Nikkomycin Z Is a Specific Inhibitor of Saccharomyces cerevisiae Chitin Synthase Isozyme Chs3 In Vitro and In Vivo

JOANN P. GAUGHAN, MARGARET H. LAI, DONALD R. KIRCH, AND SANFORD J. SILVERMAN*

Molecular Genetic Screen Design, Agricultural Research Division, American Cyanamid, Princeton, New Jersey 08543

Received 25 January 1994/Accepted 11 July 1994

Nikkomycin Z inhibits chitin synthase in vitro but does not exhibit antifungal activity against many pathogens. Assays of chitin synthase isozymes and growth assays with isozyme mutants were used to demonstrate that nikkomycin Z is a selective inhibitor of chitin synthase 3. The resistance of chitin synthase 2 to nikkomycin Z in vitro is likely responsible for the poor activity of this antibiotic against Saccharomyces cerevisiae.

Chitin synthesis and degradation are considered excellent targets for pharmaceutical and agricultural pathogen management (12). Several compounds that affect chitin synthase have been identified. Nikkomycins and polyoxins are antifungal antibiotics that are competitive inhibitors of chitin synthase (Chs) enzymes (8, 13). Polyoxin D is used as an agricultural antifungal agent to treat rice sheath blight and pear black spot (16, 17). Unfortunately, compounds that affect in vitro chitin synthase activity are not necessarily effective antifungal agents (19). While permeability and intracellular stability of these compounds have been discussed as barriers to in vivo efficacy, differential effects on chitin synthase isozymes could influence a drug’s effectiveness. Investigations of the specific effects of chitin synthase inhibitors on the different Chs isozymes have been hampered by the complex enzymology of chitin synthases in fungi (20, 25).

In vitro effects of nikkomycin Z. The availability of mutant strains that express or overexpress single chitin synthase activities makes it possible to obtain extracts containing individual chitin synthase enzymes. We prepared digitonin-treated cell extracts from wild-type S288C cells (14) (Chs1 is the predominant chitin synthase activity found in extracts of wild-type cells treated with trypsin) and cells that contain high-copy-number CHS2 (SYY563-9B or EYC36-3D [YEp352-CHS2]) (Table 1). For a 250-μg cell pellet (an original culture volume of ~100 ml is usually sufficient), 750 μl of 1% digitonin–25 mM MES (morpholineethanesulfonic acid; pH 6.3) was added and the cells were shaken at 30°C for 15 min. They were centrifuged at ~12,000 × g for 5 min, and the pellet was washed with 2.7 ml of 25 mM MES, pH 6.3. The pellet was resuspended in a total volume of 750 μl of 25 mM MES, pH 6.3–33% glycerol. Membranes from strain EYC36-3C were prepared for Chs3 assays by a modification of the method of Orlean (20). Cells were washed once in 50 mM Tris-HCl, pH 8.0–5 mM MgCl2, and after membranes were prepared, they were resuspended in the same buffer containing 33% glycerol.

Enzyme assays were performed to measure the in vitro sensitivity of the three chitin synthases to nikkomycin Z and/or polyoxin D (both from Calbiochem). The cells were first treated with about 2 μl (experimentally determined to achieve maximum activity) of trypsin (2 mg/ml) by shaking at 30°C for 15 min. A 1.5-fold excess of soybean trypsin inhibitor was added, and the reaction was continued by the addition of putative inhibitors to a standard 50-μl assay mixture of (final concentration) 1 mM UDP-[(U-14C)]N-acetylglucosamine (400,000 cpm/μmol; Amersham)–32 mM N-acetylglucosamine plus either 50 mM Tris-HCl, pH 8–5 mM cobalt acetate (for Chs2) or 25 mM MES, pH 6.3–5 mM MgCl2 (for Chs1). Incubations were carried out at 30°C with shaking. The reaction was stopped after 90 min by the addition of 10% trichloroacetic acid, and the amount of product was quantified by filtration and scintillation counting.

