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Design and synthesis of new drugs inhibitors of Candida albicans hyphae and biofilm formation by upregulating the expression of TUP1 transcription repressor gene

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Abstract

Candida albicans is a common human fungal pathogen that causes disease ranging from superficial to lethal infections. *C. albicans* grows as budding yeast which can transform into hyphae in response to various environmental or biological stimuli. Although both forms have been associated with virulence, the hyphae form is responsible for the formation of multi-drug resistance biofilm. Here, new compounds were designed to selectively inhibit *C. albicans* hyphae formation without affecting human cells to afford sufficient safety. The newly designed 5-[3-

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Author contributions

RH synthesized the compounds. SS designed and planned the manuscript. AA run the anticandida and cytoxic activities. BF run the gene expression analysis. AH helped on running the anti-*Candida* activities. SE run the QSAR and computational studies. MH helped on running the antimicrobial activities. AI helped on designing the manuscript. SS and AI supervised and provided the required resources of the manuscript. All authors helped on data interpretation, writing the manuscript and revising the final version.

Declaration of Competing Interest

The University of Sharjah has filed intellectual property rights concerning SR compounds.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2020.105327.

substitued-4-(4-substituedbenzyloxy)-benzylidene]-2-thioxo-thiazolidin-4-one derivatives, named SR, showed very specific and effective inhibition activity against *C. albicans* hyphae formation. SR compounds caused hyphae inhibition activity at concentrations 10–40 fold lower than the concentration required to inhibit *Candida* yeast and bacterial growths. The anti-hyphae inhibition activities of SR compounds were via activation of the hyphae transcription repressor gene, *TUP1*. Correlation studies between the expression of *TUP1* gene and the activity of SR compounds confirmed that the anti-*C. albicans* activities of SR compounds were via inhibition of hyphae formation. The newly designed SR compounds showed 10–40% haemolytic activity on human erythrocytes when compared to 100% haemolysis by 0.1% triton employed as positive control. Furthermore, theoretical prediction of absorption, distribution, metabolism, excretion, and toxicity (ADMET) of SR compounds confirmed their safety, efficient metabolism and possible oral bioavailability. With the minimal toxicity and significant activity of the newly-designed SR compounds, a future optimization of pharmaceutical formulation may develop a promising inhibitor of hyphal formation not only for *C. albicans* but also for other *TUP1*- dependent dimorphic fungal infections.

Keywords

Candida albicans ; TUP1 gene; Hyphae; Biofilm; Rhodanine; Thiazolidine-one

1. Introduction

C. albicans mostly causes superficial easily treated infections (Sydnor and Perl, 2011). However, it can hematogenously disseminate to become life-threatening particularly with immunocompromised patients or those receiving broad spectrum antibiotic treatments (Edwards et al., 1978; Elewski, 1998). In fact, *C. albicans* is one of the most common causes of healthcare-associated bloodstream infections (Geffers and Gastmeier, 2011; Wenzel and Gennings, 2005). *C. albicans* can switch between two major forms, yeast and hyphal forms (Brand, 2012). The switch from yeast to hyphae is recognized as one of the most virulent traits of *C. albicans* (Brand, 2012), since it is associated with the ability of the fungus to invade host tissues and form drug-resistance biofilms. Compared to yeast forms, *Candida* biofilms are 30–2000 times more resistant to various antifungal drugs (Hawser and Douglas, 1995; Seddiki et al., 2015). The mechanisms of resistance developed by *Candida* biofilms are very complicated. These mechanisms can include changes in critical gene expression, changes in the phenotype and formation of extracellular polymers; that affect the influx of antifungal agents (Douglas, 2003). Therefore, the majority of antifungal drugs show limited anti-biofilm activity (Touil et al., 2020).

Biofilms are architecturally complex structures consisting of basal yeast cells and poly-layers of hyphae encapsulated in extracellular matrix (Choi et al., 2016). Thus, biofilms are inherently resistant to most antifungal treatments, provide haven for *C. albicans* from host defenses and constitute a reservoir for recurrent infection (Cavalheiro and Teixeira, 2018; LaFleur et al., 2006). Consequently, biofilm structures are often the cause of failed antifungal treatment, and indeed are associated with greater mortality of patients with candidemia (Blankenship and Mitchell, 2006; Tumbarello et al., 2007). The contribution

of biofilm formation to *C. albicans* pathogenicity and drug resistance emphasizes the need for new anti-hyphae agents that can inhibit *Candida* biofilm formation and hence prevents recalcitrant infection.

Quorum sensing (QS) molecules accumulated in the extracellular *Candida* environment can affect the morphological characteristics of the pathogen, forcing the formation of hyphae and thereby favoring biofilm formation and resistance to antimicrobial agents. The best characterized QS molecule involved in *C. albicans* biofilm formation is farnesol, which suppresses filament formation and reduces biofilm size (Deveau and Hogan, 2011). Taken in consideration the specific behavior of QS molecules, we have been able to design new compounds with farnesol-like function, despite of structural differences.

It has been reported that thiazolidinedione derivatives (Fig. 1A) exert significant inhibitory activities against *C. albicans* hyphal growth (Chauhan et al., 2012). Similarly, rhodanine derivatives showed inhibitory effects on *C. albicans* biofilm (Orchard et al., 2004), bacterial biofilm (AbdelKhalek et al., 2016) and numerous fungal targets including mannosyl transferase 1 (PMT1) (Feldman et al., 2014). Furthermore, aromatic compounds such as 1,2-benzisothiazolinone (Alex et al., 2012) and pyridine-rhodanine (Orchard et al., 2004) derivatives (Fig. 1A) showed potent anti-*Candida* activities. Inspired by all these facts, we designed a hybrid structure made of rhodanine analogues coupled with a side chain made of two fused aromatic structures containing lipophilic group with electron withdrawing substitutions in order to generate high potent anti-*Candida* activities (Fig. 1B).

ADMET studies play a critical role to predict the successful discovery and development of a drug candidate whereas it provides important information that significantly reduces the time and cost required (Di et al., 2018). ADMET score used to predict the drug likeness of a compound. It is estimated that almost 50% of the drug candidates failed because of unaccepted efficacy and inappropriate ADMET properties at the therapeutic dose (Di et al., 2018).

2. Material and methods

2.1. General chemistry

Most chemicals and solvents employed in chemical synthesis were of analytical grade and, when necessary, were purified and dried by standard methods. Reactions were monitored by thin-layer chromatography (TLC) using pre-coated silica gel plates (kiesel gel 60 F254, BDH), and spots were visualized under UV light (254 nm). Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. Column chromatography was performed with Merck silica gel 60 (40–60 μM). ^{1}HNMR and ^{13}CNMR spectra were recorded on a Bruker spectrometer at 500 MHz. Chemical shifts were expressed in parts per million (ppm) relative to tetramethylsilane, and coupling constant (J) values were represented in hertz (Hz) and the signals are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectroscopic data were obtained through Electrospray ionization (ESI) mass spectrum. Detailed synthesis of SR compounds can be found at Supplementary Data file.

