

Doxycycline induces SLT2 phosphorylation in *Candida glabrata*

Hala Almshawit* and Ian Macreadie

School of Applied Sciences, RMIT University, 124 La Trobe St, Melbourne VIC 3000, Australia

Abstract

The tetracycline promoter (*tet*) has been manipulated in several systems as an inducible regulator of gene expression. In this study the effect of doxycycline on *C. glabrata* viability has been examined in water and according to CLSI standard method. In addition, flow cytometry analysis was performed to detect SLT2 protein activation by sub-inhibitory concentrations of doxycycline. Our findings show that 100 µg/ml of doxycycline is the maximum tolerated dosage in media; however, *C. glabrata* was sensitive to 40-50 µg/ml in water. 50 µg/ml of doxycycline induced SLT2p phosphorylation and our results support the idea of involvement of doxycycline in alteration of *C. glabrata* oxidative status. These results should be considered when the *tet* promoter system is used in yeast molecular studies.

Publication History:

Received: March 08, 2017

Accepted: June 25, 2017

Published: June 27, 2017

Keywords:

Levofloxacin, Chitosan, EDTA, *S. aureus*, MRSA, Antibiotic resistance, Antimicrobial study

Introduction

Various factors including the increased numbers of immunocompromised patients (e.g. AIDS and cancer patients), a growing aged population, the increased use of indwelling medical devices and antibiotic misuse have led to the increase in *Candida* infection incidence. *C. glabrata* is one of non - *C. albicans* species which has become of concern because of its increased incidence and ability to develop resistance towards common antifungals [1].

Detailed genetic studies of *C. glabrata* are now commonplace thanks to the regulatable promoters which are available to the researchers to induce or repress gene expression in *C. glabrata*. These promoters, such as *tetO*, *MET3* and *PDC1* promoters [2,3] control expression of the gene of interest upon incubation of cells in growth media containing the activating or the repressing substance. The tet-regulatable promoter is directed affected by tetracycline, induced by addition of doxycycline to the growth medium with induction being reversed by doxycycline removal. This system has been successfully used for functional analysis studies of essential genes with unknown function in *C. glabrata* [2]. Doxycycline was described to have no detectable effect on global gene expression in *Saccharomyces cerevisiae* at certain concentration [4]. However, there are no studies that examine the effect of doxycycline on *C. glabrata* global gene expression.

In mammalian cells doxycycline was shown to be an ERK1/2 inhibitor [5]. Abioinformatic database analysis (*Candida* Genome Database (CGD)) indicates that *Homo sapiens* ERK2 shows 48% identity and 65% similarity to the *C. glabrata* SLT2, the MAPKKK protein of cell wall integrity mitogen activated protein kinase signaling pathway (CWI MAPK). Based on that, we set out to determine the effect of doxycycline on SLT2 phosphorylation status in *C. glabrata* and on its survival in general. We believe that research community in the field of yeast molecular genetics may benefit from the conclusions of our studies.

Materials and Methods

Susceptibility of *C. glabrata* to doxycycline

C. glabrata ATCC90030 and *C. glabrata* ATCC2001 strains were used in this study. Susceptibility testing to doxycycline was performed using three methods. The first method was the standardized broth

microdilution assay as defined by CLSI (2008) using RPMI1640 medium (Sigma) and *C. glabrata* ATCC2001. Doxycycline concentrations ranged from 6.2 to 200 µg/ml. After incubation for 24 h at 35°C the minimum inhibitory concentration was determined visually. Susceptibility was also observed by spotting 5 µl from serial dilutions of both strains onto YEPD agar plates [1% (w/v) yeast extract, 2% (w/v) bacteriological peptone, 2% (w/v) dextrose and 1.5% bacteriological agar (Oxoid)] contain 100 or 200 µg/ml doxycycline.

The sensitivity of *C. glabrata* to doxycycline was tested in water as well. High density cells in water (about 3-8 x 10⁶ CFU/ml) were treated with different concentrations of doxycycline ranging from 0 to 400 µg/ml. Preparation of *C. glabrata* cells for doxycycline treatment in water was carried out as described previously in [6]. Doxycycline was dissolved in RPMI1640 medium or added directly to water.

To examine the effect of sorbitol and antioxidants such as ascorbic acid and reduced glutathione on inhibition of 50 µg/ml of doxycycline toxicity in water, 1 M of sorbitol or 10 mM of ascorbic acid or reduced glutathione were added to about 3 x 10⁵ CFU/ml of *C. glabrata* ATCC90030 together with doxycycline.

