CHAPTER V

Role of NAD-GDH in yeast - hypha transition in *B. poitrasii* and in development of antifungal agents
5.1 Introduction

Most of the human or plant pathogenic fungi are capable of dimorphic switch between yeast to filamentous hypha. This saprophytic to pathogenic change is the most important event in causing infection to the host cell. Most of the antifungal drugs available are known to act on the cell wall synthesis which takes longer time due to the ability of fungus to tackle it by the vegetative growth. It has also known to escape the host immune system by changing the morphological forms. In human pathogenic fungi like \textit{Candida albicans, Histoplasma capsulatum, Paracoccidioides brasiliensis}, etc. dimorphism plays crucial role during the pathogenesis. The yeast form prevails in the pathogenic phase of fungi like \textit{Paracoccidioides brasiliensis, Histoplasma capsulatum, Sporothrix schenckii} and \textit{Blastomyces dermatitidis}, whereas, \textit{Candida albicans} and \textit{Wangiella dermatitidis} appear in both the forms within the host tissue (Deshpande, 1996). Similarly, plant pathogenic fungi such as \textit{Ceratocystis ulmi, Ustilago maydis, Taphrina weisneri}, etc. also exhibit morphological shift during the pathogenesis (Nadal et al, 2008). This morphological change is triggered by host body temperature, blood sugar level, serum and the nature of host tissue. The resulting morphological forms are usually budding yeasts, hyphae, spores or sclerotic bodies.

The polyene antibiotics, azole derivatives and the allylamines are the major three groups of antifungals used against fungal pathogens (Ghannoum and Rice, 1999; Gooday, 1995). There are reports of resistance developed to azole drugs and about toxic effects of polyene antibiotic Amphotericin B, (Hunter, 1995).

The effective way to control dimorphic fungal pathogen is to prevent morphological transition, i.e. saprophytic to an invasive form. To identify the potential target the study of vital cellular functions in the pathogens is important. Since fungi are eukaryotes like mammalian cells and therefore, the agents that inhibit fungal cellular processes are likely to affect the same in the patient, producing toxic side effects. In this regard, morphological change can be used as a selective event to visualize their potential as an ideal target for antifungal drugs.

Various biochemical correlates are known for the morphological transition and for pathogenesis: enzymes of cell wall synthesis and degradation, enzymes of
carbon and nitrogen metabolism, intracellular effectors like polyamines, cAMP, Ca-
calmodulin, etc. (Deshpande, 1998 a). However the correlation of biochemical
changes with morphogenesis is still not clear, probably due to the strain dependent
variations in stimuli inducing such changes. The major structural component of the
fungal cell wall, a determinant of the shape is chitin. Both carbon and nitrogen
metabolism pathways are involved in the chitin synthesis. The ammonia assimilating
enzymes NAD- and NADP-GDH act at the interface of carbon and nitrogen pathway
of the chitin synthesis. Both NAD-GDH and NADP-GDH are present in the yeast and
hyphal (Khale et al., 1992) of B. poitrasii. Moreover, there is form specific
expression of two NADP-GDHs in B. poitrasii and NAD-GDH is common for both
the forms (Amin et al., 2001). As these enzymes showed correlation with Y-H
transition in B. poitrasii, they could be used as potential targets. In the present section
purified NAD-GDH was used to screen the inhibitors and their effect were observed
on Y-H transition in B. poitrasii.

In the present study chemically synthesized compounds such as triazole-
linked β-lactam–bile acid conjugates (B-compounds), bile acid dimers linked with
triazone and bis- β-lactam (D-compounds) and cholic acid derivatives (S-compounds)
were screened. Azoles are the largest class of antifungal agents reported in clinical
use. 1,2,3-triazole moieties are attractive connecting units, as they are stable to
metabolic degradation and capable of hydrogen bonding, which can be favorable in
binding of biomolecular targets and solubility (Dalvie et al., 2002). Bile acids were
reported as an effective component in the preparation of new pharmaceutical drugs
because of their chemical and biological properties (Virtanen and Kolehmainen,
2004). In the present work compounds were tested for the inhibitory activity against
Y-H transition and purified NAD-GDH of B. poitrasii.
5.2 Result

