Initial studies on the lytic effects of burkholdines upon mycotic agents

Abstract
Exposure of yeast and fungi to burkholdines, metabolites produced by an isolate of Burkholderia ambifaria, lead to 3-log reductions (99.9% killing) in colony counts of Saccharomyces cerevisiae in 30min. Burkholderia ambifaria also resulted in rapid (30min) lysis and release of the intracellular enzyme maltase of cells of either S. cerevisiae or Aspergillus niger. Viability of S. cerevisiae, as measured by the LIVE/DEAD® Yeast Viability test, was abolished within 30min of burkholdine exposure. Loss of colony-forming units, loss of viability, and release of maltase were highly correlated over time, suggesting that the basis for the anti-fungal activity of burkholdines is the loss of membrane integrity.

Keywords: burkholdines, Burkholderia ambifaria, yeast, fungi, killing, lysis

Introduction
It has become clear that there is a need for novel anti-fungal agents whether for medicine or agriculture. For example, new anti-fungal agents are needed for the treatment of plant pathogenic fungi due to the emergence ofazole-resistant isolates of plant pathogenic fungi.1 Burkholdines, produced by an isolate of Burkholderia ambifaria are novel anti-yeast and antifungal compounds.2 The burkholdines are lipopeptide antibiotics and strain 2.2 N produces as many as 8 related Burkholdines of molecular weight 1097–1229.3 4 Field trials showed activity against a wide variety of fungi that infect crops; for example Septoria tritici and Septoria nodorum in wheat, Phytophthora infestans in tomato, and Pyricularia oryzae in rice and tomato.5,6 In addition, burkholdines have strong anti-fungal activity against Mycosphaerella fijiensis,7 a pathogen of bananas that is responsible for considerable economic loss.8

In addition to displaying anti-fungal activity against plant pathogenic fungi, the burkholdines have strong activity against the medically important yeast Candida albicans and Cryptococcus neoformans and the filamentous fungus, Aspergillus niger.9 Infections caused by yeast and fungi have a significant impact on human health in the United States. For example, the emergence of azole-resistant Candida albicans leads to significant increases in morbidity and mortality of those infection.8 Further, a population-based survey in the United States demonstrated that the incidence of hospital-acquired Candida spp. infections was 8 cases per 100,0009 and based on an estimate of the cost of treating each Candida spp. infected patient, the total cost of nosocomial Candida spp. infections in the United States would be $800million per year.10 In addition to azole-resistance in Candida spp., azole-resistance has emerged in Aspergillus fumigatus.11 Further, azole-resistance in A. fumigatus has been linked with agricultural use of azole fungicides.1

Microscopic observations of suspensions of S. cerevisiae exposed to burkholdines demonstrated significant short term (<30min) lysis. That observations prompted an investigation to determine the mechanism of the basis for the anti-fungal activity of the burkholdines.

Materials and methods

Burkholdines
The source of burkholdines was a 1% (wt/vol) suspension of spray dried cells of B. ambifaria strain 2.2N in sterile tap water. One gram of the spray dried cells had an equivalent activity of 0.006gm of purified burkholdines3,4 and had an MIC of 0.5µg/mL against S. cerevisiae and C. albicans and 1.0µg/mL against A. niger.

Microbial strains and growth
Saccharomyces cerevisiae, Candida albicans, and Aspergillus niger cultures2 were grown to mid log phase in 10mL of Yeast Extract-Peptone-Maltose Broth (YEMP) for 6 hr at 32°C. Cultures were streaked on Yeast Extract Peptone Dextrose (YPD) agar and incubated at 32°C for 24-48hr. to confirm purity and colony morphology. Yeast or fungal mycelium was collected by centrifugation (5,000xg for 20min) and after discarding the supernatant medium, cells or mycelium were washed in 10mL of sterile tap water and finally suspended in an equal volume of sterile tap water.

