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R. F. Hector

Phyllis C. Braun
Fairfield University, pcbraun@fairfield.edu

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Synergistic Action of Nikkomycins X and Z with Papulacandin B on Whole Cells and Regenerating Protoplasts of *Candida albicans*

RICHARD F. HECTOR[1]†* AND PHYLLIS C. BRAUN[2]

Miles Pharmaceuticals, West Haven, Connecticut 06516,[1] *and Department of Biology, Fairfield University, Fairfield, Connecticut 06430*[2]

Combinations of nikkomycin X (NX) or nikkomycin Z (NZ), known inhibitors of chitin synthesis in fungi, together with papulacandin B (PB), an inhibitor of beta-glucan synthesis, were tested for synergistic activity against four isolates of *Candida albicans* by using the broth microdilution checkerboard technique and a method to assess the regeneration of cell wall material in protoplasts. The construction of isobolograms from the data generated by the checkerboard determinations revealed a synergistic effect for the two classes of compounds against all strains. The combination of NX and PB was more effective than the combination of NZ and PB, perhaps reflecting the lower K_i value of NX. While the presence of NX and NZ reduced chitin synthesis, as determined by staining with calcofluor white and assaying with a microfluorimeter, cells treated with PB demonstrated an increased synthesis of chitin. Protoplast regeneration experiments using similar concentrations of the two classes of compounds resulted in comparable findings. The combination of NX and PB resulted in a greater inhibition of chitin synthesis than did equivalent combinations of NZ and PB. These data suggest that combinations of agents active against cell wall synthesis in fungi may prove more useful as chemotherapeutic agents than such compounds used singly.

Compounds inhibitory to the synthesis of fungal cell wall material have been reported recently to have demonstrable effects against fungi of medical importance (1, 4, 11, 19, 22, 23). Of these compounds, two classes are the inhibitors of chitin synthesis (nikkomycins [9, 18] and polyoxins [7, 12]) and inhibitors of beta-glucan synthesis (papulacandin B [PB] [13, 21], aculeacin A [16, 17], and echinocandin B [22]). While these agents have been suggested as possible chemotherapeutic agents, experiments with polyoxin D against *Candida albicans* have shown that the MICs far exceed the calculated K_i value for the compound (1). Attempts to reduce the MICs by modifying the parent compound have been unsuccessful (19, 23).

While chitin is a logical target for a chemotherapeutic agent against the majority of fungi, it may be less appropriate for *C. albicans* because of the relatively low chitin content of this yeast, which has been reported to be approximately 1% (3). Beta-glucan, however, constitutes at least 39% of the cell wall (3). Because of the availability of agents able to block the synthesis of both of these wall components, we sought to determine if combinations of nikkomycin X (NX) or nikkomycin Z (NZ) and PB would act synergistically against *C. albicans*.

MATERIALS AND METHODS

Organisms and conditions of culture. In this study, four isolates of *C. albicans* were used. Strain C-6 was obtained from M. D. Anderson Medical Center, Houston, Tex.; strain C-215 was obtained from Hartford Medical Center, Hartford, Conn.; strain C-DCH was obtained from a case of diaper dermatitis from the son of one of the authors (R.F.H.); and strain B-311 was originally obtained from H. Hasenclever, National Institutes of Health, Bethesda, Md. All isolates were maintained by periodic transfer on Sabouraud dextrose agar

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* Corresponding author.

† Present address: Cutter Biologicals Division, Miles Laboratories, Berkeley, CA 94701.

and were kept at 4°C until needed. The cultures for experiments were initiated by inoculating flasks of glucose-yeast extract broth and incubating them with shaking at 30°C.

Susceptibility testing. NX and PB or NZ and PB were tested for possible synergistic activity by a checkerboard technique, using a 96-well microtiter format and yeast-extract peptone glucose broth (25). NX and NZ were a gift from K. Schaller (Bayer AG, Institute for Chemotherapy, Wuppertal, FRG), while PB was a gift from K. Scheibli (CIBA-GEIGY Corp., Basel, Switzerland). NX and NZ were dissolved directly in the yeast extract-peptone-glucose broth. PB was prepared as a 10% (wt/vol) solution in 95% ethanol and further diluted into the yeast extract-peptone-glucose broth. Wells contained an inoculum of 5×10^4 cells in a final volume of 200 µl. Plates were incubated at 37°C for 12 h before being read. Endpoints were defined as MICs resulting in the complete inhibition of visible growth.

