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The Effects of the F-Actin Inhibitor Latrunculin A on the Pathogenic Yeast *Cryptococcus neoformans*

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Key Words
Yeast · Latrunculin A · Freeze-substitution · Electron microscopy · Cell ultrastructure

Abstract
Background: This basic research aimed to investigate the effects of the actin inhibitor latrunculin A (LA) on the human pathogen *Cryptococcus neoformans*, by freeze-substitution (FS) and electron microscopy (EM), to determine whether the actin cytoskeleton can become a new antifungal target for inhibition of cell division. ***Methods:*** Cells treated with LA for 20 h in yeast-extract peptone dextrose medium were investigated by phase-contrast and fluorescent microscopy, FS and transmission EM, counted in a Bürker chamber and the absorbance was then measured. ***Results:*** The disappearance of actin patches, actin cables and actin rings demonstrated the response of the cells of *C. neoformans* to the presence of the actin inhibitor LA. The removal of actin cables and patches arrested proliferation and led to the production of cells that had ultrastructural disorder, irregular morphology of the mitochondria and thick aberrant cell walls. Budding cells lysed in the buds and septa. ***Conclusion:*** LA exerts fungistatic, fungicidal and fungilytic effects on the human pathogenic yeast *C. neoformans*.
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Introduction

Cryptococcus neoformans is a human yeast pathogen [1], rich in the microtubules required for nuclear division and with an actin cytoskeleton required for cell division [2], along with a typical fungal ultrastructure [2, 3]. The yeast is distributed worldwide causing cryptococcosis, meningitis, infections of the inner organs, bones and skin, atypical pneumonia and fungemia, and it occurs in both immune-deficient patients and immune-competent people. Therapy is difficult, with the frequent development of resistance and considerable

M.K. and M.Y. participated in this work equally and should both be considered as first authors.

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mortality [4–6]. Recently, we studied the effects of microtubules and actin inhibitors on *C. neoformans* to examine whether its cytoskeleton can become a new antifungal target for the inhibition of cell division. The actin inhibitor LA was the most efficient: the actin structures [7] and cytoplasm disappeared and the inhibited cells died, with only their cell walls persisting [8]. Here, we investigated the early effects of LA on *C. neoformans* cells using freeze-substitution (FS) and transmission electron microscopy (EM) to identify how and why inhibited cells die.

Table 1. Number of cells treated with 100 μM of LA and the OD of the cells

	T_0	At 20 h
Number of cells		
Control cells	1×10^6 ml^{-1}	2.25×10^8 ml^{-1}
Cells treated with LA	1×10^6 ml^{-1}	1.75×10^6 ml^{-1}
OD		
Control cells	0.03	1.2
Cells treated with LA	0.03	0.125

Materials and Methods

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 [9] was used.

Media and Cell Cultivation

The strain was maintained in 2.0% (w/v) agar containing YEPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose] at 23°C. To obtain an exponential culture, cells cultivated in YEPD medium [1% (w/v) yeast extract, 1% (w/v) peptone and 1% (w/v) dextrose] on a shaker overnight at 23°C (about 16 h) were diluted to 5×10^5 to 1×10^6 cells ml^{-1} by 1% YEPD and used for the application of LA [7, 8].

LA Treatment. 10 mM stock solution was prepared by dissolving 100 μg of LA (Molecular Probes, USA) in 25 μl of DMSO, kept at −20°C and then added to cells in 1% YEPD to a final concentration of 100 μM that contained 1% DMSO [7, 8, 10, 11]; 500-μl volumes of cultures in test tubes were shaken in the dark in a water bath. Samples for EM were taken before the LA treatment, i.e. at T_0, and after 20 h of the LA effect, were fixed with 3% glutaraldehyde in PBS of pH 7.4 [3] and sent from Brno to Chiba for FS and EM. For fluorescent microscopy, the cells were fixed with 5% paraformaldehyde [2, 7].

FS and transmission EM were conducted as previously described [3, 12]. The image processing software Adobe Photoshop CS5 and Adobe InDesign CS5 for Windows were used for electronic arrangement of the figures.

Results

Proliferation of C. neoformans Cells Treated with LA

See table 1. The number of control cells at T_0, i.e. 1.0×10^6 ml^{-1} increased to 2.25×10^8 ml^{-1} after 20 h. The optical density (OD) of 0.03 at T_0 increased to 1.20 after 20 h of inhibition.

