Repositioning of Solifenacin and Hydroxyzine as Antibiofilm Agents in Candida Albicans.

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Abstract

Biofilm growth and drug resistance by Candida albicans can cause serious health problems in immunocompromised patients. Therefore there is a necessity for developing novel drugs. One of the strategies is repositioning of drugs. Solifenacin is a muscarinic receptor antagonist used for overactive bladder treatment. While Hydroxyzine is an anti-allergy drug and also it has mild anti-muscarinic receptor effect. In this manuscript, the effect of Solifenacin and hydroxyzine against C. albicans virulence factors is reported. It is found that Solifenacin and Hydroxyzine inhibited biofilm formation, adhesion, growth and morphogenesis in C. albicans. C. albicans Rrp9, an essential protein is found to exhibit identity and similarity with muscarinic receptor M1. A docking study between Hydroxyzine and C. albicans Rrp9 revealed good binding energy. Molecular docking studies between human muscarinic M1 receptor and muscarinic receptor antagonists, Solifenacin and hydroxyzine were carried out. It was found that both Solifenacin and Hydroxyzine could bind with muscarinic M1 receptor. Based on this study, it is suggested that Solifenacin and Hydroxyzine could be repositioned as anti-biofilm as well as anti-virulence agents in the human pathogen, C. albicans.

Keywords: Candida Albicans, Biofilm; Solifenacin; Hydroxyzine; Molecular docking

Introduction

The opportunistic fungus, Candida albicans can easily form biofilm on host tissues and implanted devices which may cause high morbidity and mortality in humans [1-4]. Prosthetic devices implanted in a patient's body such as artificial heart valves, venous catheters, central nervous system prostheses, intrauterine devices, urinary catheters, denture materials, joint prostheses and contact lenses are susceptible to be colonized by C. albicans [5]. Biofilm formation and drug resistance are a serious concern among immune-compromised individuals, hospitalized patients, and those using various medical implants [6]. C. albicans develops biofilm on implanted devices, which can release free cells in the body, which can colonise other places causing serious complicated infections [7]. Currently used antifungal drugs can cause toxic side effects [8]. The side effects due to the toxicity of available antifungal drugs make it a necessity to search for novel molecules with potential anti-biofilm activity. One of the strategies is repositioning of drugs. Human muscarinic acetylcholine receptors (M1-M5) are G protein coupled receptors, which have important roles in human physiology [9-17]. Muscarinic receptors play crucial roles in various physiological processes in humans [18]. Many drugs act on muscarinic receptors and contribute to the treatment and management of various diseases. Solifenacin is antimuscarinic receptor antagonist that can target muscarinic M3 receptor as well as M1 receptor [19]. It can stop the Overactive Bladder (OAB) by blocking muscarinic receptor activity on muscle cells. Hydroxyzine is an antihistamine medicine that can target histamine H1 receptor and it has mild antagonistic activity against muscarinic receptor [20]. Aim of this study was to reposition Solifenacin and Hydroxyzine as anti-biofilm agents.

Materials and Methods

Media and chemicals

Solifenacin succinate and Hydroxyzine hydrochloride tablet forms were purchased from medical store. A 2, 3-bis (2-methoxy-4-nitrosophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and menadione were purchased from Sigma-Aldrich Chemicals Ltd. Components of media and plates were purchased from HIMEDIA Chemicals Ltd, Mumbai, India.

Candida albicans culture

The standard strain, C. albicans (ATCC 90028) was obtained from the Institute of Microbial Technology (IMTECH) Chandigarh, India. C. albicans (GMC-16) strain was obtained from Government Medical College, Nanded, Maharashtra, India. The culture was kept up on Yeast Peptone Dextrose, (YPD) agar slant at 4°C. One single colony was transferred from the YPD agar plates into YPD broth in 250-ml conical flask and incubated overnight at 30°C at 100 rpm in an orbital shaker incubator. The cells were collected by centrifugation at 2000 rpm and washed thrice with sterile 0.1 M phosphate-buffered saline pH 7.4 [21].