Under these conditions, we determined the Km for Chs1 to be ~0.23 mM and that for Chs2 to be ~0.67 mM. Both compounds were most active against Chs1 (Fig. 1; Table 2). Similar to data from previous reports, Chs2 in the presence of Co2+ was found to be quite resistant to nikkomycin Z (Table 2) (8).

To measure Chs3 activity, two methods (5, 11, 20) were employed. For the first method (6, 20), 20 μl of membranes is incubated in the presence of putative inhibitors in a standard 50-μl assay mixture: 1 mM UDP-[(U-14C)]N-acetylglucosamine (400,000 cpm/mg)–40 mM N-acetylglucosamine–50 mM Tris-HCl, pH 7.5–3 mM MgCl2 at 25°C. Under these conditions, the Km for Chs3 was 0.83 mM. For the second method (11), membranes from EYC36-3C were treated with detergent and subsequently incubated with trypsin in the presence of substrate. These conditions resulted in an enzyme with a Km of ~0.3 mM. The results show that Chs3 was inhibited by much lower concentrations of nikkomycin Z (Fig. 1; Table 2) than are inhibitory to Chs2. The sensitivity of Chs3 to polyoxin D (Fig. 1; Table 2) was greater than that found previously for Chs2. Comparable levels of sensitivity to either compound are seen whether Chs3 is prepared by conventional methods or the newly describedzymogen protocol (Table 2) (11).

Chs3 is somewhat less sensitive to these inhibitors than is Chs1 (8, 20). However, Chs1, which is believed to catalyze septum repair during bud separation (10), will not support cell viability in the absence of other chitin synthases (26). Thus, it seemed unlikely that the major in vivo effects of nikkomycin Z and polyoxin D are produced as a result of actions on Chs1. In addition, Chs3 is inhibited by much lower concentrations of nikkomycin Z than had been shown for Chs2 (8), which...
TABLE 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECY36-3C*</td>
<td>MATa chs1-23 chs2::LEU2 ura3-52 leu2-2 trp1-1</td>
<td>26</td>
</tr>
<tr>
<td>ECY36-3D*</td>
<td>MATa chs1-23 calB1(chs3*) ura3-52 leu2-2 trp1-1</td>
<td>26</td>
</tr>
<tr>
<td>SSY638-3B</td>
<td>MATa chs1-23 calB1(chs3*) ura3 leu2 trp1-1 ade2-1 his3-11,15</td>
<td>This report</td>
</tr>
<tr>
<td>CGY161</td>
<td>MATa ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15</td>
<td>D. Challeff</td>
</tr>
<tr>
<td>LFY115-30</td>
<td>MATa his3 trp1 ade2 ade3 leu2 his2 ura3 cmk1∆1::HIS3</td>
<td>M. Pausch</td>
</tr>
<tr>
<td>SSY640-10A</td>
<td>chs1::HIS3 chs2::TRP1 his3 trp1 ade2 ade3 leu2 ura3 (possible cmk1∆1::HIS3)</td>
<td>This report</td>
</tr>
<tr>
<td>SSY563-9B</td>
<td>MATa chs1-23 chs2::LEU2 ura3 leu2-2 trp1-1 [YEp352-CHS2]</td>
<td>29</td>
</tr>
<tr>
<td>S288C</td>
<td>MATa SUC2 mal mel gal2 CUP1</td>
<td>27</td>
</tr>
<tr>
<td>Y294</td>
<td>MATa leu2-3,112 ura3-52 his3D trp1 Gal* [cir+]</td>
<td>J. Broach</td>
</tr>
<tr>
<td>JW17-11/1A-FOA</td>
<td>MATa chs1::ura3(3) chs2::LEU2 ura3-52 leu2-3,112</td>
<td>Reference 28, this report</td>
</tr>
</tbody>
</table>

* leu2 allele originally misidentified as leu2-3,112 (26).

suggests that Chs3 may be the critical target for nikkomycin Z action in vivo.