The effect of the LA treatment was an increase in the number of cells from 1.0×10^6 ml^{-1} at T_0 to only 1.75×10^6 ml^{-1} after 20 h of inhibition; the OD at T_0 was 0.03, and after 20 h of inhibition it was only 0.125. This indicated that 100 μM LA inhibited the growth and division of *C. neoformans* cells.

Phase-Contrast and Fluorescent Microscopy of C. neoformans Cells Treated with LA

On phase-contrast microscopy, the control cells at T_0 were spherical budding cells (fig. 1a), which, after 20 h of cultivation, reached a stationary phase of growth and had no buds (fig. 1b).

After 20 h of LA effect, cells were blocked in the budding stage (fig. 1c) or at cytokinesis (fig. 1d) or lysed at the bud apex or septum region (fig. 1e–g). Cytoplasm was released from lysed cells to a culture medium. This indicated that the F-actin cytoskeleton is required for budding, bud growth and formation of the septum.

On fluorescent microscopy, the control cells had actin patches (AP) which accumulated in the buds and at the septum region, but were also present on the mother cells; actin cables (AC) proceeded from mother to bud and actin rings (AR) occurred at the cytokinesis region (fig. 1h) [2].

Cells inhibited by LA for 20 h had no actin cytoskeleton structures (fig. 1i); this is similar to other yeasts inhibited by LA [10, 11, 14].

FS and Transmission EM of Cells Treated with LA

Control cells were spherical in shape and reproduced by budding. The cytoplasm contained the nucleus, endoplasmic reticulum, mitochondria, electron-dense vacuoles, groups of electron-transparent 'cortical vesicles' and ribosomes [2, 3]. The cell walls had an inner electron-dense layer, a middle electron-transparent layer and an outer electron-denser capsule (fig. 2a, b).

Cells inhibited by LA for 20 h had a greatly affected ultrastructure (fig. 3a–f). They contained many small mitochondria. Their pairs had middle constriction, suggest-

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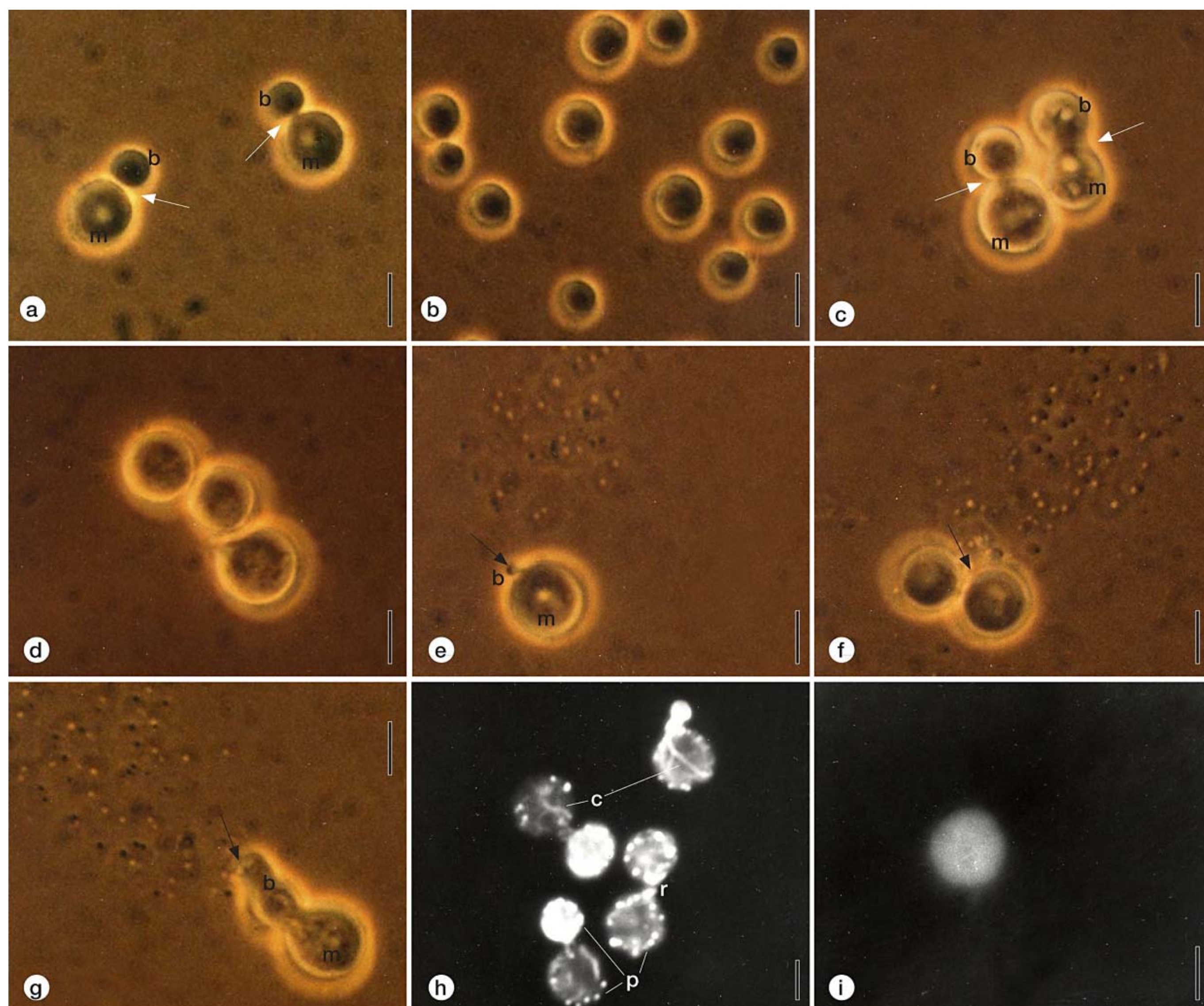