Hyphal formation assay

Yeast to hyphal form transition assay was carried out in microtitre plates (96 wells) [22]. From C. albicans cells stock, a 1×10^6 cells/mL was prepared in 5% serum. The concentrations of Solifenacin and Hydroxyzine were prepared and added in each well separately. The controls were kept without drugs. 200 µL was final volume in each well. Plates were incubated at 37°C at 120 rpm for 2 hrs. After incubation, cells were observed microscopically by using an inverted light
microscope (Metzer, India) to note yeast and hyphal form transformation. All the experiments were done in triplicates.

**Antifungal activity**

Minimum Inhibitory Concentration (MIC): The standard methodology M27 A2 as per CLSI guidelines was utilized to determine the MIC. Serial double dilution of Solifenacin and Hydroxyzine concentrations and 1×10^3 cells/mL of *C. albicans* cells stock was prepared in RPMI-1640 medium and added in the 96-well microtitre plates. Two hundred microlitre of RPMI-1640 medium was kept in each well as a final volume. The wells without addition of drugs saved as a control. The microtitre plates were held and incubated at 37°C for 48 hrs. After incubation period, plates were held on the microplate reader (Multiscan Ex, Thermo Electronic Corp, USA) to take the absorbance. A 50% reduction in the absorbance was compared to the control and it was considered as the Minimum Inhibitory Concentration (MIC) [21]. All the experiments were done in triplicates.

Minimum Fungicidal Concentration (MFC): Determination of MFC was carried out by selecting the wells of MIC and above this concentration of Solifenacin and Hydroxyzine against *C. albicans* growth. Contains of wells were mixed. A 10 µL of cells suspension was transferred and spread on YPD agar plates. Plates were incubated at 30°C for 24 hrs. After incubation period, the colonies growth was observed. Absence of visual colonies growth was considered as fungicidal concentration [23]. All the experiments were done in triplicates.

**Adhesion assay**

Adhesion of cells on polystyrene surface was studied using 96-wells plates. Double dilutions of various concentrations of Solifenacin and Hydroxyzine were prepared in sterile PBS and added in each well. A 50 µL was added in each well to get 1×10^3 cells/mL. Wells without Solifenacin and Hydroxyzine were kept as a control. The plates were incubated at 37°C for 90 min at 100 rpm. Then, the wells were washed by sterile PBS to expel nonattached cells. XTT metabolic assay was used to analyses metabolic activity of cells in the early and mature biofilms. All the experiments were done in triplicates.

**Biofilm formation**

*C. albicans* biofilms were developed on polystyrene surface of 96-wells plates as per standard method. Hundred microlitre of cells suspension (1×10^3 cells/mL) was added in each well. The plates were incubated at 37°C for 90 min at 100 rpm. The cells were washed by sterile PBS to remove non adhered cells. For, the early biofilm formation, double dilution of serial concentrations of Solifenacin and Hydroxyzine were prepared in RPMI-1640. Then, 200 µL was added in each well. The controls were kept without drugs. Then, the plates were incubated at 37°C for 48 hrs for allowing of biofilm formation. After incubation, the plates were washed with sterile PBS and were observed under the inverted light microscope (Metzer, India) to watch the presence of biofilm. For mature biofilm, two hundred of RPMI-1640 was added in each well included adhered cell. The plates were incubated at 37°C for 24 hrs to permit of biofilm formation. After that the plates were washed with sterile PBS. Various concentrations of Solifenacin and Hydroxyzine were prepared in RPMI-1640 by double dilution. Then, 200 µL was added in each well and plates were incubated at 37°C for 48 hrs. Again, the plates were washed using sterile PBS [21]. The XTT metabolic assay was used to analyse metabolic activity of cells in the early and mature biofilms. All the experiments were done in triplicates.