Susceptibility of *C. glabrata* to doxycycline

Detection of SLT2 phosphorylation by using of flow cytometry was carried out using phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology). Cells of *C. glabrata* ATCC2001 from an overnight YEPD plate were used to inoculate YEPD liquid medium and incubated 4-5 h at 30°C with agitation (200 rpm). Cells were harvested and washed with RPMI1640 liquid medium and resuspended in the same medium at density of about OD₆₀₀=1.

To examine SLT2 phosphorylation induction by doxycycline, *C. glabrata* ATCC2001 exponential phase cultures were examined 30 min

*Corresponding Author: Dr. Hala Almshawit, School of Applied Sciences, RMIT University, 124 La Trobe St, Melbourne VIC 3000, Australia; E-mail: hala.almshawit@gmail.com

Citation: Almshawit H, Macreadie I (2017) Doxycycline induces SLT2 phosphorylation in *Candida glabrata*. Int J Clin Med Microbiol 2: 122. doi: <https://doi.org/10.15344/2456-4028/2017/122>

Copyright: © 2017 Almshawit et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

after addition of 0, 25 or 50 µg/ml doxycycline. Cells were fixed, permeabilised and antigens detected according to the primary antibody manufacturer's instructions. In brief, one ml samples were collected from each treatment and 100 µl of 37% formaldehyde was added. The samples were incubated at 37°C for 10 min, chilled on ice for 1 min and centrifuged (9000 rpm) for 1 min. The pellets were resuspended in 100 µl PBS. Permeabilisation was carried out by adding 900 µl of 100% cold methanol (-20 °C) and incubated on ice for 30 min. For immunostaining, cells were harvested and washed with PBS. Pellets were resuspended in blocking buffer by vortexing and incubated for 10 min at room temperature. Then the cells were harvested and resuspended in blocking buffer containing 1:200 diluted phospho-p44/42 MAPK primary antibody (thr202/tyr204; Cell Signaling Technology #9101). Cells were incubated for 1 h at room temperature. After washing with PBS the cells were suspended in 1:1000 diluted anti-rabbit HRP secondary antibody (Sapphire, Australia) in blocking buffer and incubated for 30 min in the dark at room temperature. After three washes with PBS, fluorescence intensity was detected by AmCyan filter in FACS Canto II (BD Biosciences) flow cytometer.

Results

Susceptibility of *C. glabrata* to doxycycline in media

The minimum inhibitory concentration of doxycycline to *C. glabrata* ATCC2001 in RPMI1640 medium was 200 µg/ml. A weak growth was visualized in the microwells which contained 100 µg/ml doxycycline comparing with growth control microwells. Aliquots of serial dilutions of both strains on YEPD agar containing 0, 100 or 200 µg/ml doxycycline showed no inhibition of growth (Figure 1).

