Microtubules and Actin Cytoskeleton of *Cryptococcus neoformans* as Targets for Anticancer Agents to Potentiate a Novel Approach for New Antifungals

Marie Kopecká

Department of Biology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

Key Words

Cryptococcus neoformans · Cytoskeleton · Microtubule inhibitors · Actin inhibitors · Microscopy

Abstract

Background: We investigated the targeting of microtubules (MT) and F-actin cytoskeleton (AC) of the human pathogenic yeast *Cryptococcus neoformans* with agents for cancer therapy, in order to examine whether this yeast cytoskeleton could become a new antifungal target for the inhibition of cell division. **Methods:** Cells treated with 10 cytoskeleton inhibitors in yeast extract peptone dextrose medium were investigated by phase-contrast and fluorescence microscopy, and growth inhibition was estimated by cell counts using a Bürker chamber and measuring absorbance for 6 days. **Results:** Docetaxel, paclitaxel, vinblastine sulfate salt, cytochalasin D and chlorpropham [isopropyl N-(3-chlorophenyl) carbamate] did not inhibit proliferation. The MT inhibitors methyl benzimidazole-2-ylcarbamate (BCM), nocodazole, thiabendazole (TBZ) and vincristine (VINC) disrupted MT and inhibited mitoses, but anucleated buds emerged on cells that increased in size, vacuolated and seemed to die after 2 days. The response of the cells to the presence of the actin inhibitor latrunculin A (LA) includ-

ed the disappearance of actin patches, actin cables and actin rings; this arrested budding and cell division. However, in 3–4 days, resistant budding cells appeared in all 5 inhibitors. Disruption of the MT and AC and inhibition of cell division and budding persisted only when the MT and AC inhibitors were combined, i.e. VINC + LA, BCM + LA or TBZ + LA. **Conclusion:** The MT and AC of *C. neoformans* are new antifungal targets for the persistent inhibition of cell division by combined F-actin and MT inhibitors.

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Introduction

Cryptococcus neoformans is a dangerous human pathogenic yeast that causes several serious diseases (e.g. cryptococcal pneumonia, cryptococcal meningitis and cutaneous cryptococcosis), not only in immune-deficient patients, but also in immune-competent people. Therapy is difficult, with frequent development of resistance and considerable mortality [1, 2].

This paper is dedicated to the memory of Miroslav Gabriel, MD, PhD and Associate Professor, with whom we initiated this research in 1999. He died on June 7, 2008.

Fig. 1. a–p Phase-contrast micrographs of *C. neoformans*. **a** Control cells cultivated with 1% DMSO at the beginning of the experiment. Cells were treated for 2 days with 100 μ M of BCM (**b**), NOC (**c**) and VINC (**d**). **e** Control cells were cultivated with 2% DMSO for 2 days. Cells were treated for 2 days with 200 μ M of DOC (**f**), PAC (**g**), CD (**h**), LA (**i**) and 100 μ M of LA (**j**). Cells were treated with 100 μ M of LAT (**k**) and BCM (**l**) for 4 days. Cells were treated with combined 100 μ M LA + 100 μ M VINC for 4 (**m**) and 6 (**n**) days. Cells were treated with combined 100 μ M LA + 100 μ M BCM for 4 (**o**) and 6 (**p**) days. Scale bar: 10 μ m.

Our basic cell biological research of *C. neoformans* identified a cytoskeleton of microtubules (MT) and actin filaments (F-actin) involved in budding, mitosis and cytokinesis [3]. Here, several agents used in cancer therapy that inhibit MT or F-actin [4–8] were studied. Their effects on the F-actin cytoskeleton (AC) and the cell division of *C. neoformans* were investigated by fluorescence and phase-contrast microscopy in the search for new antifungal agents for the inhibition of cell division. Previously, *C. neoformans* was studied by electron microscopy [3], and the effects of some of these inhibitors were monitored [9, 10]. Electron microscopy has also been used for examining inhibition in other yeasts [11–15].

Materials and Methods

The yeast strain *C. neoformans* var. *neoformans*, IFM 41464 (CUH 34, 48-9943, 881, skin, serotype A), from the Medical Mycology Research Centre, Chiba University, Japan [16] was used. Media and cell cultivation were identical as described [9, 10].

Inhibitor Treatments

Methyl benzimidazole-2-ylcarbamate (BCM), vincristine (VINC) and latrunculin A (LA) were used as described [9, 10]. Further inhibitors were nocodazole (NOC), thiabendazole (TBZ), docetaxel (DOC) and vinblastine sulfate salt (VINB) from Fluka, paclitaxel (PAC; BioChemica), and chlorpropham [isopropyl N-(3-chlorophenyl) carbamate] (CIPC) and cytochalasin D (CD) from Sigma [11].