**XTT assay for biofilm quantitation**

Wells of early and mature biofilms were washed using sterile PBS. Eighty microliters of sterile PBS was added in each well, and then, 20 µL of XTT-menadione reagent was added. The plates were incubated in dark condition at 37°C for 3 hrs to allow of colour formation. The absorbance of colour formation was taken at 450 nm using a micro plate reader (Multikan Ex, Thermo Electron Corp. USA). Wells without Solifenacin and Hydroxyzine were kept as a control. The concentration of Solifenacin and Hydroxyzine which caused ≥ 50% reduction of biofilm formation was considered as MIC [21]. All the experiments were done in triplicates.

**Kill curve assay**

The effect of Solifenacin and Hydroxyzine against *C. albicans* (ATCC90028) was examined using time dependent kill curve assay. The MFC of Solifenacin as well as Hydroxyzine were added separately in each 10 mL sterile RPMI-1640 which has a 2.5×10^3 cells/mL. The mixture was incubated at 30°C at 100 rpm. Then, 500 µL was taken at several time intervals ( zero time, 15 min, 30 min, 1 hr, 2 hrs, 4 hrs, 5 hrs, 6 hrs, 7 hrs and 8 hrs), and washed with sterile PBS to remove a drug carryover effect. The collected pellet was re-suspended in 50 µL sterile PBS and was spread on YPD agar plates and incubated at 30°C for 48 hrs. After incubation the colonies were observed and counted. Growth of colonies on YPD agar plates was compared with the plates which have no drugs [24]. All the experiments were done in triplicates.

**Scanning electronic microscopy of *Candida albicans* biofilm formation on silicon discs**

*C. albicans* biofilm was developed on sterile or pharyngeal silicon rubber airway discs seeded in microtitre plates [22]. A 2 mL of 1×10^3 cells/mL was put in each well with sterile disc. The plate was incubated at 37°C for 90 min at 100 rpm to permit of cells adhesion on discs. After incubation, the discs were washed thrice using sterile PBS. The discs were transferred gently to sterile wells. Then, concentration of 1 mg/mL from Solifenacin and concentration of 0.5 mg/mL from Hydroxyzine were prepared in RPMI-1640. RPMI-1640 without drugs kept as a control. Plates were incubated for 48 hrs at 37°C at 60 rpm. Then, the discs were washed again using sterile PBS and fixed in 2.5% glutaraldehyde for 24 hrs at 4°C. After that the discs were post-fixed in 2% aqueous solution of osmium tetroxide for 4 hrs. Then, the fixed discs were dehydrated in series of graded alcohols and finally dried to a critical drying point. The discs were held over stubs, and coated by gold using an automated gold coater (Model: JOEL JFC-1600, JOEL Limited, Akishima, Tokyo, Japan) for 5 min. Then, the coated discs were held on scanning electron microscope objective (Model: JOEL-JSM 5600, JOEL Limited, Akishima, Tokyo, Japan) and photographs were taken. All the experiments were done in triplicates.
Docking Study

Ligand preparation

The structure of Hydroxyzine and Solifenacin was retrieved from Pubchem database. The structures were followed by 2D structure cleaning, 3D optimization and viewing is done using Marvin View and saved in Mol2 file format. Mol2 files were converted to PDBQT file format using Auto dock Tools version 1.5.6rc2 [25].

Molecular docking

Auto dock Tools package version 1.5.6rc2 was utilized to produce docking input files. All the non-polar hydrogen’s were combined and the water molecules were evacuated. Before docking, all the water molecules were expelled from the protein structures and followed by addition of hydrogen atoms to the receptor and combined non-polar hydrogen’s. The modeled three dimensional structure (3D) of *C. albicans* Rrp9 [26] and the structure of the ligand Hydroxyzine were changed over to PDBQT format. Also, the 3D structure of human muscarinic receptor M1 [26] and the structures of the ligands Solifenacin and Hydroxyzine were changed over to PDBQT format. Molecular docking study of hydroxyzine with *C. albicans* Rrp9 was done using Auto Dock®. Also, molecular docking studies of solifenacin and Hydroxyzine with muscarinic M1 receptor were carried out by using AutoDock® [27]. Default optimization parameters were done utilizing Lamarckian Genetic Algorithm with a population size of 150 dockings. Autodock® tools produced sixty possible binding conformations, i.e. sixty runs for each docking by utilizing Genetic Algorithm (GALS) searches. The grid box was utilized for indicating the search space was set at 60×60×60 centred on proteins with a default grid point spacing of 0.375 Å. Autogrid was used to get pre calculated grid maps. After completion of docking, most appropriate conformation was chosen based on lowest docked energy. Chosen conformations were analyzed by Autodock tool [25].

