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Short Communication

An in vitro repositioning study reveals antifungal potential of chloroquine to inhibit growth and morphogenesis in *Candida albicans*

Ravikumar B. Shinde, Sandeep B. Rajput, Jayant S. Raut, and S. Mohan Karuppayil*

School of Life Sciences, SRTM University, Nanded (MS), India

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Antifungal drugs available to treat infections caused by *C. albicans* are mainly limited to the azole, polyene and echinocandin classes of molecules. However these drugs are unable to treat *Candida* infections because of multiple drug resistance and alternate survival strategies like biofilm formation. Side effects due to toxicity is another problem associated with these drugs. Hence, there is a need to search for novel molecules with antifungal activities (Mishra et al. 2007; Öncü, 2011). Designing a new drug is a time- and money-consuming process. Drug repurposing/repositioning, which involves revealing novel bioactivities of drugs already being used in therapeutics (for treatment of a different ailment), is an attractive strategy (Ashburn and Thor, 2004). For example, cisplatin, which was originally discovered as an antimicrobial, proved to be an effective tumor inhibitor. Azole antifungals were shown to have good anti-mycobacterium activities, while anticancer agents were reported to possess potential anti-*Candida* properties (Routh et al., 2011). Chloroquine (CQ), a synthetic derivative of quinine, is a popular antimalarial agent and has been extensively used against malaria since 1940.

* Corresponding author: Dr. S. Mohan Karuppayil, Department of Biotechnology, DST-FIST & UGC-SAP sponsored School of Life Sciences, SRTM University, Nanded (MS), India, 431606.
Tel: +91-9028528438; +91-2462-222154
Fax: +91-2462-229245
E-mail: prof.karuppayil@gmail.com

Increased incidence of CQ resistant *Plasmodium falciperum* infections has suspended its use for malaria treatment in most of countries. However, attempts are being made to explore the unknown bioactivities of chloroquine (Savarino et al., 2003). It was used in clinical trials as an investigational antiretroviral agent for HIV-1 infection in humans, particularly when survival has been prolonged by other antiretroviral treatments.

Furthermore, in a study by Sotelo and co-workers, chloroquine was added to a conventional therapeutic protocol of surgery, radiotherapy and chemotherapy to treat glioblastoma in HIV-1-seronegative adults and found to be synergistic (Sotelo et al., 2003). Chloroquine was also found effective against the yeasts *Histoplasma capsulatum* and *Cryptococcus neoformans* (Khan et al., 2005). Effects of CQ in the human fungal pathogen *C. albicans* have not been reported except for one conference abstract (Park et al., 2003). In this systematic study, we report the effect of CQ on growth as well as yeast-to-hyphae transition which is an important virulence factor of *C. albicans*. In order to explore the mechanism of action, ergosterol profiling of *C. albicans* was done.

A standard strain of *Candida albicans* (ATCC 90028) was obtained from the Institute of Microbial Technology, Chandigarh, India. Cultures were stored on Yeast extract-Peptone-Dextrose (YPD) agar at 4°C. Activation of cultures was done by inoculating a single colony into 50 ml of YPD broth in a 250 ml Erlenmeyer flask. The flasks were incubated at 30°C on a rotary

incubator shaker, for 24 h. Cells were harvested by centrifugation and washed with sterile Phosphate Buffer Saline (PBS). RPMI-1640 medium, pH 7.0 (with L-glutamine, without sodium bicarbonate; buffered with 0.165 mM MOPS), was filter sterilized. All the media components and chemicals were purchased from Hi-Media Pvt. Ltd., Mumbai. Chloroquine (CQ) and fluconazole were purchased from Ipca Pharm. Pvt. Ltd., India and Cipla Pharm. Pvt. Ltd., Mumbai, India, respectively. Susceptibility to drugs was studied by using the broth micro dilution method as per the guidelines of CLSI (Routh et al., 2011). Briefly, various concentrations of CQ ranging from 2,000 to 31.2 μg ml^{-1} were prepared in RPMI-1640 by double dilution in 96-well microplates (Costar, Corning Corp., USA), while wells without drugs served as controls. Medium containing cells was added so that the final volume of the assay system in each well remained 200 μl, with a cell density of 1×10^3 cells ml^{-1}. The standard drug fluconazole (8–0.125 μg ml^{-1}) served as another control. Plates were incubated at 35°C, for 48 h and the absorbance of wells was read at 620 nm wavelength using a microplate reader (Multiskan- Ex, Thermo Electron Pvt. Ltd., Banglore, India). Minimum inhibitory concentration (MIC) for growth was determined as the lowest concentration causing 50% reduction in the absorbance of cells compared to that of the control.