After the MICs were determined, the cells were stained with calcofluor white (Poly Sciences) and processed as described below for the regenerating protoplasts. Cells were transferred to black microtiter plates before the plates were read with the epifluorescent reader.

Formation of protoplasts. Yeasts from the early exponential growth phase were centrifuged and washed twice with phosphate-buffered saline, pH 7.2. Approximately 1 ml of the packed cell volume of each strain to be converted to protoplasts was transferred to 250-ml flasks containing 50 ml 0.5 M sodium thioglycolate (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M Tris, pH 8.7 (Eastman Kodak Co., Rochester, N.Y.). Flasks were incubated at 37°C with shaking (100 rpm) for 30 min. After centrifugation, the cells were washed once with a 0.2 M phosphate–0.1 M citrate buffer containing 0.6 M KCl as an osmotic stabilizer. The cells were suspended in 4.5 ml of this buffer in a 50-ml flask, and 0.5 ml of β-glucuronidase, type H-2 (Sigma), was added. Flasks were incubated with shaking (100 rpm) at 37°C for 1 h. The degree of formation of protoplasts was assessed by the observation of lysis in hypotonic solution by using a light microscope.
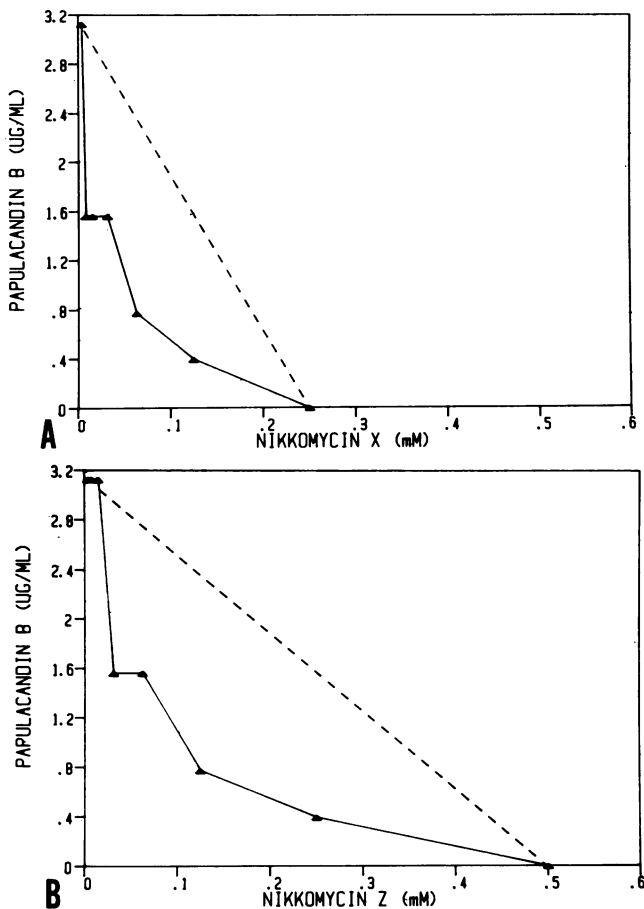
FIG. 1. Isobologram representations of combinations of nikkomycins and PB against *C. albicans* strain DCH. (A) NX and PB; (B) NZ and PB. Dotted line represents additive line.

Preparations not yielding >99% lysis were not used. Protoplasts were washed three times in phosphate-citrate buffer containing KCl and were stored at 4°C until required for use.

