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Solasodine-3-*O*-β-D-glucopyranoside kills *Candida albicans* by disrupting the intracellular vacuole

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ABSTRACT

The increasing incidence of fungal infections and emergence of drug resistance underlie the constant search for new antifungal agents and exploration of their modes of action. The present study aimed to investigate the antifungal mechanisms of solasodine-3-*O*-β-D-glucopyranoside (SG) isolated from the medicinal plant *Solanum nigrum* L. In vitro, SG displayed potent fungicidal activity against both azole-sensitive and azole-resistant *Candida albicans* strains in Spider medium with its MICs of 32 μg/ml. Analysis of structure and bioactivity revealed that both the glucosyl residue and NH group were required for SG activity. Quantum dot (QD) assays demonstrated that the glucosyl moiety was critical for SG uptake into *Candida* cells, as further confirmed by glucose rescue experiments. Measurement of the fluorescence intensity of 2′,7′-dichlorofluorescin diacetate (DCFHDA) by flow cytometry indicated that SG even at 64 μg/ml just caused a moderate increase of reactive oxygen species (ROS) generation by 58% in *C. albicans* cells. Observation of vacuole staining by confocal microscopy demonstrated that SG alkalized the intracellular vacuole of *C. albicans* and caused hyper-permeability of the vacuole membrane, resulting in cell death. These results support the potential application of SG in fighting fungal infections and reveal a novel fungicidal mechanism.

1. Introduction

The application of broad-spectrum antibiotics, implantable devices and immunosuppressive agents in recent decades has led to an increase in the incidence of fungal infections (Corti et al., 2009). Invasive fungal infections are difficult to manage and are important factors in morbidity and mortality among immunocompromised patients (Gudlaugsson et al., 2003; Miller et al., 2001). A recent epidemiological survey revealed that *Candida* species were the third most common pathogen among hospital patients (Hidron et al., 2008). Current clinically used antifungal agents are mainly divided into four categories according to their modes of action: polyenes, azoles, echinocandins and flucytosine (Pappas et al., 2009). However, the latter three categories of therapeutics are often associated with drug resistance, highlighting the need for extensive efforts to develop new antifungal agents and therapeutic strategies.

Natural products, particularly traditional Chinese medicine, provide a rich pool for drug discovery due to the therapeutic efficacy and versatile structures of secondary metabolites (Harvey, 2008). *Solanum nigrum* L. (SNL), which belongs to the nightshade Solanaceae family, is traditionally used as a herbal plant, and its ripe fruits are edible. SNL produces a class of steroidal alkaloids and glycosides that possess a variety of biological activities, including antifungal, antiviral, antibacterial, molluscicidal and anticancer activities (Kumar et al., 2009; Simons et al., 2006; Sun et al., 2010; Thorne et al., 1985). Due to these diverse bioactivities, the chemical synthesis, biosynthesis and bioactivity of the steroidal alkaloids have been the focus of several recent studies (Cárdenas et al., 2015, 2016; Jiang et al., 2015; Sharpe and Johnson, 2016).

The glycosidic residues are considered crucial for the bioactivity of glycosides. For example, the steroidal glycoalkaloid α-tomatine produced by tomato exerts antimicrobial effects for self-defense. However, removal of this glycosidic residue by the tomatinase of *Septoria lycopersici* abolishes this antimicrobial activity (Bouarab et al., 2002). Moreover, attachment of the glycosidic moiety

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Abbreviations

AMB	Amphotericin B
CLSM	Confocal laser scanning microscopy
CMAC	Cell Tracker Blue CMAC Dye
CLSI	Clinical and Laboratory Standards Institute
DCFH- DA	2′,7'-dichlorofluorescin diacetate
DMSO	Dimethyl sulfoxide
MIC	Minimal inhibitory concentration
FM4-64	N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide
PI	Propidium iodide
QD	Quantum dots with streptavidin conjugate 605 nm
ROS	Reactive oxygen species
SNL	*Solanum nigrum* L.
SG	Solasodine-3-O-β-d-glucopyranoside
Spider-Glu	Spider medium with glucose instead of mannitol
Spider-Rha	Spider medium with rhamnose instead of mannitol
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
YPD	Yeast-peptone-dextrose

increases the hydrophilicity of the molecule, which influences pharmacokinetic properties such as circulation, elimination and concentrations in body fluids (Kren and Martínková, 2001).

We previously reported that solasodine-3-*O*-β-D-glucopyranoside (SG) displays anti-virulence activity against *C. albicans* by inhibiting cell adhesion, morphological transition and biofilm formation (Li et al., 2015b). However, little is known about the antifungal activities of SG analogues and the essential functional groups in the structure of SG. Moreover, the mode of action of SG remains unclear.