The effect of CQ on yeast-to-hyphae transition in *C. albicans* was studied by microplate-based standard assay (Chauhan et al., 2011). FBS and RPMI-1640, two different inducers of germ tube formation, were used. Various concentrations of CQ were separately prepared by double dilution in these inducers and tested for their activity against germ tube formation. Cells were added to get 1×10^6 cells ml^{-1} in 200 μl of assay system in each well. Wells without CQ were kept as a control and plates were incubated at 37°C, at 200 rpm for 2 h. Plates were observed using an inverted microscope (Metzer, India) for the presence of yeast and hyphae. Every time 100 cells were counted, and the percentage of germ tube formation in the presence of the drug compared to that of control was calculated. Ergosterol profiling was performed as per the method given by Arthington-Skaggs et al. (1999). Briefly, cultures were incubated overnight in the presence of varying concentrations of CQ, at 35°C and 100 rpm. Cells were harvested and wet weight of the cell pellet was determined. Cells were suspended in 25% ethanolic KOH and incubated at 85°C for 1 h. Sterols were extracted in 75% v/v *n*-heptane and the extracts were diluted 1 : 5 with ethanol.

Spectrum was taken at the wavelengths 240–300 nm using the UV-Vis Spectrophotometer (model UV-1800, Shimadzu, Inc.). The presence of ergosterol and the late intermediate 24 (28) DHE (dehydroergosterol) in the extracted sample results in a characteristic four-peak curve. The height of the absorbance peaks was indicative of the ergosterol concentration. Experiments were done three times and the values mentioned are means with standard deviations. Values of the treatment and control groups were compared by using Student's *t*-test and a p value < 0.05 was considered significant.

The aim of this study was to explore the potential of repositioning the antimalarial drug chloroquine against the human pathogen *C. albicans*. CQ inhibited growth in a concentration-dependent manner. Around 25% of growth was inhibited at 500 μg ml^{-1}, while a significant ($p < 0.05$) inhibition of growth was exhibited at 1,000 μg ml^{-1}. CQ prevented morphogenesis (an important virulence attribute) of *C. albicans* at very low concentrations. The number of cells switching from yeast to hyphae forms, induced by two standard inducers (i. e. serum & RPMI-1640 medium) was reduced with increasing CQ concentration. CQ, at a 31.2 μg ml^{-1} concentration, showed significant (more than 60%) inhibition of Y-to-H form switching (Fig. 1). Complete inhibition of germ tube formation was obtained at 250 μg ml^{-1} and only yeast-form cells were observed (Fig. 2F). Morphogenesis of *C. albicans* was sensitive to CQ at concentrations much lower than the

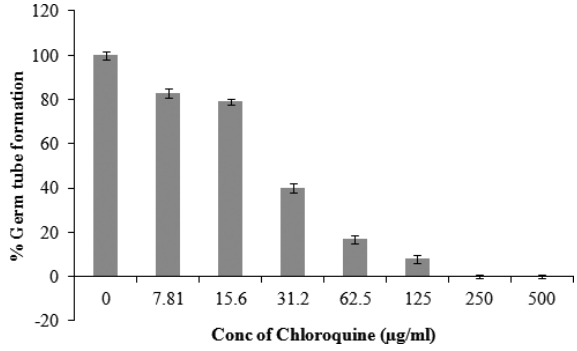

Fig. 1. Effect of chloroquine on serum-induced yeast-to-hyphae dimorphism in *Candida albicans*.

The assay was performed by a microplate-based method. Various concentrations of CQ ranging from 7.81 to 500 μg ml^{-1} were added to the induction medium and the effect on morphogenesis was analyzed after 2 h. Percentage of germ tube formation was calculated compared with the control.