In order to define the structural geometry of the compound SR7, the relative stability of the isomers was optimized by MOE software. Energy minimization and different descriptors were calculated including the heat of formation (HF), potential energy (E), angle bend energy (E-ang), Dipole moment (DM) and the Total energy.

2.2. Organisms and growth conditions

Collections of bacterial and fungal strains were obtained from the laboratory of Dr. Ibrahim at Harbor-UCLA medical center, Torrance, California. The bacterial strains used in this study were the Gram-negative bacterium *E. coli* and the Gram-Positive bacterium methicillin-resistant *Staphylococcus aureus* (MRSA) LAC300. All bacterial strains were cultured on sterile Luria-Bertani medium (LB). The fungal strain used in this study was *C. albicans* SC5314. *C. albicans* was inoculated into sterile yeast potato dextrose broth (YPD) and incubated for 24 h at 37 °C (This was used as an initial culture for all studies described), and incubated in two different media, Yeast Nitrogen Base (YNB) broth or Roswell Park Memorial Institute medium (RPMI-1640). Colistin, vancomycin and Ketoconazole were purchased from (Sigma–Aldrich).

2.3. Determination of the antimicrobial activity

The antimicrobial activity of compounds **SR1–7** on agar plates, liquid broth media and MIC were measured according to modified Clinical and Laboratory Standards Institute (CLSI) and as described by Soliman et al., 2017 (Soliman et al., 2017a, 2017b). Briefly, 0.1 mL containing 10^5 CFU/mL was spread on Luria-Bertani (LB) agar plates. The plates were then incubated at 37 °C for 34 h with filter discs (8 mm diameter) saturated with different dilutions of the compounds (1–400 μg/mL).The inhibition zones (mm) were measured by determining the diameter of the clear area. Similarly, the activity in liquid media was measured by incubating the aforementioned concentrations of the compounds into LB broth media inoculated with 10^5 CFU/mL in 96-well microplates at 37 °C for 24 h. The turbidity representing the microbial growth was measured by microplate reader (DYNEX technologies) at OD_{600}. The MIC was the lowest concentration of the compound that prevented microbial growth (showed no turbidity). At low MIC values, the concentration was repeated with increasing/ decreasing the concentrations by 1 μg/mL at a time until the MIC was identified. At high MIC values, the concentration was repeated with increasing/ decreasing the concentrations by 5 μg/mL at a time until the MIC was identified. Each test was performed in triplicate. Ketoconazole, colistin and vancomycin were employed as positive controls against C. albicans, *E. coli* and *S. aureus* bacteria, respectively. Cultures without the compounds or antimicrobials were employed as negative control.

2.4. Inhibition of Candida biofilm formation

The MICs for cells forming biofilm were determined by microtiter plate assay as described previously by Soliman et al., 2017(Soliman et al., 2017a, 2017b). Briefly, 96-well polystyrene microtiter plate was seeded with 200μL of RPMI-1640 containing 10^6 C. albicans cells in the presence of the compound of interest at concentration range of 1–100 μg/mL. After 24 h of incubation at 37 °C, the biofilms were washed and the fungal viability was analyzed using 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide (MTT)

(Sigma) and the final absorbance was measured at 540 nm. The assay was performed in triplicate and repeated three times. The MIC of the compounds causing 100% inhibition of C. albicans biofilm formation was determined by measuring the metabolic activity of biofilm compared to solvent control.

2.5. Quantitative real time RT-PCR

To quantify the expression of *C. albicans* hyphae-specific genes (Table 1), *Candida* cells were grown in either yeast (YNB) or hyphae/biofilm (RPMI 1640 media) growing conditions as described previously in the presence of compounds **SR1–7** at10 μg/mL. The cultures were incubated in 6-well plates for 24 h at 37 °C. For yeast growing conditions, the cultures were collected separately, centrifuged and the pellets were washed by PBS prior to RNA extraction, cDNA synthesis and gene expression analysis. For hyphae growing conditions, the cultures were centrifuged, and the isolated hyphae are washed with PBS. RNA extraction was performed using Ribopure™ RNA purification kit (ThermoFisher) following the manufacturer's manual. Contaminating genomic DNA was removed from RNA samples by treatment with 4μL of Turbo-DNaseI (Invitrogen) for 30 min at room temperature. DNase was then removed using DNase inactivation reagent (Invitrogen). First-strand cDNA synthesis was performed using the Retroscript first-strand synthesis kit (Ambion). The amplification efficiency was determined by serial dilution experiments, and the resulting efficiency coefficient was used for the quantification of the products (Pfaffl, 2001). Gene expression was analyzed by an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the Power SYBR green PCR master mix (Applied Biosystems). PCR conditions were 10 min at 90 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Single PCR products were confirmed with the heat dissociation protocol at the end of the PCR cycles. The amount of gene expression was normalized to 18S rRNA (Morschhäuser et al., 2007) and then quantified using the $2^{(-\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001). All reactions were performed in triplicate, and the mixture included a negative no-reverse transcription (RT) control in which reverse transcriptase was omitted.

2.6. Correlation between anti-C. albicans activities of the compounds and TUP1 gene expression

In order to determine how strong the relationship was between *TUP1* gene expression and either the anti-yeast or anti-hyphae activities of the synthesised SR compounds, a formula was generated using Graph Pad 5.0 to produce what is referred to as the coefficient value (Zar, 1984). A Pearson's correlation attempts to draw a line of best fit through the data of two variables (*TUP1* gene expression and anti-*C. albicans* activities of the compounds), where each row represented a different compound, the X column represented the *TUP1* gene expression and the Y columns represented the anti-*C. albicans* activities of the compound against either yeast or hyphae forms. The Pearson correlation coefficient, *r*, indicates how far away all these data points are to this line of best fit. A value of −1 is representing a perfect negative linear relationship, while +1 for a perfect positive linear relationship.

2.7. Quantitative structure–activity relationship (QSAR) studies

To explore the correlation between the 3D structures of the training set (**SR1–7**) and their biological activities, a predictive 3D QSAR model was built. Comparative Molecular Field Analysis (CoMFA) was used to build the 3D QSAR model using Py-CoMFA 3D QSAR service. CoMFA calculates steric and electrostatic properties of each molecule on 3D cubic lattice with grids pacing of 2°A in *x, y,* and *z* directions using the Tripos force field (Clark et al., 1989). A sp3 carbon atom probe with a Vander Waals radius of 1.52°A and a charge of þ1.0 was used to generate the steric and electrostatic (Coulombic potential) field with distance-dependent dielectric at each lattice point. The steric and electrostatic energy values were truncated at 30.0 Kcal/mol. The minimum sigma (column filtering) was set to 0.05 Kcal/mol. Equal weights for CoMFA were assigned to steric and electrostatic fields using CoMFA STD scaling option. The regression analysis was carried out using the partial least squares (PLS) analysis and the cross validation was carried with leave-one-out (LOO) method (Bush and NachbarJr., 1993) in which one compound is removed from the data set and its activity is predicted using the model derived from the rest of the molecules in the dataset.

