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Short Communication

Cancer drugs inhibit morphogenesis in the human fungal pathogen, *Candida albicans*

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Abstract

Candida infections are very common in cancer patients and it is a common practice to prescribe antifungal antibiotics along with anticancer drugs. Yeast to hyphal form switching is considered to be important in invasive candidiasis. Targeting morphogenetic switching may be useful against invasive candidiasis. In this study, we report the antimorphogenetic properties of thirty cancer drugs.

Key words: *Candida albicans*, morphogenetic switching, cancer drugs, antifungal antibiotics.

Candida albicans is an important opportunistic fungal pathogen of the humans. Systemic candidiasis is a serious situation in patients undergoing treatment for cancer (Safdar and Armstrong, 2002). *Candida* species now rank among the ten most prominent pathogens in leukemia patients, accounting for 75% of fungal infections in general and these infections result in 25-60% mortality (Winston *et al.*, 2000). In the last two decades, use of azole antifungals has rapidly led to the development of drug resistance in patients with advance cancers, AIDS, organ transplantations, and surgeries etc. *C. albicans* cells exist in different morphological forms, including yeast, pseudohyphal and true hyphal forms (Pauw, 2004). Yeast to hyphal form morphogenesis in *C. albicans* is believed to be related to its virulence, since mutants defective in hyphal growth are less virulent in mouse models than their wild-type (Lo *et al.*, 1997). There are various reports on drugs inhibiting yeast to hyphal form switching. For example, 6-Amino-2n-pentylthiobenzothiazole an antifungal agent is reported to inhibit hyphal growth (Fabry *et al.*, 1999). Antimetabolite class of anticancer agents are also tested for their hyphal inhibitory activity along with mRNA, DNA synthesis and protein synthesis inhibitors. Among all these, cyclohexamide showed most potential activity against hyphal induction (Imanishi *et al.*, 2004). The known actin inhibiting drugs, latrunculin-A and jasplakinolide inhibited yeast to hyphal form transition in a dose dependent and reversible manner (Toenje *et al.*, 2005). Undecylenic acid inhibits the switch from yeast form to hyphae, in sublethal concentrations (McLain *et al.*, 2000). Earlier we have reported the potential morphogenetic role for ethyl alcohol and its first oxidation product acetaldehyde in *C. albicans*. Both of them, inhibited yeast to hyphal form morphogenesis induced by four standard inducers in a concentration dependent manner (Chauhan *et al.*, 2010, 2011). Most of the commonly used anticancer drugs inhibit fundamental steps involved in eukaryotic metabolism or cell cycle like inhibition of DNA, RNA and protein synthesis (Liscovitch and Lavie., 2002). Some of the anticancer drugs targeting tubulin function, DNA synthesis, induction of apoptosis, DNA replication, protein synthesis, estrogen receptors etc, are known to have *in-vitro* anti-*Candida* activity (Davies *et al.*, 2000; Kesavan *et al.*, 2005). Amino acid induced yeast to hypha formation was inhibited by treatment with Actinomycin-D, Bleomycin, 5-Flourouracil and Hydroxyurea at varying concentration in the range of 100-1000 μg/mL in *C. albicans* 5685 strain (Land *et al.*, 1980). However no direct roles for antimorphogenetic properties for anticancer drugs are reported in *C. albicans*. Considering the eukaryotic nature of *C. albicans*, it can be expected that anticancer drugs may exert anti-*Candida* activity also. Recently, we reported antifungal activity of thirty commonly prescribed anticancer drugs. Most of the drugs are tested for the first time. These drugs exhibited growth inhibitory effects on fluconazole sensitive as well as fluconazole resistant strains (Routh *et al.*, 2011). Here in this comprehensive study we report the inhibitory effect of thirty anticancer agents on yeast to hyphal form transition of *C. albicans*.