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Fig. 1. a–g Phase-contrast micrographs of *C. neoformans.* **a** Control cells were cultivated with 1% DMSO at the beginning of the experiment. **b** Control cells were cultivated with 1% DMSO for 20 h. **c** Cells were treated with 100 μM LA for 20 h (the white arrow shows the bud neck). **e**, **g** Black arrow shows the lysed bud. **f** Black arrow shows the lysed cytokinesis region. b = Bud; m = mother. **h**, **i** Fluorescent micrographs of *C. neoformans.* **h** Control cells were cultivated with 1% DMSO without inhibitor for 20 h. **i** Cells were treated with 100 μM LA for 20 h. Cells were fixed and stained for actin with rhodamine phalloidin. c = Actin cables; p = actin patches; r = actin rings.

ing fragmentation of the mitochondria (fig. 3a1). Some mitochondria had affected cristae and variable size and looked like disappearing ruins (fig. 3b, c). Vacuoles were electron-transparent. There were ‘cortical vesicles’ in only a few cells (fig. 3d, f). Almost all cells had aberrant cell walls penetrating to the cytoplasm as wall thickenings or wall ridges. Plasma membrane was invisible in many dying or dead cells and the cytoplasm was diminished. Some small buds contained cell wall material instead of cytoplasm (fig. 3f) as actin mutant cells with disrupted AC [13]. Among many dying or dead cells, only one cell (fig. 3d) was similar to the control cells. It had a standard cytoplasm and cell wall, small mitochondria only and the ‘cortical vesicles’ were aggregated, which could block exocytosis and abnormal wall formation; this cell was probably partially resistant to LA.

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Discussion

LA in C. neoformans Inhibits Cell Growth and Division and Triggers Uncontrolled Formation of an Aberrant Cell Wall and Cell Lysis

Untreated control cells containing AC and AP formed a standard cell wall because AC direct the secretory pathway and cell growth to the bud, and subcortical actin can stabilize the plasma membrane in the growing areas. In addition, AP may be required for the synthesis and assembly of the rigid microfibrils of the cell wall in the growing buds, as in *Saccharomyces cerevisiae* [14]. Disruption of AP and AC by LA blocked the directing of wall growth to the bud and septum. After the disruption of AP in the buds, wall-synthesizing enzymes that had accumulated in the buds probably escaped, diffusing randomly along the plasma membrane to the mother cell and synthesizing the aberrant cell wall. *S. cerevisiae* actin mutant cells *(act1–1)*, having disrupted the AC [15], formed a similar aberrant cell wall [13, 16, 17]. This indicates that the F-actin cytoskeleton is required for the controlled (regulated) formation of the yeast cell wall in space and time. A disrupted actin cytoskeleton, however, triggers the uncontrolled formation of an aberrant cell wall and cell lysis in the regions of the growing buds and septa.