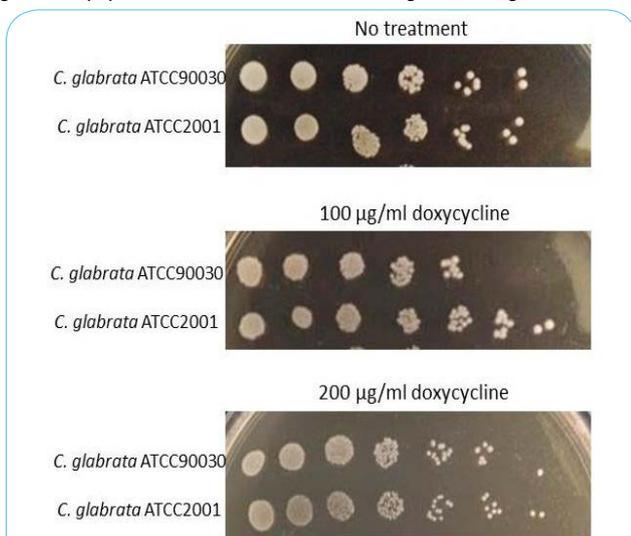
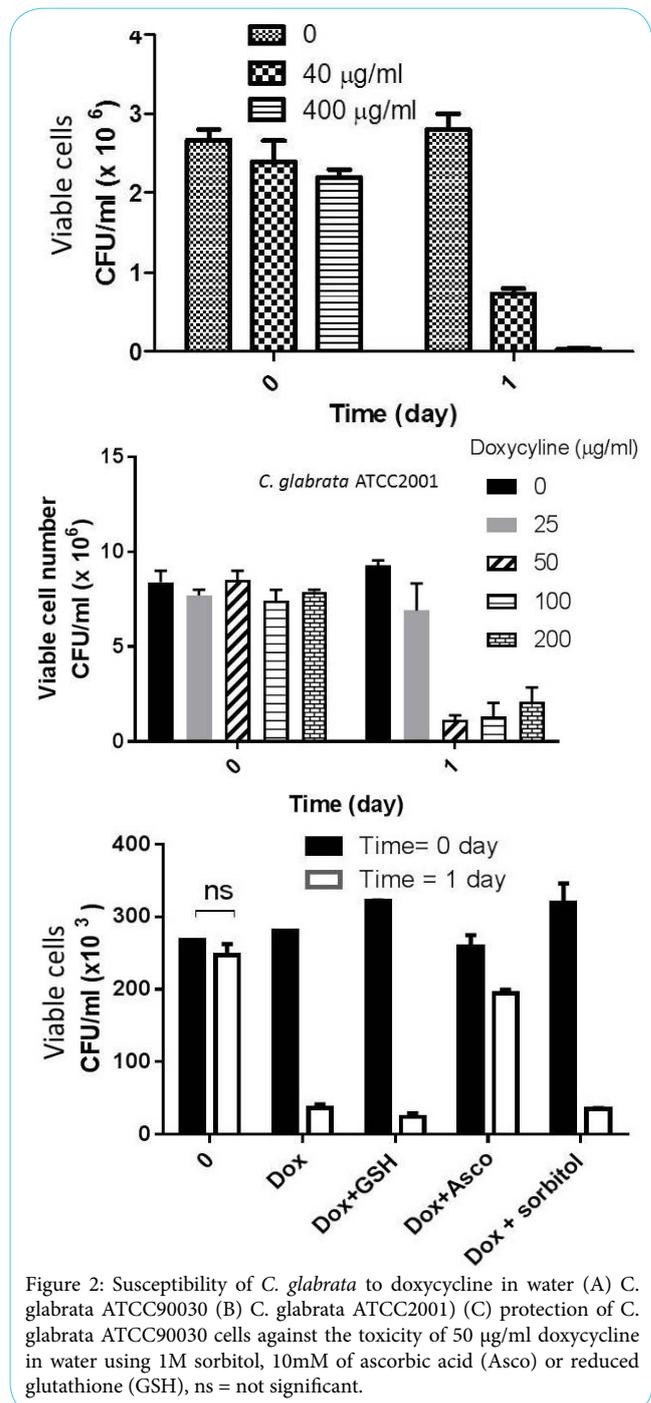


Figure 1: Susceptibility of *C. glabrata* ATCC2001 to doxycycline by spotting serial dilutions of *C. glabrata* ATCC2001 and ATCC90030 on YEPD agar plates contain 0, 100 or 200 µg/ml doxycycline.

Susceptibility of *C. glabrata* to doxycycline in media

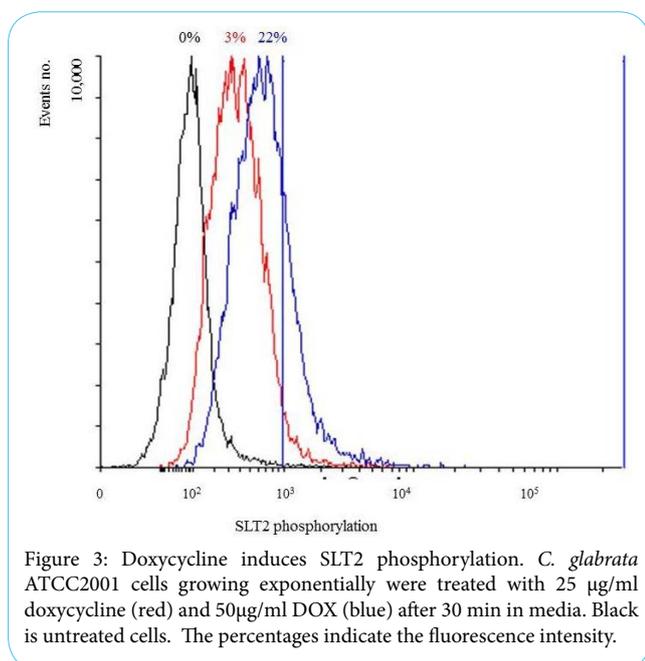
C. glabrata high density cell populations (~3 x 10⁶ CFU/ml) of ATCC90030 strain or 8 x 10⁶ CFU/ml of ATCC2001 strain in water began to lose more than 60 and 80% of their viability respectively after treatment with 40 µg/ml or 50 µg/ml of doxycycline (Figure 2a and Figure 2b). The toxicity of doxycycline in water could be partly abrogated (20% loss in viability) by adding 10 mM ascorbic acid but not reduced glutathione (> 80 % loss in viability). Sorbitol also could not save cells from doxycycline toxicity in water (Figure 2c).