C. albicans, ATCC 90028 was used throughout the study. Methodology for growth and storage of *C. albicans* were same as per described previously (Chauhan *et al.*, 2010, 2011). 10% Horse serum was used for germ tube in-

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duction. Thirty anticancer drugs from different classes were used for this work. All the drugs were purchased from the local market. To study the effect of anticancer drugs on germ tube formation, *Candida* filamentation assay was done using microtiter plate assay in 96 well microtiter plates (Chauhan *et al.*, 2010, 2011). Cells from a stock solution were inoculated in 10% serum to get 1 x 10^6 cells/mL. Various concentrations of anticancer drug (ranging from 0.781 μg/mL to 50 μg/mL) were added to the wells. Wells without drugs were kept as control. Final volume in each well was kept 200 μL. The plates were incubated at 37 °C at 200 rpm on an orbital shaker for two hours. After incubation cells were observed microscopically. Every time 100 cells were counted and numbers of yeast and germ tube forms are noted. Percentage of germ tube inhibition was calculated by comparing with control (without drug). As shown in Table 1 and Figure 1, anticancer drugs effectively prevent yeast to hyphal form transition induced by 10% serum. Among the four antimicrotubule anticancer agents, Docetaxel significantly inhibited (around 80%) morphogenesis at 25 μg/mL, while 50 μg/mL showed complete inhibition of filamentation (Figure 1 B). Vinblastine did not alter morphogenesis upto 50 μg/mL. Whereas Paclitaxel caused inhibition of yeast to hyphal form morphogenesis at 50 μg/mL, while Vincristine at same concentration caused 80% inhibition of filamentation (Table 1). Platinum analogs showed efficient activity. Cisplatin, Oxaliplatin and Carboplatin (Figure 1J) completely blocked germ tubes at 50 μg/mL (Table 1). Cyclophosphamide inhibited filamentation at 50 μg/mL. At 6.25 μg/mL it inhibited almost 50% morphogenesis. Busulfan and Ifosfamide also blocked 60-80% switching at 12.5 μg/mL and complete inhibition at 50 μg/mL. Melphalan (Figure 1D) and Carmustine exherted inhibition of germ tubes at 50 μg/mL (Table 1).

Among the three antimetabolites tested, Methotrexate completely restricted morphogenesis at 25 μg/mL, followed by Gemcitabine which inhibited complete morphogenesis at 50 μg/mL (Figure 1E). Hydroxyurea was not effective upto 50 μg/mL, where 100% germ tubes were seen (Table 1).

Among various antitumor antibiotic drugs, Doxorubicin and 5-flurouracil were most efficient agents which inhibited morphogenesis around 80% at 12.5 μg/mL, while 25 μg/mL of Doxorubicin (Figure 1 C) and Daunorubicin completely inhibited filamentation. 5-flurouracil, Bleomycin, Mitoxantrone, Epirubicin and Mitomycin-C were found to exhibit morphogenetic switching inhibitory potential at 50 μg/mL (Table 1). Etoposide which is an epipodophyllotoxins exhibited inhibition of morphogenesis at 25 μg/mL (Figure 1G). Non classic alkalyting agent Dacarbazine completely halted filamentation at 50 μg/mL (Figure 1I). Leuprolide (Figure 1H), Tamoxifen (Figure 1L), Irinotecan (Figure 1K) and Formestane (Figure 1F) completely inhibited serum induced morphogenesis of *C. albicans* at drug concentration 50 μg/mL. Leucovorin (Figure 1A) did not have inhibitory effect on serum induced yeast to hyphal form morphogenesis (Table 1).