We previously isolated solasodine (**1**) and its glycoside SG (**2**) from SNL and synthesized several analogues of SG including solasodine conjugated with β-L-rhamnopyranose (**3**), β-D-xylopyranose (**4**), β-L-arabinopyranose (**5**), or β-D-ribopyranose (**6**), thio group substituted SG (**7**), and oxic group substituted SG (**8**) (Fig. 1) (Sun et al., 2010; Cui et al., 2012; Zan et al., 2014). In the present study, the in vitro antifungal activities of SG and its analogues were evaluated. SG exhibited fungicidal activity against *C. albicans* in Spider medium, whereas the other analogues were inactive. Structure-activity analysis revealed that both the NH group and glucosyl residue were necessary for SG activity. Further investigation demonstrated that SG penetrated the *C. albicans* cell membrane via the glucosyl residue. SG elicited the alkalization of vacuoles, which resulted in increased vacuole permeability and, consequently, triggered the death of *C. albicans*.

2. Materials and methods

2.1. Strains, culture and chemicals

The *C. albicans* wild type strain SC5314 and ten clinical isolates including five azole-sensitive and five azole-resistant isolates (Li et al., 2015a) were used in this study. The strains were stored routinely and propagated on yeast-peptone-dextrose (YPD) agar plates (2% tryptone, 1% yeast extract, 2% glucose and 2% agar) before each experiment. Following incubation for 24 h at 30 °C, the cells were inoculated in YPD broth (2% tryptone, 1% yeast extract, 2% glucose) for overnight culture at 30 °C and 200 rpm.

Solasodine (**1**) and SG (**2**) were previously isolated from SNL and several analogues of SG (**3**–**8**) were synthesized in our lab (Fig. 1) (Cui et al., 2012; Zan et al., 2014). To synthesize these glycosides, we first prepared glycosyl bromide as the glycosyl donor from the corresponding benzoylated monosaccharide using a solution of 45% HBr/HOAc in CH_2Cl_2. The glycosyl donors were condensed with aglycones such as solasodine via silver trifluoromethanesulfonate catalysis. The resultant intermediates were then hydrolyzed by MeOH/MeONa or CH_3NH_2 to remove the benzoyl group, finally providing the target compounds. Biotinylated-glucose (**9**) was previously synthesized in our lab (Fig. 1) (Wang et al., 2011). The vacuole-specific dyes Cell Tracker Blue CMAC Dye (CMAC) and *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl)hexatrienyl)pyridinium dibromide (FM4-64) were purchased from Invitrogen. Commercial quantum dots with streptavidin conjugate 605 nm (QD) were obtained from Invitrogen. The QD were composed of streptavidin covalently attached to a fluorescent nanocrystal (Qdot® nanocrystal). Qdot® nanocrystals are prepared from a nanometer-scale crystal of a semiconductor material (CdSe), which is coated with an additional semiconductor shell (ZnS) to improve the optical properties of the material. Streptavidin has a very high binding affinity to biotin or biotin conjugates, which permits the specific detection of a variety of proteins. Quinacrine, propidium iodide (PI), 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 2′,7'-dichlorofluorescin diacetate (DCFH-DA) and amphotericin B (AMB) were purchased from Sigma. Quinacrine, PI, DCFH-DA and AMB were prepared in dimethyl sulfoxide (DMSO) at 10 mg/ml and stored at −20 °C. In each assay, the content of DMSO was less than 1%.

2.2. Antifungal susceptibility test

The minimal inhibitory concentration (MIC) values of glycosides against *C. albicans* were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M27-A3) (CLSI, 2008). Briefly, the antifungal agents were 2-fold serially diluted in cell suspensions in RPMI 1640 medium or Spider medium (1% nutrient broth, 0.5% mannitol, 0.1% K_2HPO_4, pH 7.2). Then, 100-μl aliquots were added to 96-well flat-bottomed microtitration plates. The plates were incubated at 35 °C for 24 h; zero visible growth was considered as the endpoint value.

2.3. Time-killing kinetics

To investigate the fungicidal activity of SG, time-killing curves were plotted by measuring the cell-survival rates. Overnight-cultured SC5314 cells were diluted to a final concentration of 1×10^5 cells/ml and treated with 32 or 64 μg/ml SG in Spider medium at 30 °C. At different time intervals, an aliquot was removed and plated on YPD agar plates to enumerate the surviving colonies.

2.4. QD visualization assay

Biotinylated-glucose (**9**) (2 μM) and QD (20 nM) were co-incubated at 4 °C for 40 min to form glucose-QD complexes (Wang et al., 2011). Prepared SC5314 cells were diluted to 5×10^5 cells/ml and incubated with the glucose-QD complexes or QD as a negative control at 30 °C for 1 h. After washing twice, the cells were imaged by confocal laser scanning microscopy (CLSM) (Carl Zeiss, LSM700, Germany) using a 63 × objective lens. The fluorescence of the QD upon excitation by a 555-nm laser was recorded in the emission spectrum of 590–700 nm.