Doxycycline induces SLT2 phosphorylation

Experiments were designed to find whether doxycycline may act as SLT2 inhibitor, and toxicity of doxycycline in water was due to this property. Cells in RPMI1640 were treated with doxycycline and examined for SLT2 phosphorylation. Concentrations of 25, 50 and 100 µg/ml of doxycycline were tested for their ability to inhibit SLT2 phosphorylation. The phosphorylation of SLT2 in *C. glabrata* ATCC2001 was measured using flow cytometry. Within 30 min of incubation in the lowest concentration of doxycycline (25 µg/ml), phosphorylation of SLT2 protein was detected. The phosphorylation was higher in the cells treated with 50 µg/ml of doxycycline, whereas

untreated cells showed no detectable positive events indicating no SLT2 phosphorylation in these cells (Figure 3).



Discussion

Doxycycline concentrations of 200 µg/ml inhibited *C. glabrata* ATCC2001 growth in the MIC assay, while a concentration of one fifth of the MIC of doxycycline was enough to cause loss in viability of this fungus in water. In our previous study >10⁴ CFU/ml of *C. glabrata* was shown to be able to keep their viability in water over at least three days [6]. This fact has been shown again in this work as 8 x 10⁶ CFU/ml of *C. glabrata* ATCC2001 and ~ 3 x 10⁵ of *C. glabrata* ATCC90030 were able to maintain their viability in water.

Many studies depend on spotting serial dilution of yeast on YEPD agar plates to examine the effect of doxycycline on promoter induction [7] or synergy with other compounds [8]. Therefore, the spotting assay was carried out in this study to observe the effect of 100-200 µg/ml of doxycycline on *C. glabrata* reference strains. Both strains were not sensitive to the highest concentration of doxycycline. However, other yeast species such as *C. albicans* was susceptible to doxycycline at 200 µg/ml [8] when it was tested by using the spotting assay. A study on *S. cerevisiae* cells treated with 40 µg/ml of doxycycline showed that there was no phenotypic effect and no significant effect on their global genome transcription levels which allow the use of *tetO* regulatable promoter system for genetic studies [4]. In our study it is clear that 200 µg/ml of doxycycline in YEPD agar plates did not cause any phenotypic effect on *C. glabrata* in media but this was not the case when this species was treated with doxycycline in water. It seems that yeast species vary in their response to doxycycline treatment as Fiori et al., [8] demonstrated by the difference between *C. albicans* and *S. cerevisiae* to doxycycline and fluconazole treatment combinations. Growth in the presence of fluconazole and doxycycline was restored by overexpression of *ERG11* in *S. cerevisiae* but not in *C. albicans* [8].

Since CWIMAPK signaling has been reported to be activated in hypo-osmotic conditions [9] and doxycycline was described as an ERK1/2 inhibitor [5], the homolog of SLT2 in *C. glabrata* (CGD), the question was asked, could this antibiotic act as a SLT2 inhibitor,

leading to cell death in water? The toxicity of doxycycline in water was not abrogated by adding the osmo-stabiliser "sorbitol". In contrast, staurosporine (a known PKC1 inhibitor) toxicity in water could be abrogated by sorbitol [10]. PKC1 is an upstream kinase of the CWIMAPK signaling pathway. This indicates doxycycline has a target other than CWI-MAPK. Flow cytometry analysis of SLT2 phosphorylation confirmed that doxycycline does not inhibit SLT2 phosphorylation: indeed doxycycline activates SLT2 phosphorylation. The activation of this protein occurred once *C. glabrata* cells were treated with sub-inhibitory concentrations of doxycycline (50 µg/ml) in RPMI1640 medium.