To demonstrate the effect of anticancer drugs on viability of *C. albicans* cell, viability plate count was done (Chauhan *et al.* 2010 and 2011). Briefly, various concentrations of drugs ranging from 25 μg/mL to 16,000 μg/mL were added to 10% Serum in 96-well microtiter plates. Each well was inoculated with a cell density of 1 x 10^6 cells/mL. The plates were incubated at 37 °C on a shaker for various time intervals (2 and 4 h, respectively). Cells from the respective wells were diluted to get countable colonies and an aliquot of sample was spread on YPD agar plates. Plates were incubated at 30 °C for 48 h and colony count was done. The germ tube inhibitory concentrations of various anticancer drugs did not alter the viability of *C. albicans* cells at various time points studied (Table 1). However higher concentrations of some antifungal drugs affects the viability. For example as shown in Table 1, Docetaxel showed significant reduction in viability at 1600 μg/mL, while Paclitaxel, Vinblastine, Vincristine, Bleomycin, Mitoxantrone, Cyclophosphamide, Melphalan, Leuprolide, Dacarbazine, Carboplatin, Cisplatin, Oxaliplatin and Irinotecan required 600-800 μg/mL of concentration to produce the similar effect at various time points. While, Vincristine, Doxorubicin and Busulfan caused significant reduction (around 50%) in viability at 200-400 μg/mL of concentration. Whereas, other drugs such as Tamoxifen, daunorubicin, 5-flurouracil, Mitomycin-C, Epirubicin, Carmustine, Ifosamide, Gemcitabine, Methotrexate, Hydroxyurea, Formestane, Etoposide and Leucovorine fails to affect the viability of *Candida* cells even at a concentration of 1600 μg/mL (Table 1).

C. albicans can exist in various morphological forms, among these hyphae are very important because cells that do not readily form hyphae often show reduced virulence. (Lo *et al.*, 1997). Currently available antifungal chemotherapy such as amphotericin, azoles and echinocandins, etc. are limited to drugs that directly inhibit growth of *C. albicans* cells but not any virulence factor. Exploration of unknown properties of existing drugs has provided a new focus to the field of antifungal chemotherapy. For example non steroidal anti-inflamatory drugs which are cycloxygenase inhibitors have been found to inhibit dimorphism and biofilm formation in *C. albicans*. Some protein synthesis inhibitors are also screened for their antimorphogenetic activity suggesting that *de novo* mRNA or protein synthesis is essential for germ tube formation (Imanishi *et al.*, 2004; Toenje *et al.*, 2005).

Our results suggest that all the anticancer drugs used in this study may be having similar targets in *C. albicans* as in mammals, because of their common ancestral heritage.

Table 1 - Effect of anticancer drugs on growth, viability and morphogenesis in *Candida albicans*

Serial number	Drug name	MIC_{50} [§] (μg/mL)	MFC_{50} [§] (μg/mL)		Concentration (μg/mL) of drugs required for inhibition of germ tubes	
			2 h	4 h	50%	100%
1	Docetaxel	100	1600	1600	12.5	50
2	Paclitaxel	100	800	800	12.5	50
3	Vinblastine	100	600	600	NA	NA
4	Vincristine	50	400	400	12.5	> 50
5	Tamoxifen	100	NA	NA	NA	50
6	Bleomycin	50	800	800	NA	50
7	Doxorubicin	50	200	200	6.25	25
8	Daunorubicin	200	NA	NA	NA	25
9	5-flurouracil	100	NA	NA	25	50
10	Mitoxantrone	100	800	800	12.5	50
11	Mitomycin-C	800	NA	NA	12.5	50
12	Epirubicin	100	NA	NA	25	50
13	Dactinomycin	100	NA	NA	NA	50
14	Busulfan	50	400	400	12.5	50
15	Carmustine	100	NA	NA	12.5	50
16	Cyclophosmamide	25	800	800	6.25	50
17	Ifosfamide	100	NA	NA	12.5	50
18	Melphalan	200	600	600	12.5	50
19	Gemcitabine	200	NA	NA	25	50
20	Methotrexate	100	NA	NA	NA	25
21	Hydrooxyurea	800	NA	NA	NA	NA
22	Formestane	100	NA	NA	25	50
23	Etoposide	100	NA	NA	NA	25
24	Leuprolide	100	800	800	25	50
25	Dacarbazine	100	800	800	12.5	50
26	Carboplatin	50	600	600	NA	50
27	Cisplatin	50	800	800	12.5	50
28	Oxaliplatin	50	600	600	6.25	50
29	Leucovorine	NA	NA	NA	NA	NA
30	Irinotecan	50	800	800	25	50

[*]MIC- Minimum Inhibitory Concentrations; MFC- Minimum Fungicidal Concentrations; NA- Not Achieved. [§]MIC values are taken from (Routh *et al.*, 2011, Chemotherapy).