Because the in vitro sensitivity of Chs2 to this compound depends somewhat on the choice of cation and the endogenous, in vivo cation requirements are unknown, we turned to in vivo tests of nikkomycin and polyoxin action.

In vivo effect of nikkomycin on calcofluor inhibition. Calcofluor white is a fluorochrome that binds to chitin fibrils in the fungal cell wall and inhibits the growth of Saccharomyces cerevisiae (23). Microscopic examination of calcofluor-inhibited cells reveals high levels of chitin deposition suggesting that calcofluor white acts by increasing the synthesis of chitin to levels that are inhibitory to cell growth and viability (13). The argument for this putative mechanism for calcofluor action is supported by the observation that mutants selected for calcofluor resistance show decreased levels of chitin synthesis (22).

Recent molecular biological studies of yeast chitin synthases (2, 5, 7, 21, 26, 28, 29) have led to the identification of three related genes (CHS1, CHS2, and CAL1 [CalB]) which exhibit calcofluor resistance [23] and is subsequently herein referred to as CHS3) that encode three enzymatically distinct chitin synthase polypeptides (Chs1p, Chs2p, and Chs3p, respectively). Several additional genes that contribute to in vivo chitin synthase activity have also been partially characterized (5, 29).

The CHS3 locus is allelic with the CSD2 and DIT101 loci (21; for a review, see reference 6) and is necessary but not sufficient for Chs3 activity and thus presumably responsible for the abnormal buildup of chitin in calcofluor-treated cells.

Since mutants with lowered chitin levels show resistance to calcofluor, it seemed likely that compounds that act in vivo to specifically inhibit Chs3 might also reverse the calcofluor inhibition. We tested the effects of nikkomycin Z and polyoxin D on the growth of strain Y294 by applying the compounds to filter disks which were then placed on calcofluor-containing plates inoculated with the yeast culture. An overnight culture of strain Y294 was grown in liquid YEPD medium (YEPD and SD growth media and genetic methods are described in reference 27) shaken at 30°C. One part of culture inoculum plus 1.25 parts of calcofluor stock (Calcofluor White [Blankophor BBH]; Mobay Co., Rock Hill, S.C.; 10 mg/ml in 0.025 M KOH) were added to 100 parts of YBGM (a neutral pH synthetic medium [24]) which had been melted and kept at −50°C. Plates were poured and 50 μg of each compound was

**FIG. 1.** (A) NIKKOMycin Z inhibition of Chs isozymes. ●, Chs2; ■, Chs3; ▲, Chs1. (B) Polyoxin D inhibition of Chs3. Bars indicate standard deviations.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chs1</th>
<th>Chs2</th>
<th>Chs3*</th>
<th>Chs3b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nikkomycin Z</td>
<td>0.21</td>
<td>890</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Polyoxin D</td>
<td>ND*</td>
<td>ND</td>
<td>8.3</td>
<td>7.4</td>
</tr>
</tbody>
</table>

* Orlean method (20).

b Choi et al. method (11).

ND, not determined.
applied to filter disks that were then placed on petri dishes. The plates were incubated at 30°C for 2 days and then examined for cell growth (Fig. 2).

Under these conditions, yeast growth was detected around filter disks carrying nikonmycin Z (Fig. 2). No growth stimulation was observed with polyoxin D or with a group of fungicial compounds with mechanisms of action other than chitin biosynthesis inhibition (Fig. 2). The inactivity of control compounds suggests that growth stimulation is not an artifact produced by secondary effects of nikonmycin, such as a slowing of growth rate. This suggests that nikonmycin Z is a selective inhibitor of Chs3 activity in vivo.