In summary, the fungicidal effect of doxycycline in water is not due to inhibition of the SLT2 activation. Doxycycline is an iron chelator leading to depletion of intracellular iron [8]. Iron depletion has been proposed to decrease ergosterol content in *C. albicans* and *S. cerevisiae*, leading to higher fluidity in cell membranes, with consequent increased passive diffusion [11,12]. Further investigation might include the examination of the effect of iron addition on *C. glabrata* sensitivity to doxycycline. Water and doxycycline might diffuse passively and cause cell death. The elimination of doxycycline toxicity with ascorbic acid might indicate an interference with the oxidative homeostasis due to iron depletion. In addition, doxycycline was shown to increase the susceptibility of *C. albicans* to amphotericin when they were treated with 50 to 200 µg/ml of doxycycline [13]. This alteration in susceptibility was suggested to be due to the inhibition of mitochondrial function in *C. albicans*, which eliminates the diauxic shift [13]. Lack of diauxic shift, or the lack of functional mitochondria alters sterol metabolism, resulting in lower ergosterol levels, consistent with the need for 12 molecules of oxygen to synthesize one molecule of ergosterol [13]. *Saccharomyces cerevisiae* was shown to require antioxidant function to resist the toxicity of doxycycline [14]: this has been also demonstrated in our study where ascorbic acid protected against doxycycline toxicity in water. However, it is not clear for us why reduced glutathione, also an antioxidant, did not protect against doxycycline toxicity.

In conclusion this study demonstrates that *C. glabrata* cells are more sensitive to doxycycline in water than in media, and doxycycline is an inducer of SLT2 phosphorylation. Furthermore, this study supports the proposed mechanism of killing of doxycycline in water may be attributed to an alteration of oxidative homeostasis in yeast which alters the cells permeability.

Competing Interests

The authors declares that they have no competing interests.

References

1. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, et al. (2012) *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. FEMS Microbiol Rev 36: 288-305.
2. Nakayama H, Izuta M, Nagahashi S, Sihta EY, Sato Y, et al. (1998) A controllable gene-expression system for the pathogenic fungus *Candida glabrata*. Microbiol 144: 2407-2415.
3. Zordan RE, Ren Y, Pan SJ, Rotondo G, De Las Penas A, et al. (2013) Expression plasmids for use in *Candida glabrata*. G3 (Bethesda) 3: 1675-1686.
4. Wishart JA, Hayes A, Wardleworth L, Zhang N, Oliver SG (2005) Doxycycline, the drug used to control the *tet*-regulatable promoter system, has no effect on global gene expression in *Saccharomyces cerevisiae*. Yeast 22: 565-569.

5. Kim HS, Luo L, Pflugfelder SC, Li DQ (2005) Doxycycline inhibits TGF-beta1-induced MMP-9 via Smad and MAPK pathways in human corneal epithelial cells. Invest Ophthalmol Vis Sci 46: 840-848.
6. Almshawit H, Pouniotis D, Macreadie I (2014) Cell density impacts on *Candida glabrata* survival in hypo-osmotic stress. FEMS Yeast Res 14: 508-516.
7. Matsuo Y, Granneman S, Thoms M, Manikas RG, Tollevery D, et al. (2014) Coupled GTPase and remodelling ATPase activities form a checkpoint for ribosome export. Nat 505:112-116.
8. Fiori A, Van Dijck P (2012) Potent synergistic effect of doxycycline with fluconazole against *Candida albicans* is mediated by interference with iron homeostasis. Antimicrob Agents Chemother 56: 3785-3796.
9. Levin DE (2005) Cell Wall Integrity Signaling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 69: 262-291.
10. Almshawit H (2016) Studies on the effect of thymoquinone on oxidative stress and cell wall integrity of *Candida glabrata*, PhD thesis, pp. 135.
11. Prasad T, Chandra A, Mukhopadhyay CK, Prasad R (2006) Unexpected link between iron and drug resistance of *Candida* spp.: iron depletion enhances membrane fluidity and drug diffusion, leading to drug-susceptible cells. Antimicrob Agents Chemother 50: 3597-606.
12. Shakoury-Elizeh M, Protchenko O, Berger A, Cox J, Gable K, et al. (2010) Metabolic response to iron deficiency in *Saccharomyces cerevisiae*. J Biol Chem 285: 14823-14833.
13. Oliver BG, Silver PM, Marie C, Hoot SJ, Leyde SE, White TC (2008) Tetracycline alters drug susceptibility in *Candida albicans* and other pathogenic fungi. Microbiol, 154: 960-970.
14. Angrave FE, Avery SV (2001) Antioxidant functions required for insusceptibility of *Saccharomyces cerevisiae* to tetracycline antibiotics. Antimicrob Agents Chemother 45: 2939-2942.