Mitomycin-C was the most efficient inhibitor of morphogenesis which inhibited yeast to hyphal form transition at $1/16^{th}$ of its MIC. This was followed by Daunorubicin which was effective at $1/8^{th}$ of its MIC value .These two anticancer agents belong to the same class of antitumor antibiotics. Mitomycin –C inhibits transcription by targeting DNA dependent RNA polymerase. It acts as an alkalyting agent to cross link DNA resulting in inhibition of DNA synthesis and function. Whereas Daunorubicin inhibits topoisomerase II by forming a cleavable complex with DNA and topoisomerase II to create uncompensated DNA helix which creates a torsional tension leading to eventual DNA breaks (Toenje *et al.*, 2005). Epirubicin, Melphalan, Gemcitabine, Methotrexate, Etoposide and Leuprolide were inhibiting 100% germtubes at $1/4^{th}$ of their MIC which suggest their good antimorphogenetic potentials without affecting growth. Some drugs like Docetaxel, Paclitaxel, Tamoxifen, 5-Flurouracil, Dactinomycin, Carmustine, Ifosfamide, Formestane, and Dacarbazine inhibited 100% germ tubes at half of their MIC values. Rest few drugs were completely inhibiting at MIC (Table 1). The two antimicrotubule i.e Docetaxel and Paclitaxel may affect microtubule formation resulting in inhibition of morphogenesis. 5-Flurouracil, the antitumor antibiotic is reported to inhibit protein synthesis in *C. albicans* which can block the proteins which are essential for filamentation (Chen *et al.*,