**In vivo effect of nikonmycin on CHS mutants.** Strains that rely on the expression of a single chitin synthase isozyme for survival were employed to correlate the in vitro effects of nikonmycin Z with its fungicidal action. Nikonmycin Z and polyoxin D were tested for growth inhibition on strains ECY36-3C or SSY640-10A (Chs3+ only) versus ECY36-3D or SSY638-3B (Chs2+ only) and S288C (wild type). Strains ECY36-3C and ECY36-3D (or SSY640-10A and SSY638-3B) were inoculated into SD medium plus nutritional supplements (0.01% [wt/vol, each] uracil, adenine sulfate, leucine, tryptophan, histidine, and lysine) and grown to stationary phase. The cultures were diluted 1/100 into YEPD medium containing 2% agar which had been maintained at ~50°C, immediately poured into petri dishes, and allowed to cool. Fifty-microgram aliquots of the compounds (except for cycloheximide and ketoconazole [5 μg]) were applied as described above for YEPD medium (Fig. 2). Plates were incubated at 30°C overnight and growth-inhibitory zones around the disks were measured (Fig. 3). Polyoxin D did not inhibit any of these strains, while nikonmycin Z selectively inhibited growth of strains ECY36-3C and SSY640-10A (not shown). A group of antifungal compounds with varied mechanisms of action were included as controls in this experiment. None of these compounds showed selective inhibition of strains ECY36-3C and SSY640-10A, suggesting that these strains are not generally supersensitive to antifungal agents.

The effectiveness of nikonmycins and polyoxins depends to some extent on peptide transport systems and intracellular proteases which degrade these compounds (see references 1, 15, 18, and 30 for discussion of peptide transport mutants). Although the MIC of nikonmycin for ECY36-3C is significantly higher than the 50% inhibitory concentration for Chs3 enzyme, *S. cerevisiae* cells expressing only Chs3 activity are sensitive to nikonmycin Z, while cells expressing only Chs2 activity are insensitive. These cell types are presumably identical in peptide transport function, as indicated by the observation that both strains remained resistant to levels of polyoxin D known to inhibit Chs isoymes in vitro. This result suggests that the resistance of Chs2 to nikonmycin Z inhibition is the major barrier to the activity of this compound in vivo. It seems likely that the in vivo effect of polyoxin D is tempered by the permeability or metabolism of this compound and that these barriers are less effective against nikonmycin Z. However, we cannot rigorously exclude the possibility that polyoxin D is ineffective in our in vivo assays because it is eight- to ninefold less effective at inhibiting Chs3 in vitro than is nikonmycin Z.

Since both Chs2 and Chs3 are inhibited by roughly comparable levels of polyoxin D, this compound might not afford rescue to calcofluor-treated cells: a level of polyoxin D necessary for rescue could lead instead to cell death due to its activity on Chs2. In addition, Chs3 has been predicted to produce ~90% of normal cell chitin (7). The known morphological effects (but not growth inhibition in our assays) of polyoxin D (4, 9) could be explained by a major effect on Chs1 or Chs2 in vivo (10, 28): polyoxin D-treated cells show calcofluor staining patterns consistent with defective septum formation (9), and in some studies such cells morphologically resemble septum-repair-deficient chs1 mutants (4). The fact that some polyoxins are effective antifungal agents against many species of filamentous fungi (e.g., *Rhizoctonia solani* [17]) may indicate the presence of differences in polyoxin uptake and/or metabolism among different fungal families, although differential enzyme sensitivity is not excluded. Although Chs3 has only modest conservation with other chitin synthases, Bowen et al. (3) found high degrees of conservation within several chitin synthase isozyme classes (and presumably their corresponding functions [31]) across diverse families of fungi. This finding supports the utility of *S. cerevisiae* as a model for investigations of such antifungal targets. Our in vivo assays should be useful in identifying the targets of new antifungal agents and thus potentially to identify novel classes of chitin synthase inhibitors.

We thank Enrico Cabib and Christine Bulawa for strains and for communication of results prior to publication. We thank Eunice Froeliger for critical reading of the manuscript.

**REFERENCES**