TBZ: 10 mM stock solution was prepared by dissolving 2 mg of the drug in 1.0 ml of DMSO, and kept at -20°C .

Table 1. Number of cells treated with 100 μM of inhibitors and optical density of cells treated with 200 μM of inhibitors

	T_0	At 6 days
<i>Number of cells^a</i>		
Control cells (1% DMSO)	5×10^5	1.5×10^8
Control cells (2% DMSO)	5×10^5	1.5×10^8
Cells treated with		
BCM	5×10^5	7.5×10^6
NOC	5×10^5	7.5×10^6
TBZ	5×10^5	7.5×10^6
VINC	5×10^5	4.5×10^7
LA	5×10^5	1.0×10^6
VINC + LA	5×10^5	7.5×10^5
BCM + LA	5×10^5	7.5×10^5
<i>Optical density</i>		
Control cells (1% DMSO)	0.015	1.5
Control cells (2% DMSO)	0.015	1.5
Cells treated with		
PAC	0.015	1.7
DOC	0.015	1.7
CIPC	0.015	1.35
CD	0.015	1.5

^a Values represent number of cells per milliliter.

NOC: 10 mM stock solution was prepared by dissolving 10 mg of the drug in 3.0 ml of DMSO, and kept at -20°C .

CIPC, PAC, DOC and VINB: 10 mM stock solution was prepared by dissolving 5 mg of the drug in 500 μl of DMSO, and kept at -20°C .

CD: 10 mM stock solution was prepared by dissolving 5 mg of the drug in 1 ml of DMSO, and kept at -20°C .

Phase-contrast and fluorescence microscopy were performed as reported earlier [3]. Image Processing Software Adobe Photoshop CS5 and Adobe InDesign CS5 for Windows were used.

Results

Phase-Contrast Microscopy

Phase-contrast microscopy showed control budding cells (fig. 1a). The MT inhibitors BCM (fig. 1b), NOC (fig. 1c), VINC (fig. 1d) and TBZ in 100 μM and 200 μM concentrations did not inhibit bud growth but when bud and mother cells reached equal size, they failed to separate. Cells with several buds increased in size and vacuolated and seemed to die after 2 days. Control unbudding cells that reached the stationary phase of growth (fig. 1e) looked similar to the cells treated with DOC (fig. 1f), PAC (fig. 1g), VINB and CIPC which did not inhibit proliferation, similar to actin inhibitor CD (fig. 1h; table 1). Actin

inhibitor LA [17] inhibited proliferation and cells lysed at areas of cell-wall growth (fig. 1i, j; table 1), as we have recently observed on electron microscopy [9, 10].

However, resistant budding cells appeared later in LA (fig. 1k), BCM (fig. 1l), NOC, TBZ and VINC. We attempted to prevent their occurrence by combining 100 μM LA + 100 μM VINC, that was then added to the cells. This inhibited cell division (fig. 1m, n), similar to using 100 μM LA + 100 μM BCM (fig. 1o, p) and 100 μM LA + 100 μM TBZ.

Fluorescence Microscopy

Control cells (fig. 2a) stained with DAPI revealed nuclei (fig. 2b), those stained by rhodamine phalloidin revealed F-actin structures (fig. 2c) and those stained by anti-tubulin antibody revealed MT (fig. 2d) [3]. Cells inhibited by BCM (fig. 2e–h) and VINC (fig. 2i–l) revealed nuclei (fig. 2f, j) and evenly distributed actin patches (fig. 2g, k), but MT disappeared (fig. 2h, l), similar to when treated with NOC and TBZ. LA-inhibited cells (fig. 2m–p) revealed nuclei (fig. 2n) and a disappearance of F-actin structures (fig. 2o), but MT persisted (fig. 2p). Only when 100 μM VINC + 100 μM LA were combined and added to the cells (fig. 2q–t), was the nucleus revealed (fig. 2r), but the F-actin structures (fig. 2s) and MT (fig. 2t) disappeared, similar to after treatment with 100 μM BCM + 100 μM LA or 100 μM TBZ + 100 μM LA.