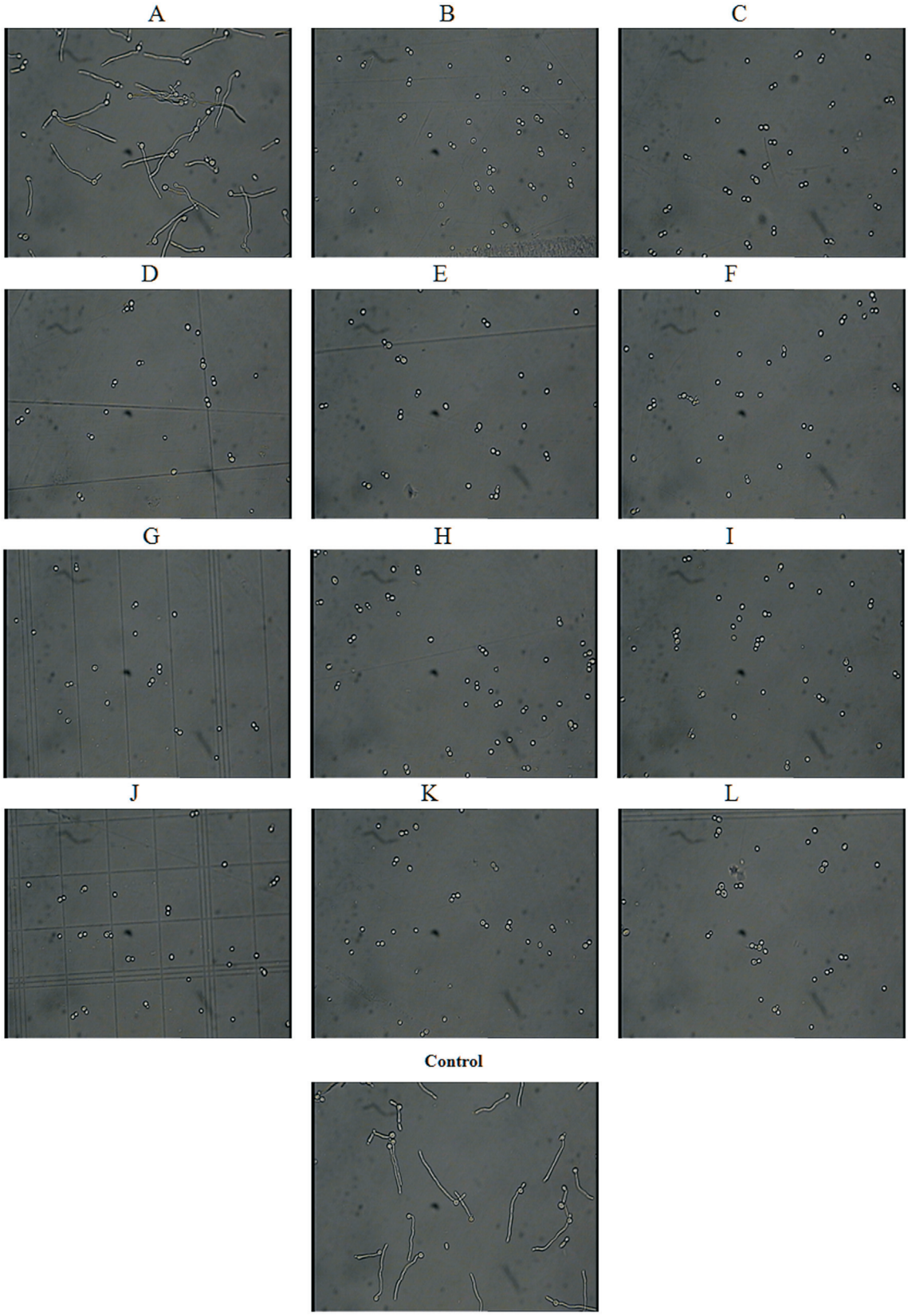

Figure 1 - Morphology of *Candida albicans* cells in presence of various anticancer drugs. A. 50 µg/mL Leucovorine; B. 50 µg/mL Docetaxel; C. 50 µg/mL Mitomycin-C; D. 50 µg/mL Melphalan; E. 50 µg/mL Gemcitabine; F. 50 µg/mL Formestane; G. 25 µg/mL Etoposide; H. 25 µg/mL Leuprolide; I. 50 µg/mL Dacarbazine; J. 25 µg/mL Carboplatin; K. 50 µg/mL Irinotecan; L. 50 µg/mL Tamoxifen. Briefly cells were incubated in serum at 37 °C for 2 h respectively and morphology was assessed after incubation and photographs were taken by Labomed imaging device. (Magnification 100x).

2011). An anti-metabolite agent, Methorexate is reported to produce higher amount of catalase, a marker for oxitatative stress which may cause inhibition of filamentation (Linaris *et al.*, 2006).

Candida infections are very common in cancer patients. As such it is a common practice to prescribe antifungal antibiotics along with anticancer drugs (Pauw, 2004). Recently we have reported the anti *C. albicans* properties of a variety of popularly used anticancer drugs (Routh *et al.*, 2011). Our study suggests rethinking on the logic of prescribing antifungal antibiotics against *C. albicans* infections in cancer patients undergoing chemotherapy. *C. albicans* is usually found in various morphological types *in vivo*. Among these, yeast to hyphal form switching is considered to be important in invasive candidiasis. Targeting yeast to hyphal form morphogenetic switching may be useful against invasive candidiasis. However such antibiotics are not in practice currently. Most of the drugs inhibited yeast to hyphal form morphogenesis at concentrations lower than their MIC and MFC. The different class of anticancer drugs may exert similar effects depending on its concentrations. At lower concentrations it may inhibit morphogenetic switching and support yeast phase growth. While high concentrations of drugs may slow down the growth rate suggesting good antifungal properties. This is the first report on the antimorphogenetic properties of thirty anticancer drugs. Our study indicates the possibility of repositioning cancer drugs as anti-morphogenetic agents in *C. albicans*.

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