Statistical Analysis

Results

Solifenacin and hydroxyzine inhibit yeast to hyphal form transition

Solifenacin and Hydroxyzine hindered yeast to hyphal form transition induced by serum in both strains of *C. albicans* (ATCC 90028 & GMC-16). Solifenacin hindered this transition in *C. albicans* (ATCC 90028) at 0.031 mg/mL (Table 1 and Figures 1a and b) and in *C. albicans* (GMC-16) at 0.125 mg/mL Table 1 and Figure 1a (B). Hydroxyzine blocked hyphal formation induced by serum in both strains of *C. albicans* (ATCC 90028 & GMC-16) at 0.125 mg/mL (Table 2 and Figure 1c).

| Sr. No. | C. albicans strain | MIC (mg/mL) | Morphogenesis induced by serum | Planktonic assay | Adhesion | Developing biofilm | Mature biofilm | MFC |
|---------|---------------------|-------------|--------------------------------|------------------|----------|--------------------|---------------|-----|
| 1       | *C. albicans* (ATCC 90028) | 0.031       | 0.5                            | 0.125            | 0.25     | 0.5                | 0.5           | 1   |
| 2       | *C. albicans* (GMC-16)   | 0.125       | 0.5                            | 0.125            | 0.25     | 0.5                | 1             |     |

Table 1: Minimum Inhibitory Concentration (MIC) of Solifenacin against virulence factors of *Candida albicans* (ATCC 90028 & GMC-16).

| Sr.no  | C. albicans Strain | MIC (mg/mL) | Morphogenesis induced by Serum | Planktonic assay | Adhesion | developing biofilm | Mature biofilm | MFC |
|--------|--------------------|-------------|--------------------------------|------------------|----------|--------------------|---------------|-----|
| 1      | *C. albicans* (ATCC 90028) | 0.125       | 0.5                            | 0.25             | 0.25     | 0.5                | 1             |     |
| 2      | *C. albicans* (GMC-16)   | 0.125       | 0.5                            | 0.25             | 0.25     | 0.5                | 1             |     |

Table 2: Minimum Inhibitory Concentration (MIC) of Hydroxyzine against virulence factors of *Candida albicans* (ATCC 90028 & GMC-16).
Figure 1a: Effect of Solifenacin and Hydroxyzine on *C. albicans* morphology induced by serum. A) *C. albicans* (ATCC 90028); B) *C. albicans* (GMC-16).

Figure 1b: Effect of Solifenacin on yeast to hyphal form transition induced by serum in *C. albicans* (ATCC 90028); A) Control; B) 0.031 mg/mL; C) 0.062 mg/mL; D) 0.125 mg/mL; E) 0.25 mg/mL; F) 0.5 mg/mL; G) 1 mg/mL; H) 2 mg/mL.

Figure 1c: Effect of Hydroxyzine on yeast to hyphal form transition induced by serum in *C. albicans* (ATCC 90028); A) Control; B) 0.031 mg/mL; C) 0.062 mg/mL; D) 0.125 mg/mL; E) 0.25 mg/mL; F) 0.5 mg/mL; G) 1 mg/mL; H) 2 mg/mL.