Cell Proliferation

The number of control cells at T_0 , i.e. $5 \times 10^5 \text{ ml}^{-1}$, increased to $1.5 \times 10^8 \text{ ml}^{-1}$ over 6 days; their optical density of 0.015 at T_0 increased to 1.5 over 6 days, similar to cells treated with PAC, DOC, VINB, CD and CIPC that did not inhibit proliferation (table 1). The effect of VINC, BCM, NOC and TBZ increased the number of cells, i.e. $5 \times 10^5 \text{ ml}^{-1}$ at T_0 to $7.5 \times 10^6 \text{ cells ml}^{-1}$ over 6 days of inhibition, and in VINC, this number increased to $4.5 \times 10^7 \text{ ml}^{-1}$. In LA, the number of cells at T_0 , i.e. $5 \times 10^5 \text{ ml}^{-1}$, increased to $1.0 \times 10^6 \text{ cells ml}^{-1}$ after 6 days. In the combined MT + actin inhibitors, the number of cells at T_0 , i.e. $5 \times 10^5 \text{ cells ml}^{-1}$, increased in VINC + LA similar to that in LA alone, and was only $7.5 \times 10^5 \text{ ml}^{-1}$ after 6 days similar to that in BCM + LA and TBZ + LA, indicating the most efficient inhibition of proliferation.

Discussion

We investigated the targeting of the cytoskeleton filaments of *C. neoformans* with agents for cancer therapy, in order to potentiate a novel therapeutic antifungal ap-

Fig. 2. **a–t** Fluorescent microscopy of *C. neoformans*. **a–d** Control cells without inhibitors. **e–t** Cells treated with inhibitors, fixed, stained with rhodamine phalloidin for actin, antitubulin antibody for MT and DAPI for nuclear staining. **a** Phase contrast (PC). **b** DAPI. **c** AC. **d** MT. **e–h** Cells treated with 100 μM of BCM for 8 h. **e** PC. **f** DAPI. **g** AC. **h** MT. **i–l** Cells treated with 100 μM of VIN for 20 h. **i** PC. **j** DAPI. **k** AC. **l** MT. **m–p** Cells treated with 100 μM of LA for 4 h. **m** PC. **n** DAPI. **o** AC. **p** MT. **q–t** Cells treated with a combination of 100 μM LA + 100 μM VIN for 4 h. **q** PC. **r** DAPI. **s** AC. **t** MT. Scale bar: 10 μm .

proach. The single MT inhibitors VIN, BCM, NOC and TBZ disrupted MT and blocked cells at the metaphase checkpoint [4, 18], monitoring joining of chromosomes to the mitotic spindle that cannot exit mitosis and realize cytokinesis. F-actin persisted, but resistant budding cells appeared later. The F-actin inhibitor LA disrupted F-actin and inhibited cytokinesis, but mitosis did not proceed either. Nakaseko and Yanagida [19] described the actin

checkpoint monitoring F-actin during the M-phase that stops mitosis when F-actin is damaged; this is why mitosis did not proceed in LA-treated cells. Even though our results seemed promising during the first 3 days, resistant cells appeared here as well. We do not know whether cells metabolized the inhibitors, whether overproduction of efflux pumps removed the inhibitors, whether overexpression of alternative cytoskeleton proteins made cells resis-

tant to the inhibitors or whether the 100 μM inhibitor concentration containing 6.022×10^{16} molecules did not inhibit all 5×10^5 cells ml^{-1} (i.e. $6.022 \times 10^{16} : 500,000 = 1.20 \times 10^{11}$ inhibitor molecules per cell). Only when a 100 μM concentration of an MT inhibitor was combined with a 100 μM concentration of an actin inhibitor, did 2.40×10^{11} inhibitor molecules inhibit all 5×10^5 cells ml^{-1} and no resistant cells appeared. The inhibition was also effective with CD and BCM in the black fungus, *Aureobasidium pullulans* [13], but the combination of CD + BCM was found to be the most efficient (M.K. and Miroslav Gabriel, unpubl. data). LA + BCM combined also gave rise to the most efficient inhibition of the proliferation of the yeast, *Fellomyces fuzhouensis* [14, 15]. It remains open to question whether combined MT + AC inhibitors can also inhibit other yeast pathogens, e.g. *Candida albicans*. VINC [20] and LA [21] alone were effective [20, 21], so, in combination, they may also inhibit *C. albicans* as well as other fungi that contain MT and actin filaments. Another open question is whether such combined MT + actin inhibitors could prevent the occurrence of resistant cells in human cancers which contain the intermediate filaments absent in fungi.

Conclusion

DOC, PAC, VINB, CD and CIPC did not inhibit the proliferation of *C. neoformans* cells. BCM, NOC, TBZ and VINC disrupted MT, LA disrupted the AC and proliferation was temporarily inhibited, but resistant cells appeared in all 5 samples. Only when we combined VINC + LA, BCM + LA and TBZ + LA, were both the MT and AC disrupted, and persistent inhibition of budding and nuclear and cell divisions persisted. These combined inhibitors should first be tested for local application on cutaneous cryptococcosis.

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