2.8. In silico pharmacokinetics and toxicological properties of SR compounds

The physicochemical and pharmacokinetics properties of the synthesized SR compounds were examined using theoretical prediction of absorption, distribution, metabolism, excretion, toxicity (ADMET) qualitative models. The compounds were built in 3D structures using MOE software and saved as sdf files. The compounds were then submitted to admetSAR software, available at (https://www.lmmd.ecust.edu.cn/admetsar1/predict/) (Yang et al., 2018). The parameters generated by the software evaluated the pharmacokinetics characteristics of the compounds. The parameters evaluated in the pharmacokinetics study included human intestinal absorption (HIA), cytochrome binding, biodegradation, acute oral toxicity, carcinogenicity, rat acute toxicity and aqueous solubility (Ramos et al., 2019).

2.9. Cytotoxicity assay

The cytotoxic assay of SR compounds was measured by quantifying the amount of hemoglobin released by lysis of human erythrocytes as previously described (Soliman et al., 2017a, 2017b). Briefly, fresh erythrocytes were re-suspended to 3×10^7cells/mL in DPBS followed by incubation with the compounds in round-bottomed 96-well plates in 200 μL final volume. Washing buffer and 0.1% Triton X-100 were used as negative and positive controls, respectively. The plate was incubated at 37 °C for 30 min, and the intact cells were precipitated by centrifugation at 500 g for 10 min at 4 °C and the hemoglobin released was measured by absorbance at 405 nm. The absorbance values for each sample were subtracted from the absorbance of the washing buffer-treated cells and the hemolytic activity (%) was calculated. The experiment was conducted in triplicate.

Blood samples were collected after obtaining a signed informed consent from healthy volunteers under an approved Los Angeles Biomedical Research Institute IRB protocol #11671.

2.10. Statistical analysis

The data was graphed using Graph Pad5.0 for Windows (GraphPad Software, La Jolla, CA, USA). The statistical significance was analyzed using one-way or two-way analysis of variance (ANOVA) using either Bonferroni's multiple comparisons test or Dunn's Multiple Comparison Test. *P*-value < 0.05 was considered significant.

3. Results

3.1. Chemistry of the hybrid SR compounds

The synthetic route of 5-[3-substitued-4-(4-substituedbenzyloxy)-benzylidene]−2-thioxo-thiazolidin-4-one (SR) compounds is described in Scheme 1 (Fig. 2). The intermediate 3-substituted-4-(4-substituedbenzyloxy) benzaldehyde (**R1–7**) was synthesized by coupling substituted-benzyl chloride (a–c) with 3-substituted-4-hydroxybenzaldehyde in the presence of K_2CO_3 and KI in acetonitrile. The obtained compounds (**R1–7**) were then reacted with rhodanine in the presence of β-alanine and glacial acetic acid via Knoevenagel condensation reactions in order to provides 5-[3-substitued-4-(4-substituedbenzyloxy)-benzylidene]−2-thioxo-thiazolidin-4-one derivatives, **SR1–7** (Fig. 2).

To predict the isomerisation (Z or E) of the compound, the molecular structure of Z and E isomers were compared (Supplementary Fig. 1) and the energy minimization parameters were predicted (Supplementary Table 1). The heat of formation of Z-isomer (−164.316) less than that of E-isomer (−161.005), and the total energy of Z-isomer is less than of E-isomer. The results revealed that the most stable structure among both isomers was Z-isomer determined by the total energy together with the heat of formation energy and other parameters identified in Supplementary Table1. This result was in consistent with previously reported isomerisation of rhodanine derivative with exocyclic double bond (Bataille et al., 2017; Fan et al., 2010); whereas Z-isomer was the major thermodynamically stable diastereomers. Furthermore, it has been reported that the main product obtained from rhodanine reaction with aromatic aldehyde is Z-isomer (Kaczor et al., 2019).

3.2. Inhibitory activities of SR compounds on Candida hyphae formation

The effect of the newly-synthesized compounds, named **SR1–7** (Fig. 2), on *C. albicans* growing either in yeast- or hyphae-forming conditions were tested separately at different concentrations including 1, 10, 50, 100 μg/mL. All tested concentrations showed weak inhibition activities against *C. albicans* yeast (Fig. 3A), while all compounds except compounds **SR6** showed significant inhibition activities (60–100%) against *Candida* hyphae in particular at concentrations 50 and 100 μg/mL (Fig. 3B). At the lower concentration of 10 μg/mL, all compounds showed inhibition of yeast at 10–20%, whereas compounds **SR1–3** and **5** showed 60–80% hyphae inhibition. Furthermore, compounds **SR4** and **6** showed ~50% hyphae inhibitory activities, while compound **SR7** showed 90% inhibition activity (Fig. 3C). These results indicated that SR compounds were more effective against *C. albicans* hyphae formation compared to their activity against yeast (two-way ANOVA, $p < 0.0001$). Concordant with these results, the minimum inhibitory concentration of the compounds that resulted in 100% killing (MIC_{100}) against *C. albicans* yeast ranged from 125 μg/mL for compounds **SR6** and **7**; 200 μg/mL for compounds **SR2, 4** and **5**; to 400

μg/mL for compounds **SR1** and **3** (Table 2). In contrast, the MIC_{100} that resulted in almost complete inhibition of biofilm formation (i.e. hyphal formation) ranged between 10–20 μg/mL (Fig. 4 and Table 2). Finally, the MIC_{100} of the tested compounds ranged from 125–400 μg/mL against *E. coli* and >400 μg/mL against *Staphylococcus aureus* (Table 2). Collectively, these results confirmed that the newly designed compounds (SR) are *C. albicans* hyphae-specific inhibitors.

3.3. The hyphae inhibitory activities of SR compounds was via TUP1 activation

To define the mechanism by which the SR inhibitors exerted their effect on *C. albicans* hyphae, we studied the effects of **SR 1–7** on the expression of genes that are known to either control *C. albicans* yeast/hyphal formation or are expressed in hyphae. The analyzed genes included those associated with: (1) adhesion/invasion/biofilm formation such as *HWP1* (hyphae cell wall protein), *ALS3* (agglutinin-like sequence), *EAP1* (extracellular adhesion protein); (2) the transcription activator *UME6* responsible for hyphae formation; and (3) the transcription repressors of *Candida* filamentation (*TUP1* and *NRG1*). At the yeast growth condition, all compounds showed significant increase in the expression level of *TUP1* gene (Fig. 5). Compounds SR4, 5, 6, and 7 caused 3–4 fold increase in the expression of *TUP1* gene compared to the expression level of 18S rRNA. Furthermore, all compounds except **SR6** caused 2-fold increase in the expression of *NRG1*. In contrast, all other tested genes were not significantly changed. At the hyphae growth condition, all tested compounds caused ~ 4–5-fold increase in the expression level of *TUP1* gene, while other genes were significantly not affected compared to the expression level of 18S rRNA gene (Fig. 5). Moreover, a correlation studies between *TUP1* gene expression levels and *C. albicans* inhibition activities by SR compounds either at yeast or hyphae growing conditions was conducted (Fig. 6). *TUP1* gene expression showed strong direct correlation with hyphae inhibition activities ($r^2 = 0.85$) but not with yeast inhibition activity ($r^2 = 0.02$) (Fig. 6). The results from these studies indicated that SR compounds primarily exert their inhibitory effect on *C. albicans* hyphae formation through activation of the *TUP1* hyphae repressor expression. Further, these studies revealed that **SR7** is the best candidate for further development of prevention of *C. albicans* hyphal growth and biofilm formation.