5.2.1 Effect of compounds on germ-tube formation in *B. poitrasii*

Yeast cells were obtained by growing in YPG (1% glucose) and incubated at 37 °C for 24 h. These cells were washed with YP medium and used as inoculum. 8 x 10⁶ cells were inoculated in the 50 ml of YPG (0.1% glucose) medium. The effect of compounds were tested on Y-H transition by observing percentage of germ-tube forming cells as mentioned under ‘Methods and Materials’ (section 2.2.1). Effect of different compounds on the yeast-hypha transition of *B. poitrasii* was studied by measuring inhibition of germ tube formation. Using potential inhibitors (Table 5.1), the transition experiments were carried out in YPG (0.1% glucose) medium at 28°C for 12 h. Among the compounds tested, triazole-linked β-lactam-bile acid conjugates (B compounds) were found to affect germ-tube formation. In remaining compounds bile acid dimers linked with triazole and bis- β-lactam (D17 and D19) were effective in inhibiting Y-H transition.
Table 5.1 Concentration required to prevent 50 % germ-tube formation.

<table>
<thead>
<tr>
<th>S. no</th>
<th>Compound</th>
<th>Concentration* (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B17</td>
<td>30.00</td>
</tr>
<tr>
<td>2</td>
<td>B18</td>
<td>39.11</td>
</tr>
<tr>
<td>3</td>
<td>B20</td>
<td>35.18</td>
</tr>
<tr>
<td>4</td>
<td>B23</td>
<td>36.08</td>
</tr>
<tr>
<td>5</td>
<td>B24</td>
<td>31.00</td>
</tr>
<tr>
<td>6</td>
<td>D15</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>D17</td>
<td>31.50</td>
</tr>
<tr>
<td>8</td>
<td>D19</td>
<td>31.23</td>
</tr>
<tr>
<td>9</td>
<td>D20</td>
<td>ND</td>
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<td>ND</td>
</tr>
<tr>
<td>14</td>
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<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>SB79</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>DS6</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Concentration required for the 50% germ-tube inhibition; ND, inhibition not detected. Compounds were dissolved in 100% DMSO (4mg/ml).
Control was taken in which DMSO was added equal to the volume of compounds.
Triazole-linked β-lactam–bile acid conjugates (B), bile acid dimers linked with triazole and bis-β-lactam (D) and cholic acid derivatives (S)
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The compounds which were found to affect Y-H transition in B. poitrasii, tested for their inhibitory activity against purified NAD-GDH. In every reaction 130 U of NAD-GDH was taken to observe inhibition in activity with different inhibitors. Among these B-compounds were effective in inhibiting NAD-GDH activity. B18 and B20 were the most potent inhibitors of NAD-GDH enzyme. Other B-compounds were less effective in inhibiting the NAD-GDH activity whereas D-compounds were not found to affect the activity of NAD-GDH enzyme.

Fig 5.1 The curve indicates the best fit for the NAD-GDH percentage inhibition data for B18 obtained, and the IC50 value was calculated from the graph. Enzyme activity was estimated by observing the reductive amination of 2-ketoglutarate at 340 nm.
Fig 5.2 NAD-GDH was assayed without (●) or with B18 at 6 μg/ml (♦) and 12 μg/ml (▲) concentrations and assayed at increasing concentrations of the substrate. The reciprocals of the rate of enzyme activity for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations. Ki was determined from the formula as per the competitive type of inhibition.
Fig 5.3 The curve indicates the NAD-GDH percentage inhibition data for B20 obtained, and the IC\textsubscript{50} value was calculated from the graph.
Fig 5.4 NAD-GDH was incubated without (●) or with B20 at 8 μg/ml (●) and 16 μg/ml (▲) concentrations and assayed at increasing concentrations of the substrate. The reciprocals of the rate of enzyme activity for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations. Ki was determined from the formula as per the competitive type of inhibition.
5.2.2 Kinetic analysis of NAD-GDH with inhibitors B18 and B20 for the determination of type of inhibition and inhibition constant (Ki).