### Solifenacin and hydroxyzine inhibit planktonic growth

The concentration of Solifenacin at 0.5 mg/mL reduced planktonic growth by 50% in both strains of *C. albicans* (ATCC 90028 & GMC-16) (Figure 1d) and it considered as MIC. The Minimum Fungicidal Concentration (MFC) of Solifenacin against *C. albicans* (ATCC 90028) growth was 0.5 mg/mL (Table 1)(Figure 1e) while against *C. albicans* (GMC-16) showed at 1 mg/mL (Table 1). At 0.5 mg/mL Hydroxyzine could reduce planktonic growth by 50% in both two strains. (Table 2 and Figure 1b) MFC of Hydroxyzine was 1 mg/mL for both strains of *C. albicans* (ATCC 90028 & GMC-16) (Table 2) and (Figure 1f).

Figure 1d: Effect of Solifenacin and Hydroxyzine on *C. albicans* planktonic growth. A) *C. albicans* (ATCC 90028); B) *C. albicans* (GMC-16).

Figure 1e: Minimum Fungicidal Concentration of Solifenacin against *C. albicans* (ATCC 90028) growth is 0.5 mg/mL.

Figure 1f: Minimum Fungicidal concentration of Hydroxyzine against *C. albicans* (ATCC 90028) growth is 1 mg/mL.

### Solifenacin and hydroxyzine inhibit cells adhesion on polystyrene surface

Solifenacin at 0.125 mg/mL reduced 50% of adhesion cells in *C. albicans* (ATCC 90028 & GMC-16) (Table 1 and Figure 1c (A,B) and it considered as MIC. Hydroxyzine inhibited fifty percent of cells adhesion at 0.25 mg/mL in both strains of *C. albicans* (ATCC 90028 & GMC-16) on polystyrene surface (Figure 2a).
Solifenacin and hydroxyzine inhibit biofilm formation

Both Solifenacin and hydroxyzine inhibited developing and mature biofilms formation of *C. albicans* (ATCC 90028 & GMC-16) on polystyrene surface. At 0.25 mg/mL Hydroxyzine and Solifenacin inhibited developing biofilm in *C. albicans* (ATCC 90028 & GMC-16). Solifenacin and Hydroxyzine at 0.5 mg/mL inhibited mature biofilms in both strains (Figures 2b and c).

Hydroxyzine and solifenacin kill *Candida albicans* cells

The MFC, 0.5 mg/mL of Solifenacin killed 99% of *C. albicans* (ATCC 90028) inoculum within 2 hr while 100% of cells were killed within 4 hrs of exposure). Hydroxyzine killed *C. albicans* (ATCC 90028) cells within 30 min of exposure at MFC of 1 mg/mL (Figures 2d, 3a and b).

Solifenacin and hydroxyzine inhibit biofilm growth on silicon discs

Both Solifenacin and Hydroxyzine inhibited biofilm formation of *C. Albicans* (ATCC 90028) on silicon discs. Hydroxyzine inhibited biofilm formation at 0.5 mg/mL. At 1 mg/mL, Solifenacin could inhibit hundred percent of biofilm formation (Figure 4).
Hydroxyzine docks with Rrp9

The molecular docking interaction between Hydroxyzine and C. albicans Rrp9 showed that Hydroxyzine could bind with Rrp9 with a binding energy of -9.24 Kcal/mol (Table 3). Hydroxyzine could form hydrogen bond interaction with the residues GLU509 and ALA232 in the active site of Rrp9 (Table 3 and Figure 5a).

\[ \text{Ligand} \quad \text{Receptor} \quad \text{Run no.} \quad \text{Interacting residues} \quad \text{Interacting atoms (Amino acid .. ligand)} \quad \text{Hydrogen formed bond} \quad \text{Binding energy (Kcal/mol)} \quad \text{Electrostatic energy} \]

| Ligand       | Receptor   | Run no. | Interacting residues | Interacting atoms | Hydrogen formed bond | Binding energy (Kcal/mol) | Electrostatic energy |
|--------------|------------|---------|----------------------|-------------------|----------------------|--------------------------|----------------------|
| Hydroxyzine  | CaRrp9     | 25      | GLU509 ALA232        | OE1—H27 HN—O2     | 2                    | -9.24                    | -0.28                |

Table 3: Molecular docking interaction of Hydroxyzine with Candida albicans Rrp9 (Ca Rrp9) and human muscarinic M1 receptor (CHRM1).