The Actin Inhibitor LA Causes a Change from Large Cylindrical Mitochondria to Small Spherical Mitochondria

LA affects the structural integrity and cylindrical shape of the mitochondria. The bacterial actin homolog MreB is required for the development of the cylindrical shape of bacteria; spherical bacteria do not require MreB protein [18]. This could mean that the F-actin cytoskeleton may have thus far unknown cytoskeleton functions in mitochondria; however, here, the affected mitochondria had features of apoptosis, together with an affected nuclear membrane, smaller nuclei, blebbing of the cortical cytoplasm and a plasma membrane that was hardly visible. The yeast requires an 'actin pathway' [16] for polarizing

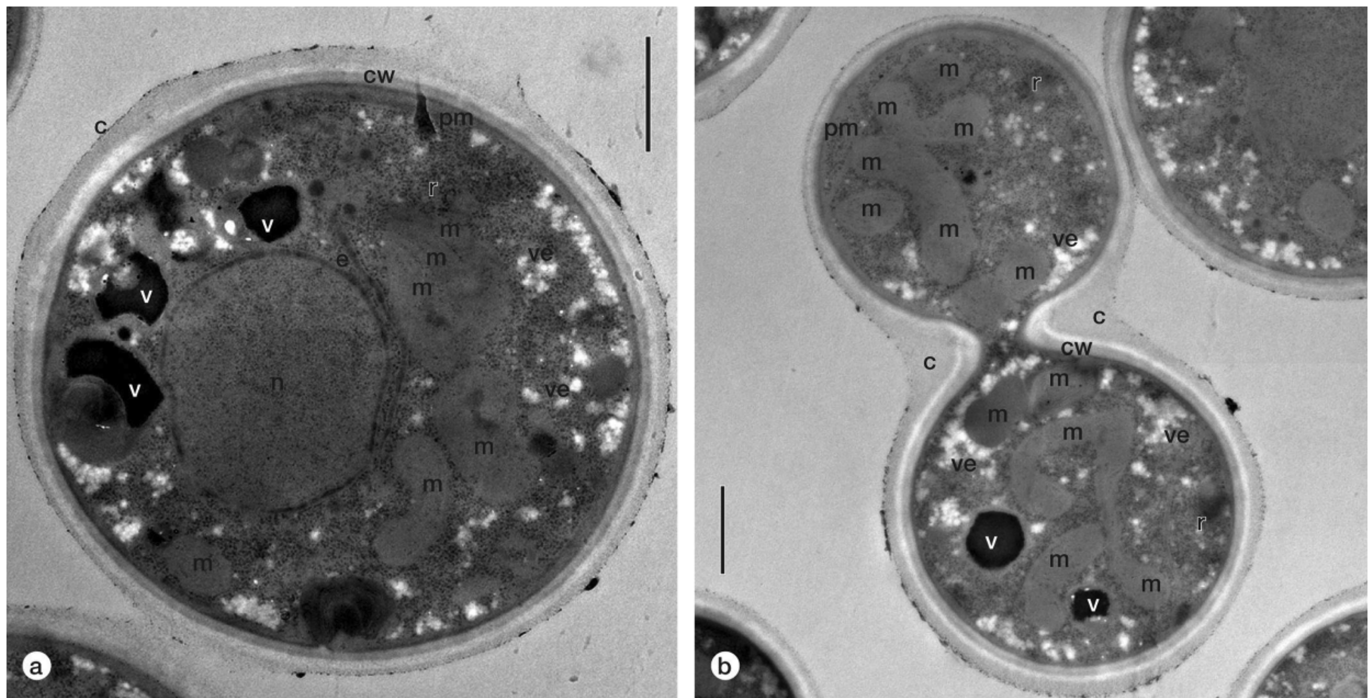

Fig. 2. a, **b** FS and transmission EM of control cells at T_0. c = Capsule; cw = cell wall; e = endoplasmic reticulum; m = mitochondria; n = nucleus; pm = plasma membrane; r = ribosome; v = vacuole; ve = 'wall vesicles' [2].

Fig. 3. a–f FS and transmission EM of cells cultivated with 100 μM LA for 20 h. **a1** Detail of fragmented mitochondria. a = Aberrant wall; c = capsule; cw = cell wall; e = endoplasmic reticulum; m = mitochondria; n = nucleus; pm = plasma membrane; r = ribosome; v = vacuole; ve = 'wall vesicles' [2]. (*For figure see next page.*)

