-F and C=C stretching frequency in the region between 1640-1690, 1230-1000, 1495–1451, 1585–1451 cm^{-1}. Moreover, C-O stretching frequency of tertiary alcohol group gives a strong peak at 1089 cm^{-1}. The shifting of C-F stretching at lower frequency from 1456 to 1421 $cm^{-1}$and C-O stretching at higher frequency from 1080 cm^{-1} to 1161 cm^{-1} suggest that both fluorine and tertiary alcohols of voriconazole undergoes in strong hydrogen bonding with glucose. Moreover, another characteristic band of C-N stretching frequency is shifted at lower frequency from 1247 cm^{-1} to 1161 cm^{-1} in azole ring. In case of amphotericin B, the spectral region between 1500 and 1800 cm^{-1} represents the stretching vibrations of the C-O, -COO-,-NH_3^+ and C-C groups. 3300–3500 cm^{-1} is characteristic for hydrogen-bonded AmpB molecules (-OH…HO-), in polyalcohol chain of molecules). The band centered at 1711 cm^{-1} is attributed to the stretching vibration of the C=O group in the ester band, at 1692 cm^{-1} and 1401 cm^{-1} for asymmetrical and symmetrical stretching vibrations of the C-O in ester group, respectively. The sharp band with a maximum centered at 1563 cm^{-1} arising together with the increase in surface pressure should be attributed to the vibrations of the - NH_3^+ group. It should be also added that the symmetrical deformational vibrations of - NH_3^+ in the amino acids in the zwitter ion form appear in the range between 1550 and 1485 cm^{-1}. This is indicated by the changes related to the increase in broad absorption at 1100-950 cm^{-1} frequency range, characteristic for vibrations of C-O-C bonds. The spectral shift from 1692 cm^{-1} to 1656 cm^{-1} is corresponding to the typical values for hydrogen bonds. The bands at 1330-1040 cm^{-1} range are assigned to the stretching and deformational vibration of C-O and C-H out of plane (*trans*-polyene) bonds, which further indicate their participation in the aggregation process. Moreover, the characteristics weak band observed at 1169 cm^{-1} shifted to 1163 cm^{-1} corresponding to the C-N stretching in ampB structure [13]. But, no significant change has been observed with anidulafungin FTIR analysis (Table S3).

^{1}H NMR experiment was next carried out to find out any H-bonding interaction between glucose and antifungal drugs. Comparison of the spectra pure glucose, pure drugs and a combined of the two, showed

definite interaction between glucose and voriconazole. This was confirmed by the appearance of a broad new peak at δH 6.5, which got exchange upon D_2O shaking (Fig. 1, lower panel). This indicates the signal to be due to a glucose hydroxyl. This signal was perhaps embedded in the upfield signals of pure glucose and underwent downfield shift on addition of voriconazole indicates slow H-bonding reaction. However, NMR spectra obtained for AmpB-glucose and anidulafungin-glucose did not show any significant effect (Fig. 2, a&b).

Furthermore, to confirm the data obtained *in vitro*, docking studies were realized with the aim to guide the resulted observed. In according with the resulted *in silico* the affinities of voriconazole and amphotericin B were analyzed forward d-glucose. For voriconazole and amphotericin B, the molecules were left close to glucose allowing random contact with the molecule. Voriconazole indicate greater affinity for the d-glucose by ITC and reinforced by NMR experiment. This was also corroborated by *in silico* studies comparing the numerous of interactions (three hydrogen bonds interactions observed), however the output energy encountered in the well defined cluster generated after data mining observed forward d-glucose was of -1.9 ± 0.1 Kcal.mol-1, while the energy observed for amphotericin B forward the d-glucose was -2.3 ± 0.6 Kcal.mol-1. The different affinity observed in silico for data obtained reflect in the length of amphotericin B, is a molecule more large than voriconazole, in this molecule was possible to observe due to length several good interactions possibilities, however all interactions not demonstrated more than two hydrogen bonds reinforcing the *in vitro* assays results. In addition, in a vision detailed of the interaction demonstrated that voriconazole form three hydrogen bonds between stabilizing the complex voriconazole-glucose. The interactions observed for voriconazole were among the hydrogen atoms (HO1, HO4 and HO6) of antifungal molecule and the atoms of nitrogen (N9, N7 and N8), with distances of 3.19, 3.09 and 3.19 Å, respectively (Fig. 2,c,i). On the another hand, amphotericin B was less interactive presenting two hydrogen bond among the oxygen atoms of amphotericin B (O) and the hydrogen of the glucose linked at carbons (C1H1 and C5H5), with distance of 2.46 and 2.78 Å, respectively (Fig. 2,c,ii).