3.4. QSAR modeling predicted the activity of compound SR7

The relation of SR compounds structure to their concentration required for 50% inhibition [pIC_{50} ($-\log MIC_{50}$)] against *C. albicans* was also calculated using Comparative Molecular Field Analysis (CoMFA) steric and electrostatic interactions of each molecule, Partial Least square (PLS) and Leave-one-out (LOO) analyses. The results showed that the squared correlation coefficient (r^2) was 0.992 and the cross-validated correlation coefficient (q^2) was 0.50, while the standard deviation error of calculation (SDEC) was 0.021 and the standard deviation error of prediction (SDEP) was 0.165 (Table 3). The experimental activities of the compounds on yeast and hyphae were listed in Table 4. The correlation between the experimental activities and the predictive activities was 0.02 (Supplementary Fig. 1). This correlation suggests the reliability and effectiveness of the established 3D QSAR model and its effectiveness in designing novel *C. albicans* inhibitors.

CoMFA steric contour maps were represented in (Fig. 7A). The steric interactions were illustrated by green and yellow contours, while electrostatic interactions were denoted by red and blue contours (Fig. 7B). A large green contour (region A) and small green contour (region B) were found near the plane of the 3-CF_3 substituent of phenyl ring (R_1) and OCH_3 group in R_2 positions (compound **SR7**), respectively (Fig. 7A). This result suggested that bulky substituent were preferred in these two regions and indicated that they were important for the activity, thus explaining the highest activity of compound **SR7**. In contrast, a yellow contour (region C) was located around the 4-CH_3 substituent of phenyl ring (R_1) of compound **SR1**, indicating that groups with low steric factor were favoured in this region to increase the activity and therefore explaining the decreased activity of compound **SR1** (Fig. 7A). Blue-colored (region A) and red-colored (region B) contours represented the regions where the positively- and negatively-charged groups enhanced the activity, respectively (Fig. 7B). These results indicated that the electron-deficient substituent at 3-position of phenyl ring (R1) were curial for the activity, and electron-rich groups near 4-position (R1) of the phenyl ring were preferred in this position.

In conclusion, the rhodanine and 3- trifluroromethyl-phenyl were employed as privilege structure, which occupied the same position in 3D QSAR results (Fig. 7). The QSAR model also suggested that other points were important in designing novel compounds with potent activity (Cruz et al., 2019). Furthermore, CoMFA study revealed that positively-charged bulky groups were favored at position number 3 of the phenyl ring (R1) and less steric, negatively-charged groups were favored at position number 4 of the phenyl ring (R1). Furthermore, bulky group at R2 position was favored for the activity.

3.5. SR compounds have limited toxicity and efficient metabolism

The cytotoxic activities of the compounds were examined by testing their hemolytic activities using human erythrocytes. Interestingly all compounds were significantly less toxic when compared to hemolysis caused by 0.1% triton (one-way ANOVA, $p = 0.0001$). Specifically, at >200 μg/mL, a concentration that is 20x the MIC_{100} required to inhibit hyphal formation, only 10–40% erythrocyte hemolysis was detected versus 100% hemolysis caused by 0.1% triton (Fig. 8). Furthermore, the physicochemical properties of the synthesized SR compounds were examined using theoretical prediction of absorption, distribution, metabolism, excretion, toxicity (ADMET) qualitative models via admetSAR server (https://www.lmmd.ecust.edu.cn/admetsar1/predict/) (Yang et al., 2018). For instance, human intestinal absorption (HIA), cytochrome binding, biodegradation, acute oral toxicity, carcinogenicity, rat acute toxicity and aqueous solubility were calculated (Supplementary Table 1). The calculations indicated that all compounds showed good intestinal absorption, good water solubility, and no inhibition of cytochromes. Hence, good metabolism and no toxicity of these compounds are predicted (Supplementary Table 1). To investigate the absorption of the drug, total polar surface area (TPSA) of rhodanine derivatives were calculated. The TPSA of the compounds understudy was less than 140 Å which indicates the compounds are likely to be orally bio-available (Shimada et al., 2014).

4. Discussion

With the growing threat due to the multi-drug resistance of *Candida* pathogen mainly because of its hyphae form, since it can generate the most-difficult to eradicate biofilms, it is urgent to develop novel compounds with novel mechanism of action. Based on the reported *Candida* inhibition activities of several synthesized thiazolidinedione derivatives (Kagan et al., 2014) including in particular the rhodanine isosteres, 1,2-benzisothiazolinone (Alex et al., 2012), rhodanine privileged structures were selected for this study (Fig. 1). A novel series of hybrid compounds (SR1–7) were designed and developed that possessing a rohdanine nucleus and lipophilic aromatic side chain with different electron withdrawing substituent to improve the cellular penetration along with the hydrophilic polar methoxy and phenoxy groups. The novel compounds showed enhanced physiochemical properties and selectivity against *C. albicans* hyphae formation.

Our results showed that the newly-synthesized compounds (**SR1–7**) are inhibitors of *C. albicans* hyphae formation by activation of the hyphae repressor factor, *TUP1. TUP1* is a critical transcription repressor that inhibits *Candida* hyphae formation by repressing the expression of hyphae-specific genes including *CPH1* and *UME6* (Braun et al., 2000; Braun and Johnson, 1997). Thus, up-regulation of TUP1 by SR compounds significantly contributed to the inhibition of *C. albicans* hyphal formation. However, we did not detect any significant changes in the expression of genes involved in *C. albicans* yeast/hyphal switch, adhesion or those expressed during hyphae growth including *HWP1, ALS3, EAP1*, and *UME6*, that is in accordance to previously reported data by (Braun et al., 2000). Although the thiazoldinedione derivatives reported before showed variable expression activities on *C. albicans* hyphae-associated genes (Feldman et al., 2014), the newly-designed rhodanines were more selectively affecting *TUP1* expression. The results indicate that SR compounds activated the expression of *TUP1* repressor which in turn inhibited *Candida* hyphae formation without affecting *C. albicans* hyphae effector genes (Fig. 9).

The transcriptional repressor, *TUP1* and the signaling molecule farnesol are both capable of negatively regulating the yeast to hyphae conversion. Specifically, it has been shown that TUP1 are up-regulated in C. albicans biofilms exposed to farnesol (Cao et al., 2005; Han et al., 2012). Therefore, we hypothesized that the mechanism of action of SR compounds is similar to farnesol, despite the fact that they have completely different structures. Our data are consistent with the critical role of *TUP1* in response to farnesol in *C. albicans* (Kebaara et al., 2008). Furthermore, it has been shown that 10–40 times the hyphae inhibitory concentrations of SR compounds were required to inhibit either *Candida* yeast or bacteria which is in accordance with the selective inhibitory effect of farnesol to *C. albicans* hyphae formation but not the yeast form (Nickerson et al., 2006; Polke et al., 2018; Ramage et al., 2002). Both results confirmed the selective inhibitory effect of SR compounds on *C. albicans* hyphae formation.