Protoplast regeneration assay. The methods used for assessing the amount of chitin synthesized by regenerating protoplasts have been described previously (Hector and Braun, submitted for publication). Briefly, the compounds to be tested were serially diluted in black plastic U-bottomed 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) by using 100-μl volumes of the defined medium of Lee et al. (14) supplemented with 0.5 M MgSO₄ as an osmotic stabilizer. Protoplasts were suspended to an optical density of 0.9 at 530 nm in phosphate-citrate buffer containing KCl and were diluted 1:10 in the growth medium. Samples of 100 μl were delivered to each well of the microtiter plates (one plate per isolate). After incubation at 30°C for 2 h, 50 μl of calcofluor white (300 μg/ml in phosphate-buffered saline), was added to each well. The plates were incubated an additional 5 min at room temperature and then were centrifuged at 800 × g for 6 min, and the supernatants were removed. The pellets were washed twice and suspended in 100 μl of phosphate-buffered saline. The plates were read immediately by using a Dynatech Microfluor reader, with fluorescence expressed as methylumbelliferone units per well.

Light microscopy. Cells from both the synergy and the

protoplast regeneration experiments were examined with a Zeiss Universal microscope (Carl Zeiss, Inc., New York, N.Y.) by using differential interference contrast and epifluorescence microscopy. Excitation was at 365 nm, and bandpass was at 420 nm. For micrographs with epifluorescence, exposures were made at equivalent settings to ensure that comparisons were valid.

RESULTS

Susceptibility testing. By using a 96-well microtiter format, four isolates of *C. albicans* were tested against combinations of NX and PB or NZ and PB in a checkerboard fashion. MICs against the single agents ranged from 0.125 to 1.0 mM for NX, 0.25 to 1.0 mM for NZ, and 1.56 to 3.13 μg/ml for PB. Isobolograms of the combinations for strain DCH demonstrate a synergistic action for the two classes of compounds (Fig. 1). Isobolograms for the other strains were similar (data not shown). Additionally, interaction indices were calculated and values of 0.5 were obtained in the majority of cases. Immediately after the plates were read, the cells from each strain were stained with calcofluor white, and the total fluorescence after excitation by UV light was measured as an indication of chitin synthesis. Treatment
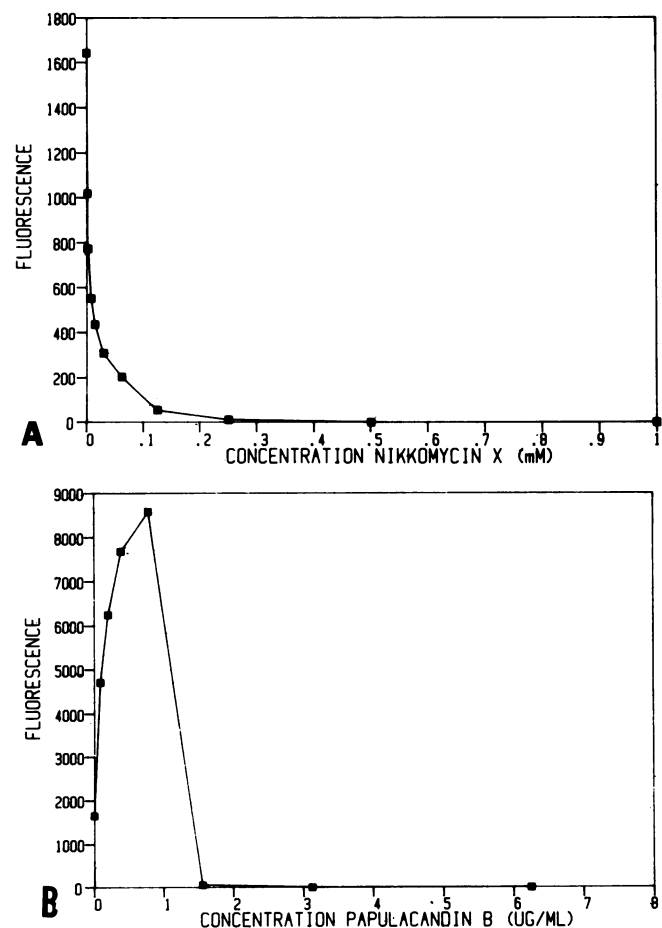