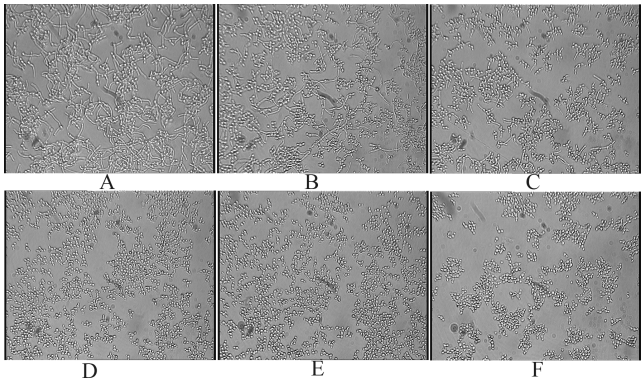

Fig. 2. Light micrographs showing the effect of chloroquine on serum-induced yeast-to-hyphae morphogenesis in *Candida albicans*.

Various concentrations of CQ, A) Control; B) 15.6 μg ml^{-1}; C) 31.2 μg ml^{-1}; D) 62.5 μg ml^{-1}; E) 125 μg ml^{-1}; and F) 250 μg ml^{-1}, were added to the induction medium and morphology of the cells was observed under an inverted microscope (magnification × 200).

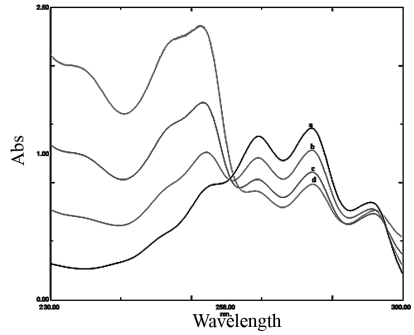

Fig. 3. Ergosterol profile of *Candida albicans* cells exposed to various concentrations of chloroquine, a) Control; b) 250 μg ml^{-1}; c) 500 μg ml^{-1}; d) 1,000 μg ml^{-1}, showing a concentration-dependent decrease in the height of the peak characteristic of ergosterol.

Note the increase in a peak specifically at 260 nm, which may correspond to an abnormal ergosterol.

growth inhibitory concentrations. This ability to inhibit a virulence factor, without killing the pathogen, may be valuable to avoid the natural selection and emergence of a drug-resistant population of microbes (Clatworthy et al., 2007).

Previous studies have reported that CQ has a potential to inhibit cholesterol biosynthesis in hepatic cells of rats. CQ was found to interfere in cyclization of squalene oxide to lanosterol, which resulted in inhibition of cholesterol (Chen and Leonard, 1984). Lowering of cholesterol may be a net result of limited synthesis of cholesterol and inhibition of re-absorption of cholesterol in intestinal as well as hepatic cells (Achudume, 2009). Along with the interruption in cholesterol re-absorption it has been shown to alter the protein-binding and permeability of various membranes (Wellems et al., 1991). It has been reported that CQ treatment leads to iron deprivation in a yeast, *Histoplasma capsulatum* (Newman et al., 1993). Interestingly, iron deprivation of yeast cells is already known to cause lowering of membrane sterols (Shakoury-Elizeh et al., 2010). We hypothesized that a similar mechanism may exist in *C. albicans*, so that CQ will interfere in ergosterol biosynthesis resulting in changes in membrane permeability and normal membrane functions. To analyze this possibility, ergosterol profiling of cells grown in the presence of varying concentrations of CQ was done. Cellular ergosterol decreased in *C. albicans* with increasing concentration (i. e. from 250 to 1,000 μg ml^{-1}) of CQ (Fig. 3). Notably one of the peaks in the characteristic four-peak spectrum was found to increase with increasing concentrations of CQ. We propose that this unknown peak may be a toxic sterol accumulated due to CQ-mediated intervention in sterol synthesis, which interferes with normal membrane functions and inhibits its growth of *C. albicans*. To summarize, our study has shown that inhibition of normal ergosterol synthesis may be one of the reasons behind CQ-mediated inhibition of *C. albicans* growth. Ergosterol also has a direct relation with morphogenesis in *C. albicans*. A mutant unable to synthesize ergosterol was shown defective in yeast-to-hyphal form conversion (Pasrija et al., 2005). Moreover, for the first time, we report its ability to inhibit morphogenesis. Prevention of this important virulence attribute, independent of growth inhibition, suggested that CQ has specific anti-morphogenesis activity. This study indicated that CQ and similar drugs have potential to be repurposed against *C. albicans*.

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