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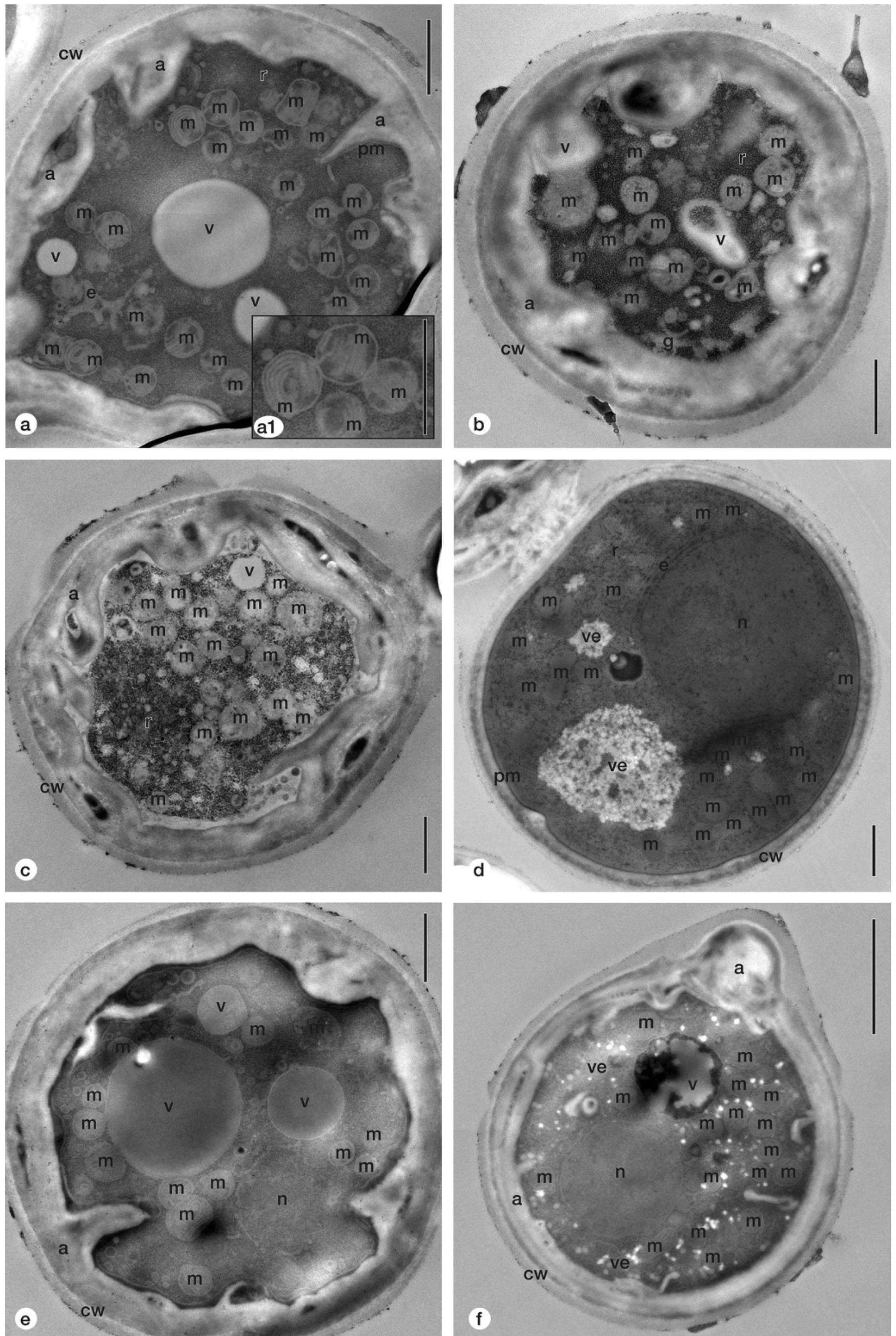

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growth, directing the secretory pathway to the bud [17], the inheritance of cell organelles and cell division of the cytoplasm [19, 20]. When AC and AP are disrupted by LA, the yeast cannot polarize growth to the bud, and wall formation proceeds randomly in the mother cells. Similarly, mouse mesenchymal cells [21], neuroblastoma cells and fibroblasts [22] have disrupted microfilaments and stress fibers when treated with LA, even at lower concentrations; the cells become spherical and are affected in other ways, but the effects are reversible. In addition, inhibited mesenchymal cells form extracellular matrix and differentiate into chondrocytes [21]. Thus, yeast cells react to disruption by LA of the actin cytoskeleton in the formation of the cell wall (as in this study) and mesenchymal cells react by forming extracellular matrix [21]. However, the inhibited yeast *C. neoformans* lyses or dies because without the actin cytoskeleton, it is apparently nonviable.

Conclusion

In *C. neoformans*, LA disrupts the F-actin cytoskeleton, inhibits polar growth and division and triggers the uncontrolled formation of the cell wall in the others, while the buds and septa lyse. LA exerts fungistatic, fungicidal and fungilytic effects on *C. neoformans* cells.

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