TUP1 is known to function with the DNA binding proteins *NRG1* to repress the expression of hypha-specific genes (Kebaara et al., 2008; Lu et al., 2014). However, our data indicated that SR compounds caused no significant increase in the expression of *NRG1*, indicating that the major effect of these inhibitors are on *TUP1* rather than *NRG1*. In contrast, *UME6*,

a key filament-specific activator, known to be down regulated by *TUP1* was not affected by SR compounds, indicative of a more likely signaling mechanism of hyphae activities by *TUP1*.

Targeting the expression of *TUP1* gene provides two important advantages over the currently available drugs, specificity and safety. To the best of our knowledge, there are no drugs developed against *C. albicans* hyphae formation by selectively affecting *TUP1* gene expression without affecting the other hyphae-associated genes including *ALS3, EAP1, HWP1, NRG1* and *UME6*. Our results showed also that activation of *TUP1* gene alone was able to inhibit *C. albicans* hyphae formation. *TUP1* gene does not exist in human and hence afford superior safety when compared to other available drugs, since most fungal genes (eukaryotes) are shared with human genome (Galagan et al., 2005). In addition to the limited number of available antifungal drugs including pyrimidine analogs, polyenes, azoles, and echinocandins, they all have different mechanisms of actions and none of them affects the expression of *TUP1* gene (Juvvadi et al., 2017). Furthermore, they all developed resistance and showed toxicities to human.

During infection, it is reported that *C. albicans* morphologies have distinct interactions with the host cells and activation of *TUP1* results in significant reduction in *C. albicans* virulence, since yeast forms can be easily cleared from tissues by host defenses much more efficiently than hyphae (Cleary et al., 2016). Our study indicated that the specificity and minimal toxicity of the newly-designed compounds can be of special value particularly against *C. albicans* resistance forms (biofilm). Furthermore, the use of the new rhodanine derivatives in combination with available anti-fungal drugs may be considered as promising strategy to enhance the treatment outcome of *C. albicans* infection.

QSAR has been widely used in drug discovery and lead optimization that effectively reduces the cost of experimental evaluation of untested chemicals and enhances the selection of best candidate compound (Tong et al., 2005). Although the reliability and importance of the QSAR results obtained in this study, the limitations associated with this study can include the number of derivatives with variable substitutions need to be increased, since the accuracy and confidence of a model's prediction depend on the size and diversity of the chemical structures. Furthermore, running a QSAR analysis on *TUP1* gene expression will improve the model's prediction. Therefore, a future study may be conducted in order to increase the number of derivatives with diverse substitutions in relation to *TUP1* gene expression.

5. Conclusion

We have designed and synthesized a series of rhodanine derivatives possessing thiazolidine core and aromatic side chain targeting *TUP1* transcription repressor and hence inhibited *C. albicans* hyphae formation. Substitutions of R1 and R2 with 3-CF_3 and OCH_3, respectively followed by 4-CH_3 and OCH_3 enhanced the hyphae inhibitory activities of the compounds. Furthermore, the compounds showed no hemolytic activities at ~20 times the concentrations showed hyphae inhibitory activities. We propose that the newly-designed compounds are candidates for the effective use of adjunctive therapy with currently used antifungal agents to ameliorate infections due to *C. albicans*. Moreover, the newly-designed compounds,

in particular compounds **SR7, 1** and **3,** may be future new quorum signaling candidates that serve as promising lead drugs for not only *C. albicans* infection but also other *TUP1*-dependent dimorphic fungal infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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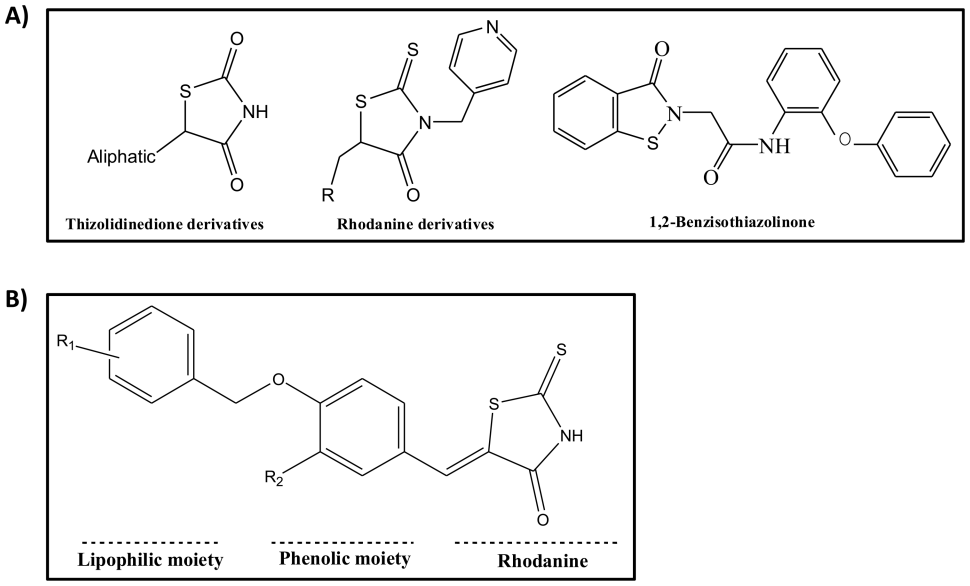
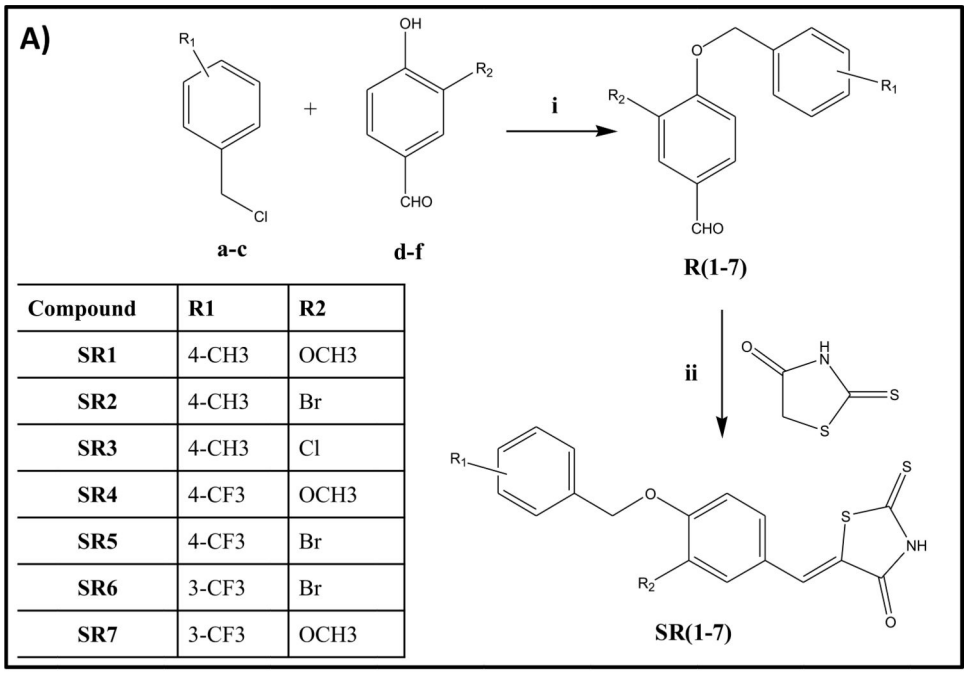


Fig. 1.