Finally, the efficacy of tested antifungal drugs was evaluated in diabetic mice and exhibits the same trends as observed *in vitro* analysis. The colony count of *C. albicans* was significantly reduced in control mice (non-diabetic) with low blood glucose level in comparison to diabetic mice (Fig. 2,d). The activity of voriconazole was reduced almost six folds whereas 2.8 folds and 1.8 folds reduction observed for AmpB and anidulafungin, respectively, in diabetic mice compared to non-diabetic mice. Voriconazole was least active drug followed by amphotericin B and anidulafingin for diabetic mice infected with *Candida*.

DISCUSSION

The genus of *Candida* is commensals to human gastrointestinal and genitourinary tract. Persons those are suffering with DM, an extra glucose level in blood stream can cause several infectious diseases ranging from superficial candidiasis to deep seated mycoses [14]. Carbon source as a major component of culture medium plays an important role in growth of microorganism including *Candia* sp. Carbon compounds are generally ranges from sugars, organic acids, alcohols, and polysaccharides etc., while microbes are prefer simple sugars such as glucose, sucrose, maltose and lactose for their rapid growth and increased the population density [15]. Fungal load or count is an important parameter of antifungal susceptibility.

Our initial approach was to investigate the antifungal drug susceptibility in culture medium containing different antifungal agents in combination with fixed concentrations of glucose in each set of experiments. The apparent failure of azole (voriconazole) followed by polyene (AmpB) antifungal agent in presence of high glucose in medium. Antifungal showed the decreased rate of susceptibility with increasing the glucose concentration in culture medium. There might have several possibilities to decrease the susceptibility of antifungals but their selectivity is interesting. An attractive

study conducted by Rodaki et al. [16] based upon the global impact of glucose on *C. albicans* transcriptome for the modulation of carbon assimilatory pathways during pathogenesis. The study revealed that glucose concentrations in the bloodstream have a significant impact upon *C. albicans* gene regulation which reflects on the elevated resistance to oxidative, cationic stresses and resistance to an azole antifungal agent, miconazole. In this study, it was observed that no significant susceptibility level was observed for anidulafungin whereas voriconazole becomes maximum resistant followed by amphotericin B. This allowed us to check whether extra glucose level decreased the susceptibility to antifungals by only genes meditated as higher expression of genes involved in drug resistance or is there any direct effect of glucose on antifungals?

Several strategies including analytical to *in silico* analyses are used to check the interaction between antifungals with glucose. Result suggests that voriconazole and amphotericin B have the strong affinity to bind with excess glucose molecules through hydrogen bonding. It seems to be occurred that the complex of voriconazole-glucose or amphotericin B-glucose may not be the effective same as their pure molecule. Anidulafungin is safe for use in DM patient which does not show any strong affinity to glucose molecule, even *in vivo* evaluation with diabetic induced mice also revealed the potentiality of anidulafungin, this is might be due to the presence of amide bonding in their chemical structure which helps in self-association other than binds to glucose molecule. But, the presence of amine (-NH_2)- in AmpB and (-F-) in voriconazole promote the association with glucose molecule by hydrogen bonding.

Excess glucose level in blood stream not only alter the genetically change for their adaptation in microenvironment but also interaction in microenvironment is a factor for increase the resistant properties of pathogens. In the light of these findings, it is flagrant to researchers and pharmaceuticals for making new antibiogram of DM patients suffering with infectious diseases as crucial to practitioners.

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FOOTNOTES

The article contains two supplemental figures and two supplemental tables. The experimental procedures are described details in supplementary section.

Abbreviations*:* AmpB, Amphotericin B; CLSI, clinical and laboratory standards institute; DM, diabetes mellitus; FTIR, fourier transform infrared; ^{1}HNMR, proton nuclear magnetic resonance; ITC, isothermal titration calorimetry; MIC, minimum inhibitory concentration; RPMI, roswell park memorial institute medium.

Figure Legends

Figure 1. ITC based thermogram and binding isotherm plot of glucose with antifungal agents. Thermogram and binding isotherm obtained from interaction between glucose with Amphotericin B (a & b); with anidulafungin (c & d) and with voriconazole (e & f), respectively. Hydrogen bonding determination with deuterium exchange by ^{1}H NMR (lower panel). Spectra were obtained from pure glucose (g), pure voriconazole (h), and combination of both in d6-DMSO (i) and after deuterium exchange of voriconazole-glucose complex in d6-DMSO (j). Arrow in spectrum shows the chemical shift due to hydrogen bonding.