The inhibition constant Ki for B18 and B20 were determined by the Lineweaver–Burk equation. For the Lineweaver–Burk analysis of NAD-GDH, it was assayed with 6 and 12 µg/ml of B18 and 8 and 16 µg/ml of B20 and at increasing concentration of 2-ketoglutarate (2-10 mM). The reciprocals of enzyme activity (1/V) for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations, and the Ki was determined by fitting the resulting data. The compound B18 was found to inhibit NAD-GDH with an IC50 value (50 % inhibitory concentration) of 14.03 µg/ml (Fig 5.1) while for B20 IC50 was 8.48 µg/ml (Fig 5.3). The inhibition of NAD-GDH with both B18 and B20 followed the same pattern of the inhibition with increasing concentrations of inhibitors. The values of inhibition constant were determined by the double reciprocal plot by the following equation for the competitive inhibition:

$$Ki = \frac{Km \times [I]}{Km' - Km}$$

Where, Km is the Km for the substrate without inhibitor, [I] is the concentration of the inhibitor and Km’ is the apparent Km for the inhibited enzyme.

The inhibition constant Ki, determined by the double reciprocal plot was 19.8 µg/ml for B18 and 13.86 µg/ml for B20. The Lineweaver–Burk reciprocal plot showed that the inhibitors were competitive inhibitors of NAD-GDH. The assays were carried out in triplicates, and the average value was considered throughout this work.

5.3 Discussion

Arrest in the dimorphic transition in the pathogenic fungi was suggested to be a useful strategy for the development of antifungal drugs (Georgopapadakou and Walsh, 1994). Various inhibitors of biochemical correlates of morphogenesis were studied in detail using different screening tests and model organisms. The yeast-hypha and reverse morphological transition exhibited by pathogens can be used as a
model system to screen antifungal agents. However, it is not always possible to use pathogens for the initial screening. Alternately, non-pathogenic dimorphic fungi exhibiting morphological transitions in response to the environmental perturbations similar to the pathogens can be the choice. \textit{B. poitrasii} shows glucose and temperature dependent reversible transition, which is easy to manipulate. In this regard, the use of \textit{B. poitrasii} was reported to demonstrate antifungal activity (Patil et al., 2001, Salunke et al., 2004).

The biochemical correlates of the morphological transition can be used as effective targets. In the present study effect of compounds on germ-tube formation of \textit{B. poitrasii} was observed. In \textit{B. poitrasii} relative proportion of NADP-/NAD-GDH is important in the morphological transition that suggested the role of both NAD and NADP-dependent GDH. As the level of NAD-GDH was higher as compared to the NADP-GDH in \textit{B. poitrasii} (Khale et al., 1992) and it is common for both the form, purification of NAD-GDH was carried out. Furthermore, purified NAD-GDH was used as a target to study the inhibition kinetics of the inhibitors. The glutamate dehydrogenases are the important class of enzyme. There are reports of the inhibition study in which GDH was used as a target (Choudhury et al., 2008; Choudhury and Punekar, 2007; Cunliffe et al., 1983). Isophthalate was the most reported inhibitor of both NAD-GDH (Cunliffe et al., 1983, Veronese et al., 1974, Stevens et al., 1989) and NAD(P)-GDH (Caughey et al., 1956, Rogers et al., 1972) of fungi. NAD-GDH from \textit{B. poitrasii} was also found to be inhibited by isophthalic acid (Doiphode, 2007). Rogers (1971) reported the use of structural analogues of L-glutamic acid such as glutaric acid, thiodiglycolic acid, oxydiglycolic acid, and iminodiacetic acid as inhibitors of bovine GDH whereas analogues of 2-ketoglutare such as 2-Oxoglutarate, 2-iminoglutarate, 2-methyleneglutarate, 2,4-pyridinedicarboxylate, 3,5-pyrazoledicarboxylate, etc. were screened for the inhibition of NADP-GDH from \textit{A. niger} in which 2-methyleneglutarate and 2,4-pyridinedicarboxylate were found to be effective (Noor and Punekar, 2005).

The structural basis was previously discussed to rationalize better inhibitory activity against GDHs. The presence of two carboxylate group, planarity of the molecule, etc. were the some of properties reported for the effective GDH inhibitors.
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In the present study, 2 compounds (B18 and B180) effectively inhibited NAD-GDH. From the double reciprocal plot it was observed that the inhibitors compete with the substrate and hence they are competitive inhibitors. This study could be useful to develop and designed antifungal drugs against dimorphic human and plant pathogen by using specific biochemical correlate as a target.