The rational design of anti-*Candida* hyphae compounds based on rhodanine scaffold. (A) Structures of thizolidinedione, pyridine-rhodanine and 1,2-benzisothiazolinone derivatives. (B) Proposed rhodanine-phenolic hybrid derivatives as novel anti-*Candida* hyphae compounds.



Compound	R1	R2
SR1	4-CH3	OCH3
SR2	4-CH3	Br
SR3	4-CH3	Cl
SR4	4-CF3	OCH3
SR5	4-CF3	Br
SR6	3-CF3	Br
SR7	3-CF3	OCH3

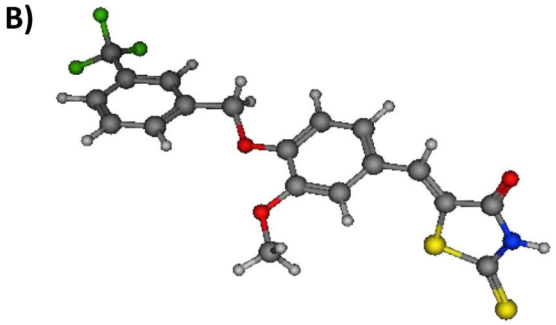


Fig. 2.

Synthesis and structure of 5-[3-Substitued-4-(4-substituedbenzyloxy)-benzylidene]−2-thioxo-thiazolidin-4-one compounds. (A) Synthesis scheme of 5-[3-Substitued-4-(4-substituedbenzyloxy)-benzylidene]−2-thioxo-thiazolidin-4-one derivatives. Reagents and conditions: i. K_2CO_3, KI, acetonitrile, overnight stirring under nitrogen; ii. β-alanine, glacial acetic acid, 100 °C, 3 h; R_1. CH_3, $3CF_3$, $4CF_3$; R_2. Cl, Br, OCH_3. (B) 2D structure of SR7.

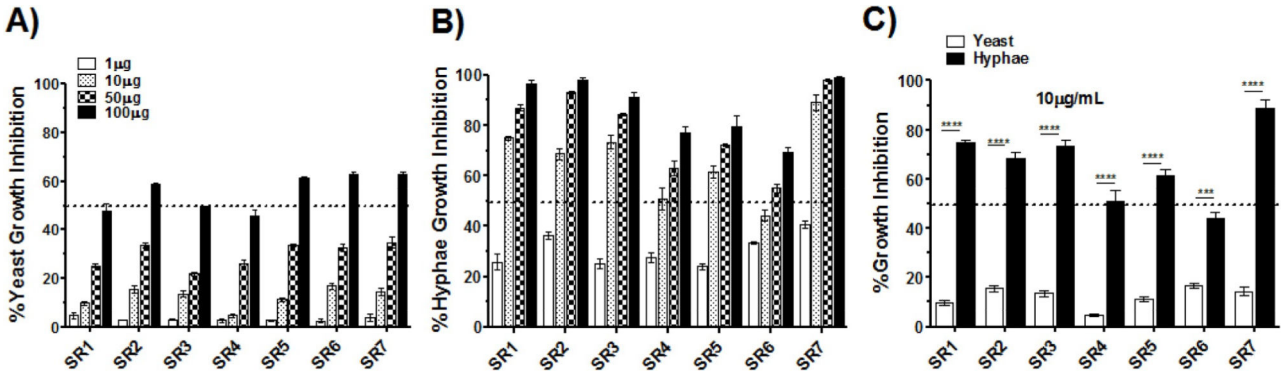


Fig. 3.

Anti-*C. albicans* activities of SR compounds. (A) *Candida* yeast growth inhibition by SR compounds at different concentration (1, 10, 50, and 100 µg/ mL). (B) Quantitative microtiter plate assay for biofilm formation using MTT assay method when *Candida* incubated with different concentration of the compounds (1, 10, 50, and 100 µg/mL) in *Candida* hyphae growth conditions (RPMI + 2% Glucose at 37 °C). (C) Growth inhibition effects of SR compounds at 10 µg/mL on *Candida* when growing at either yeast or hyphae conditions. The effects of compounds were tested on *C. albicans* compared to solvent as negative control. The data was analyzed using two-way ANOVA and statistical significance was calculated with Bonferroni's multiple comparisons test and significance level indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$: ***, $P < 0.001$; ****, $P < 0.0001$). The data display the mean ± standard error (SEM) of three replicas.

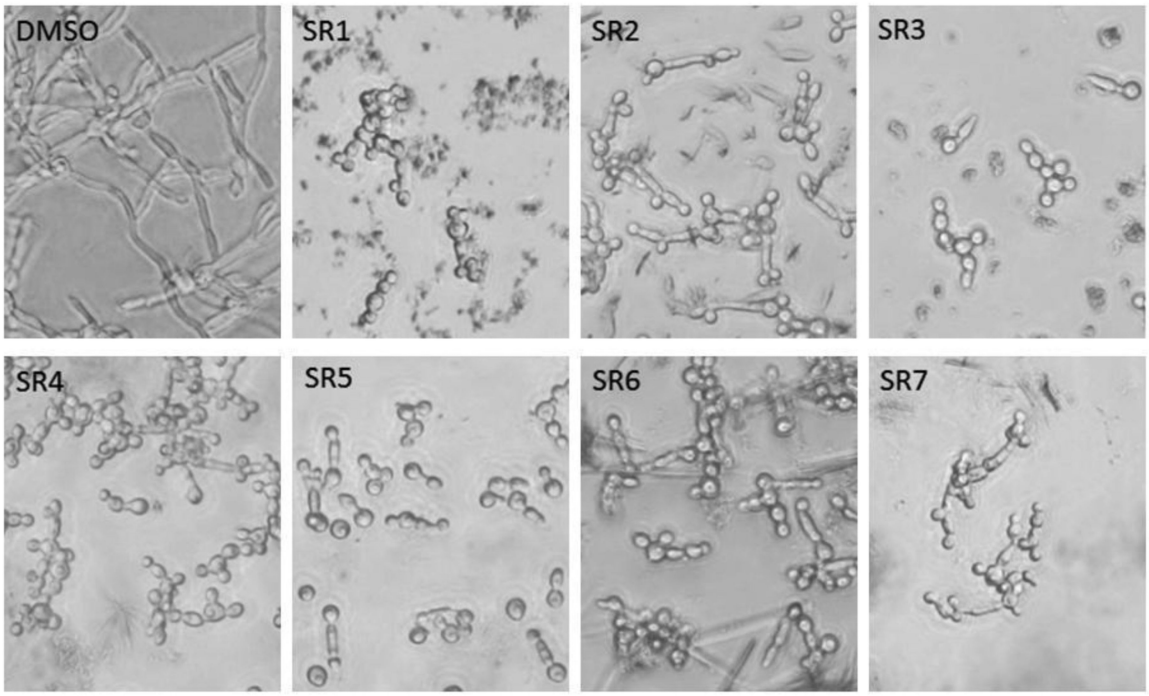


Fig. 4.