Measurement of killing, lysis, and membrane damage
Killing of S. cerevisiae and C. albicans cells was measured as the reduction in colony-forming units (CFU) over time. In a 125mL flask, 20mL of a cell suspension was mixed with 2.0mL of the burkholdine suspension and immediately and at 1, 2, and 3hr CFU were measured by spreading 0.1mL (in triplicate) of the undiluted suspension and dilutions in sterile tap water on YEPD agar and incubating at 32°C. Results were expressed as CFU/mL and survival calculated as a percentage of the initial count. Lysis of S. cerevisiae and A. niger was measured as release of the intracellular enzyme maltase.13 Using the same suspension as used for measuring CFU reduction, 0.5mL of the reaction was transferred to the filter housing of a Spin-X tube containing para-nitrophenyl maltose and centrifuged at 16,000 x g for 2min. The filter housing with cells was removed and the filtrate in the Spin-X tube incubated at 37°C for 30min, the reaction stopped and the red color of para-nitrophenol intensified by adding 0.25mL of...
1M Na2CO3, and the absorbance at 450nm measured. The positive control for maltase activity was a 0.45μm pore size-filtered sonicated suspension of S. cerevisiae or A. niger. Results are reported as the percent of maltase activity of the appropriate sonicated controls. Viability of S. cerevisiae cells was assessed using the LIVE/DEAD Yeast Viability Kit (Molecular Probes, Eugene, OR) as described by the manufacturer using the same suspension as used for measuring CFU reduction and lysis.

Results

Killing, lysis, and viability of S. cerevisiae exposed to burkholdines

In Table 1 are displayed the data on survival/killing, lysis, and viability of S. cerevisiae cells upon exposure to the burkholdines.

| Exposure | Survivala | Percent killingb | Percent lycia | Percent deadc |
|----------|------------|-------------------|---------------|---------------|
| 0        | 100        | 0                 | 0             | 43            |
| 30min    | 0.21       | 99.79             | 0             | 62            |
| 60min    | 0.088      | 99.91             | 3.95±0.21     | 68            |
| 120min   | 0.065      | 99.935            | 11.6±0.14     | 98            |
| 180min   | 0.058      | 99.942            | 19.85±1.48    | 96            |

*a*Expressed as surviving fraction of CFU of untreated control  
*b*Expressed as percent of CFU reduction  
*c*Expressed as percent of released maltase activity of sonicated control  
*d*Expressed as percent of inviable (red) cells of untreated control

Table 1 Burkholdine-mediated killing, lysis and loss of viability of S. cerevisiae

Using the LIVE/DEAD® Yeast Viability Kit (Molecular Probes, Eugene, OR) loss of viability as expressed as percent dead (red) S. cerevisiae cells was quite rapid (Table 1). The high percentage of dead cells in the 0 exposure control was likely due to the fact that the assay required 30min incubation after addition of the dyes before epifluorescence microscopy and examination of cells. As noted for both the reduction in colony-forming units and the increase in maltase activity released from cells, as the duration of burkholdines increased, the percentage of dead cells increased (Table 1). In addition, the extent of killing as loss of CFU and viability as percent dead cells for S. cerevisiae were correlated (r2=0.6394).

Burkholdine-induced lysis and loss of viability of Aspergillus niger cells

To confirm that the lytic activity of the burkholdines were not restricted to yeast, lysis and viability of A. niger hyphae were measured. Although it was not possible to reproducibly measure the killing of A. niger cells as colony-forming units, it was possible to measure the effect of burkholdine exposure on the release of intracellular maltase and the loss of viability of A. niger cells. Washed hyphae of A. niger were exposed to burkholdines and hyphae-free filtrates assayed for maltase activity. Released maltase activity, expressed as a percentage of a sonicated hyphae control was 25%. Measurement of the viability of burkholdine-exposed hyphae showed that the percentage of dead (red) A. niger hyphae was 100% after 30min exposure.

Discussion

The source of burkholdines was a sonicated 1% (wt/vol) suspension of spray dried cells of B. ambifaria strain 2.2N. The cells were spray dried at 170°C inlet temperature and contained no viable cells (i.e., <10 CFU/gm) and lacked all but anti-fungal and anti-yeast activity. Although a single purified burkholdine was not employed, to date all members of the burkholdine family produced by strain 2.2N have equal specific activity. In the sonicated suspension used here, anti-yeast and anti-fungal activity would have been due to all related burkholdines; no synergistic activity has been demonstrated between individual burkholdines.

Killing of yeast cells as measured by decrease of colony-forming units coincided with the release of intracellular maltase and loss of viability (Table 1). The short time needed to exhibit killing, lysis, and loss of viability suggests that they reflect the primary result of burkholdine activity, not a secondary consequence of some other effect (e.g., ATP reduction). Based on the observation that burkholdine exposure results in rapid lysis of both S. cerevisiae and A. niger cells suggests that the primary target is a common element of the cell membranes of both.

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None.

Conflict of interest

The author declares no conflict of interest.

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