Figure 2. Interaction and *in vivo* susceptibility analysis of antifungal agents with glucose. . ^{1}H NMR spectra were obtained from pure glucose, pure anidulafungin, and combination of both in d6-DMSO (a) and similarly, from pure glucose, pure AmphotericinB (AmpB), and combination of both in d6-DMSO (b). Docking studies of voriconazole and amphotericin B forward d-glucose. The detailed interactions of the drugs toward d-glucose highlighting (i) three hydrogen bonds and (ii) two hydrogen bonds stabilizing the complex formed (c). Differential *in vivo* susceptibility of antifungal agents in diabetic mice during *Candida* infection (d). The streptozotocins induce diabetic Swiss albino mice model was used in this study. Bar diagram represent the efficiency of antifungals agents treated for candidal infection in diabetic mice and non-diabetic mice. The light grey color and light dark color bar with stretch line representing the diabetic and non-diabetic mice, respectively for each data set. The fungal load was significantly higher in diabetic mice in comparison to non-diabetic mice. Above bar represent group mean ± SD. *, $P < 0.05$ and **, $P < 0.01$ (diabetic mice versus non-diabetic mice).

Figure 1.

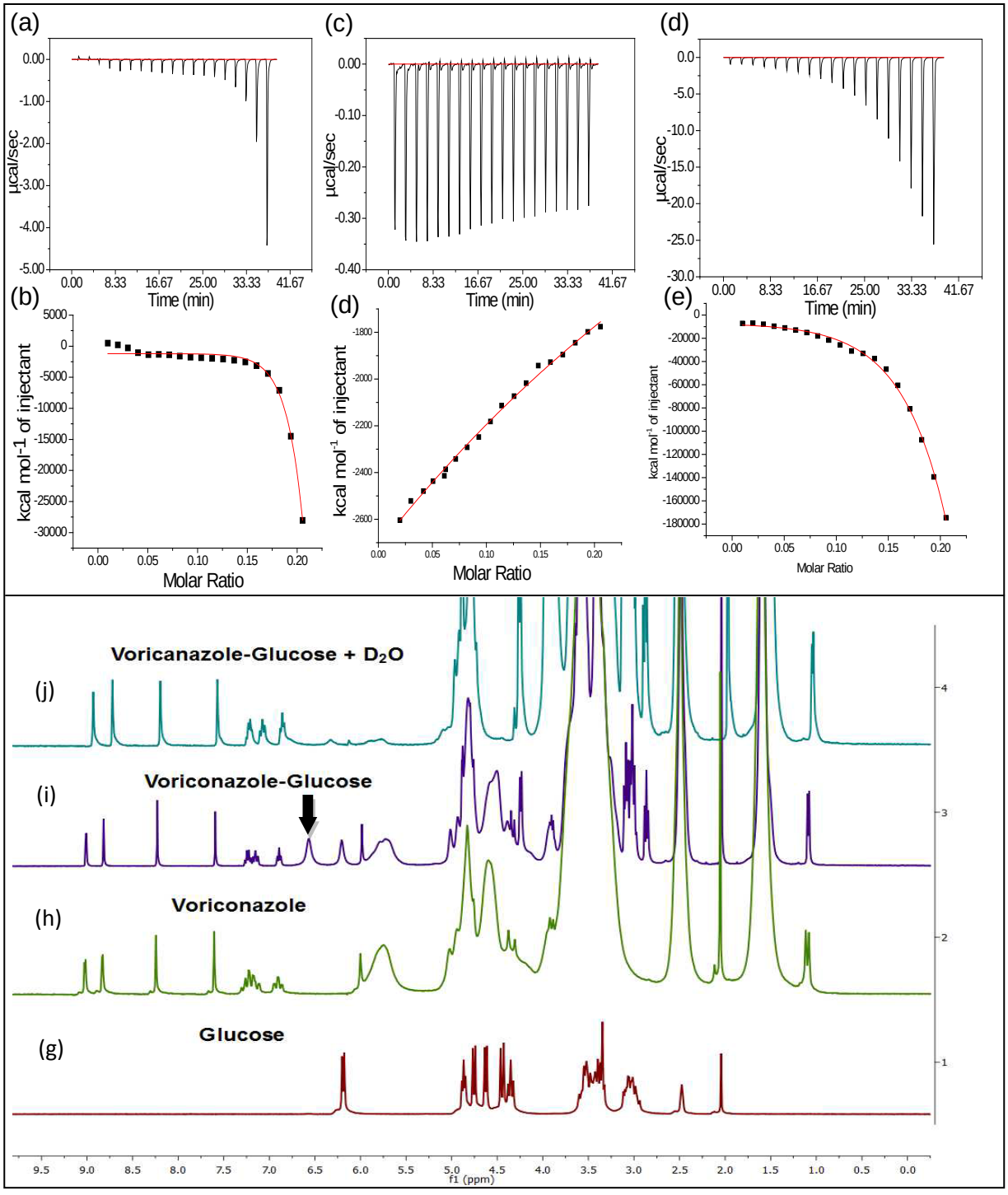
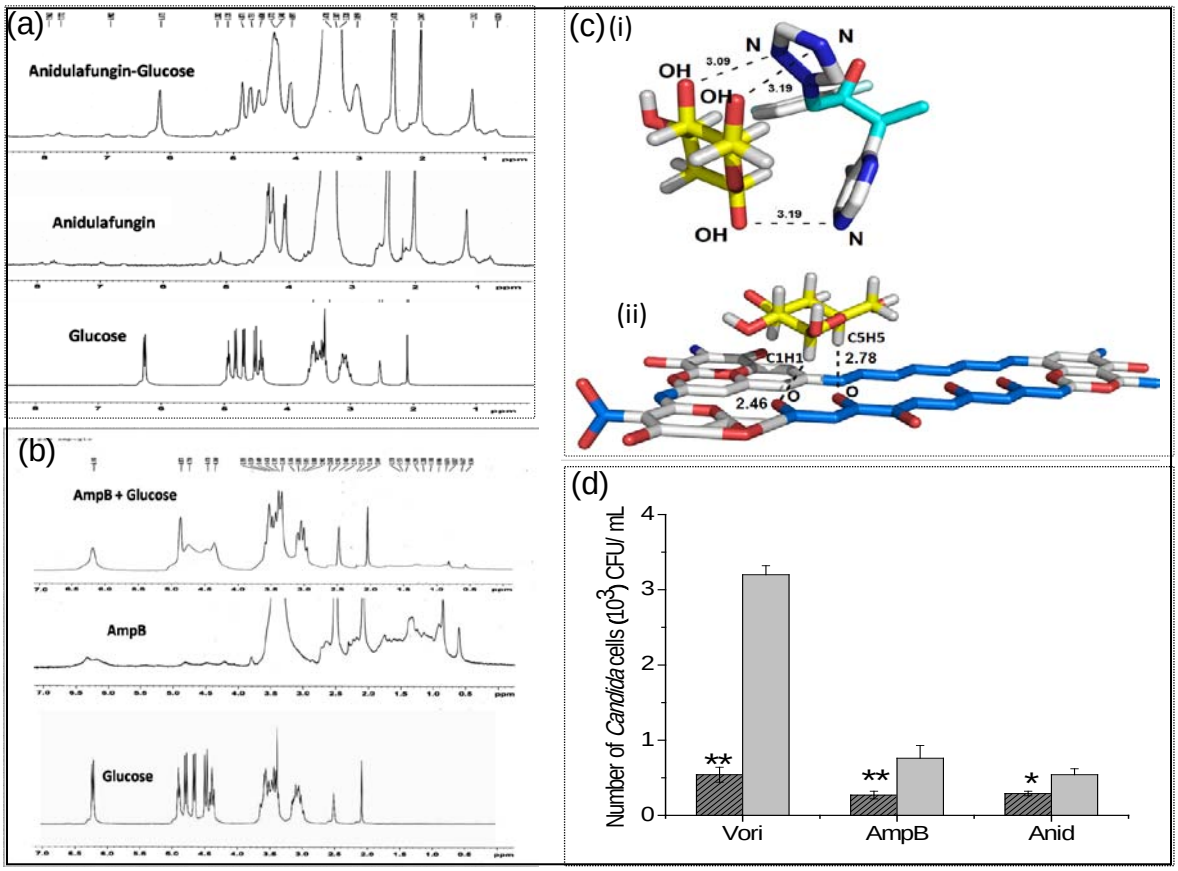


Figure 2.



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