Activity of SR compounds on hyphae formation of *C. albicans. Candida* at hyphae growth conditions were treated with 10 μg/mL of SR compounds and then incubated for 24 h at 37 °C. The hyphae growth of *C. albicans* was followed by picturing using inverted microscopy. DMSO was employed as negative control.

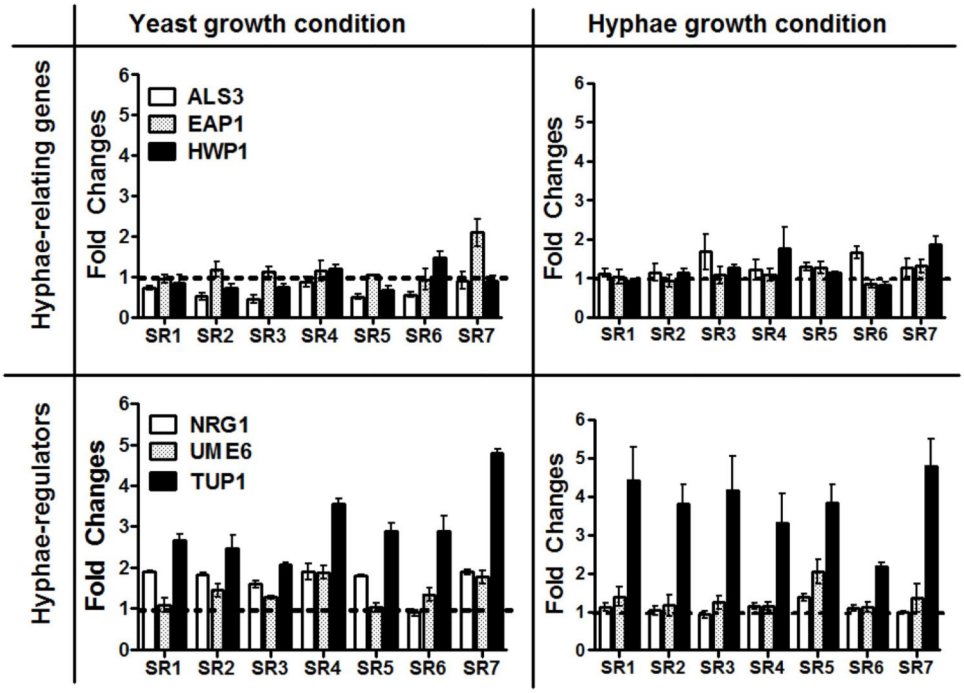


Fig. 5.

Gene expression analysis of *Candida* hyphae specific genes. *Candida* at either yeast or hyphae growth conditions were incubated with 10 μg/mL of SR compounds for 24 h. Quantitative real time PCR were performed using mRNA isolated from each condition. *AL3*; agglutinin-like sequence, *EAP1*; extracellular adhesion protein, *HWP1*; hyphae cell wall protein, *TUP1* and *NRG1* are transcriptional repressor of filamentation and *UME6* is transcriptional activator of filamentation. All data represented relative to fold changes compared to 18S rRNA housekeeping gene. Each figure has e transfers dotted line (equal 1) that represent the expression level of 18S rRNA housekeeping gene. The standard error represents the mean of three replicates.

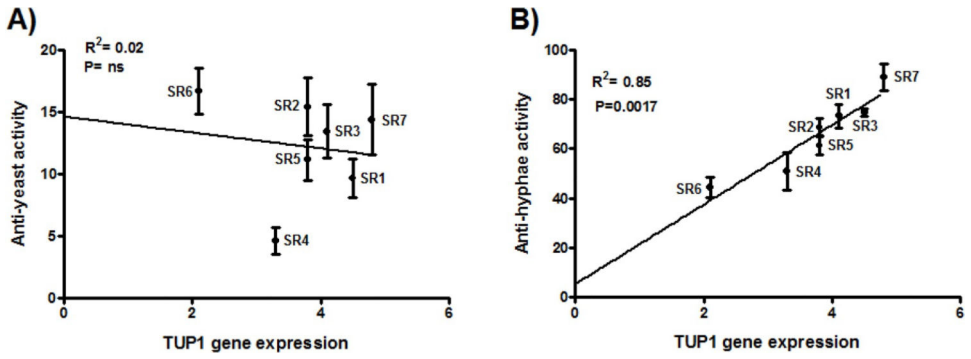


Fig. 6.

Correlation between *TUP1* gene expression and the anti-*C. albicans* activity. *Candida* at either yeast (A) or hyphae (B) growth conditions were incubated overnight with 10 μg/mL of SR compounds. In parallel, quantitative microtiter plate assay of the *Candida* activity at the end of experiment were measured by MTT viability assay. Gene expression data represented fold change compared to negative control while anti-*Candida* activities represented% growth inhibition compared to DMSO solvent as negative control. The data was analyzed using Pearson correlation and data considered normal distribution. *P* value <0.05 was considered significant.

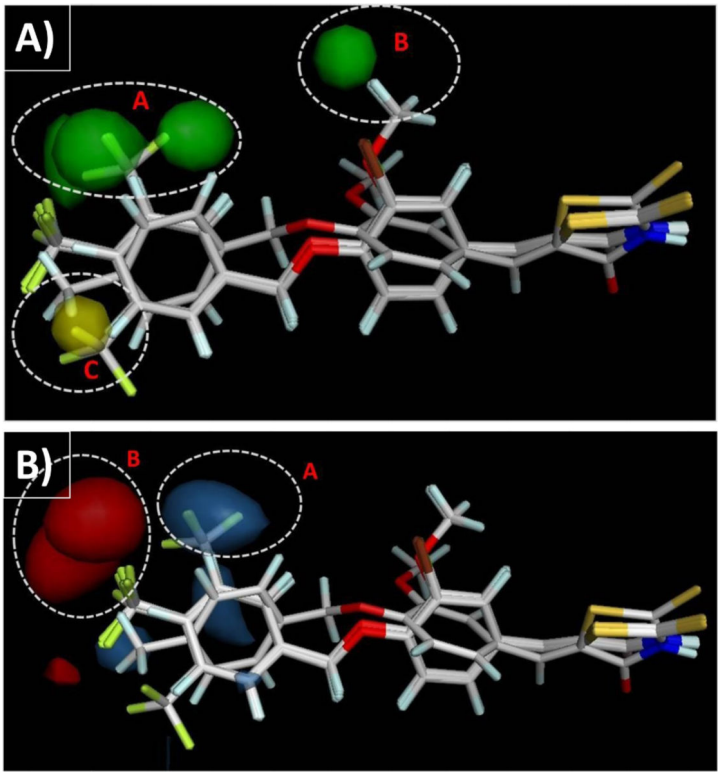


Fig. 7.

3DQSAR contour map of synthesized compounds, the fields were shown as surfaces, and the compounds were aligned together. (A) Sterically-favored area was shown in green-colored contours and sterically-unfavored area was shown in yellow-colored contours. (B) Positively-potential favored areas are shown in blue-colored contours and negatively-potential favored areas are shown in red-colored contours.