Solifenacin and hydroxyzine interact with human muscarinic M1 receptor

Solifenacin and Hydroxyzine bound with muscarinic M1 receptor with binding energies of -7.58 and -6.68 Kcal/mol respectively. Solifenacin could interact with the amino acid residue ARG123 at the active site region of muscarinic M1 receptor by forming hydrogen bond interaction Hydroxyzine interacted with the residue ASN422 in the active site region of muscarinic M1 receptor (Table 4 and Figures 5b and c).

\[ \text{Ligand} \quad \text{Receptor} \quad \text{Run no.} \quad \text{Interacting residues} \quad \text{Interacting atoms (Amino acid .. ligand)} \quad \text{Hydrogen formed bond} \quad \text{Binding energy (Kcal/mol)} \quad \text{Electrostatic energy} \]

| Ligand       | Receptor   | Run no. | Interacting residues | Interacting atoms | Hydrogen formed bond | Binding energy (Kcal/mol) | Electrostatic energy |
|--------------|------------|---------|----------------------|-------------------|----------------------|--------------------------|----------------------|
| Solifenacin  | CHRM1      | 52      | ARG123               | HH21—O2           | 1                    | -7.58                    | -0.14                |
| Hydroxyzine  | CHRM1      | 12      | ASN422               | HD21—O1,O2        | 1                    | -6.68                    | 0.01                 |

Table 4: Molecular docking interactions of Solifenacin and Hydroxyzine with human muscarinic M1 receptor (CHRM1).
Discussion

Biofilm formation and drug resistance associated with *C. albicans* infection create serious health problems in patients who have implanted devices and in immune-compromised patients. In this manuscript, an attempt is made to reposition muscarinic receptor antagonists, Solifenacin and Hydroxyzine against virulence factors of *C. albicans*. It is shown that these molecules can inhibit biofilm formation, adhesion, yeast to hyphal form conversion, and planktonic growth in *C. albicans* (Tables 1, 2 and Figures 1-5). In *C. albicans* (ATCC 90028), Solifenacin can inhibit morphogenesis (Tables 1, 2 and Figure 1a (A) and adhesion of cells more than Hydroxyzine. Solifenacin have more effect against adhesion than Hydroxyzine in both strains. The essential protein, *C. albicans* Rrp9 implicated in pre-ribosomal processes has been reported to share identity and similarity with muscarinic M1 receptor [26]. Solifenacin is reported to bind with *C. albicans* Rrp9 protein [28]. It was found that Hydroxyzine also can bind with Rrp9 (Table 3 and Figure 6(a). The molecular docking interaction between Hydroxyzine and *C. albicans* Rrp9 showed that Hydroxyzine formed two hydrogen bond interactions with the residues GLU509 and ALA232 (Table 3 and Figure 6) while Solifenacin is found to have one hydrogen bond interaction with the residue GLU509 [28] in the active site of Rrp9. This is may affect the function of Rrp9 which may led to killing of *C. albicans* cells faster than Solifenacin (Figure 1f). Both Solifenacin and Hydroxyzine can bind with human muscarinic receptor M1 (Table 4 and Figure 6c). In humans, Hydroxyzine can target selectively histamine H1 receptor [29, 20] and it has affinity to muscarinic receptors [30] and also it is weakly antagonist to other receptors like serotonin 5-HT2A receptor, dopamine D2 receptor [31, 29]. Hydroxyzine may have targets other than muscarinic receptor like proteins in *C. albicans*. The selective muscarinic M1 receptor antagonist, dicyclomine is known to targets signal transduction genes and inhibits virulence factors in *C. albicans* [32]. Docking studies showed that dicyclomine can bind with *C. albicans* Rrp9 and human muscarinic receptor M1 [26]. This study confirms the hypothesis that *C. albicans* may have proteins like muscarinic receptors which may play a role in virulence. It is suggested that Solifenacin and Hydroxyzine could be repositioned as anti-biofilm as well as anti-*Candida albicans* agents.

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