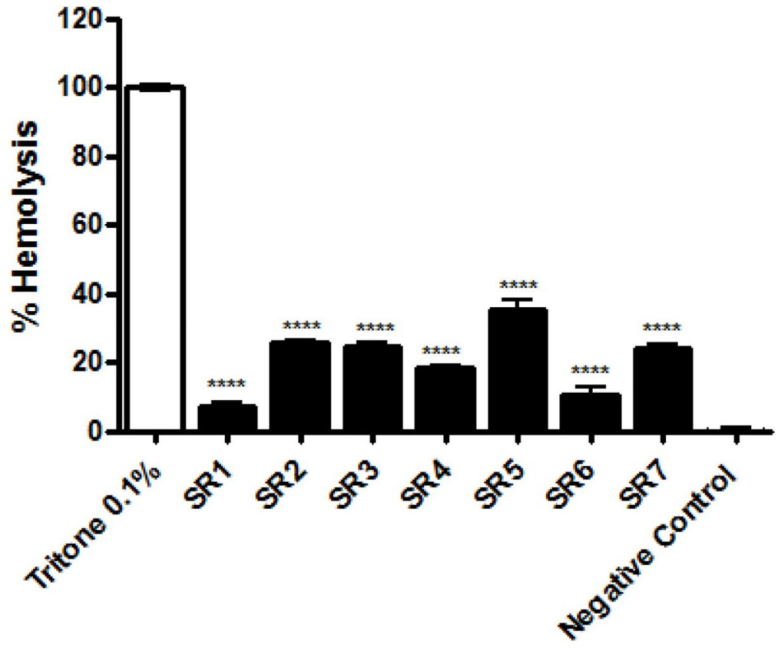


Fig. 8.

Hemolytic activity of SR compounds. DPBS-washed erythrocytes (3×10^6 cells per well) were incubated in 96-well plate with 200 μg/mL of SR compounds at 37 °C for 30 min. The hemoglobin released from lysed erythrocytes was measured using micro-plate reader at 405 nm. The absorbance value for each sample was subtracted from the absorbance value of cells treated only with washing buffer and the hemolytic activity (%) was calculated. The data display the mean ± standard error (SEM) of three independent measurements. The experiment was conducted in triplicate. **** $P < 0.0001$ vs. 0.1% triton.

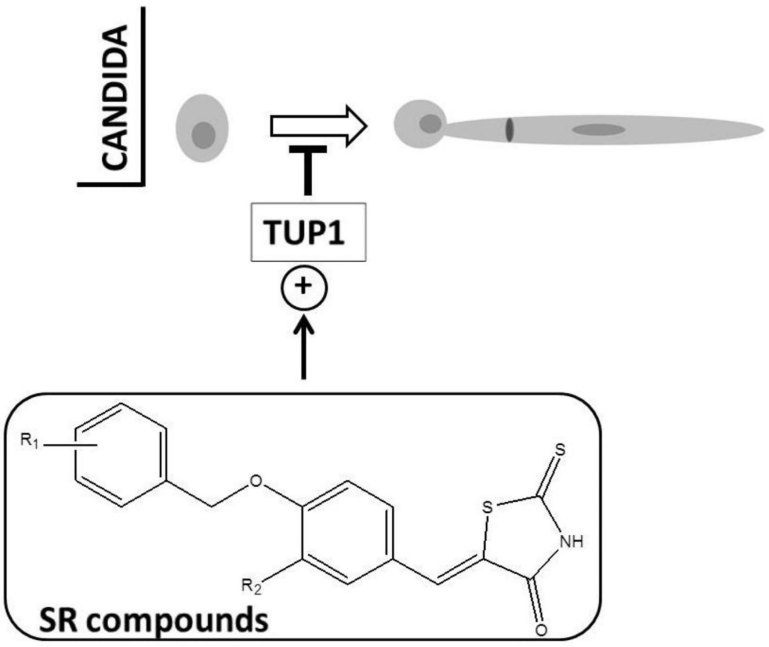


Fig. 9.

Model representing the inhibition effect of SR compounds on *C. albicans* hyphae growth. SR compounds caused significant up-regulation of *TUP1* gene, which in turn inhibit the hyphae growth of *C. albicans*.

Table 1

Oligonucleotides used in this study.

Primer name	Primer sequence (5′–3′)	Ref
ALS3	Forward; CAACTTGGGTTATTGAAACAAAAACA	(Uppuluri et al., 2009)
	Reverse; AGAAACAGAAACCCAAGAACAACC	
EAP1	Forward; TGTGATGGCGGTTCTTGTTC	(Samaranayake et al., 2013)
	Reverse; GGTAGTGACGGTGATGATAGTGACA	
NRG1	Forward; CCAAGTACCTCCACCAGCAT	(Holcombe et al., 2010)
	Reverse; GGGAGTTGGCCAGTAAATCA	
TUP1	Forward; CTTGGAGTTGGCCCATAGAA	(Holcombe et al., 2010)
	Reverse; TGGTGCCACAATCTGTTGTT	
HWP1	Forward; GCTCCTGCTCCTGAAATGAC	(Holcombe et al., 2010)
	Reverse; CTGGAGCAATTGGTGAGGTT	
UME6	Forward; ACCACCACTACCACCACCAC	(O'Connor et al., 2010)
	Reverse; TATCCCCATTTCCAAGTCCA	
18S rRNA	Forward; CACGACGGAGTTTCACAAGA	(Morschhäuser et al., 2007)
	Reverse; CGATGGAAGTTTGAGGCAAT	

Table 2

Minimum inhibitory concentration (MIC) values in μg/mL of SR compounds against *C. albicans* and bacteria in 24 h incubation period. MIC is the lowest concentration of the compound that inhibited 100 of microbial growth.

Compounds	**MIC (μg/mL)**			
	C. albicans		**Bacteria**	
	Yeast	**Hyphae**	***E. coli***	***S. aureus***
SR1	>400 ± 15	20 ± 5	100 ± 10	>400
SR2	200 ± 15	10 ± 2	100 ± 10	
SR3	>400 ± 10	20 ± 5	400 ± 10	
SR4	200 ± 10	20 ± 5	100 ± 10	
SR5	200 ± 15	20 ± 3	400 ± 20	
SR6	125 ± 10	10 ± 5	400 ± 10	
SR7	125 ± 10	10 ± 3	100 ± 5	
Ketoconazole	3	NA	NA	
Colistin	NA	NA	5	NA
Vancomycin	NA	NA	NA	3

Table 3

Summary of CoMFA results.

PLS statistics	CoMFA
r^2	0.99
q^2	0.50
SDEC	0.02
SDEP	0.16

Table 4

Experimental activities ($PMIC_{50}$ M/L) of the training set molecules (yeast versus Hyphae).

No	$pMIC_{50}$ Yeast	$pMIC_{50}$ Hyphae
SR1	2.968	4.268
SR2	3.323	4.622
SR3	2.975	4.273
SR4	3.341	4.327
SR5	3.328	4.327
SR6	3.579	4.764
SR7	3.579	4.764