FIG. 2. Effects of varyious concentrations of NX (A) or PB (B) on whole cells of *C. albicans* C-215. Fluorescence levels were determined by microfluorimetry after staining with calcofluor white. Fluroescence is expressed as arbitrary units of methylumbelliferone.
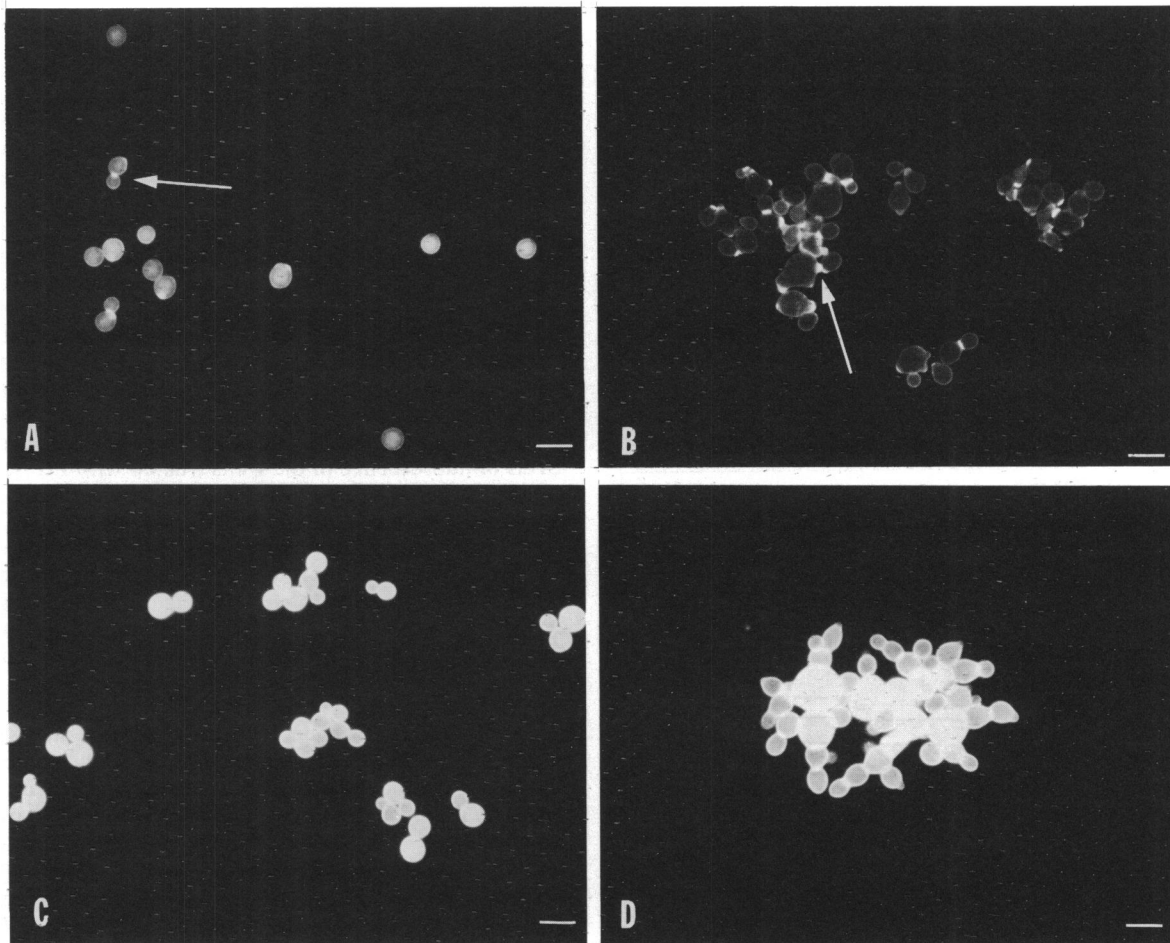
FIG. 3. Epifluorescence micrographs of whole cells of strain C-215 from MIC checkerboard determinations. (A) Micrograph of untreated controls showing moderate fluorescence over the entire cell surfaces, with concentration of fluorescence at septa and bud scars (see arrow). (B) Micrograph of cells treated with 0.0156 mM NX showing fluorescence restricted to septa and bud scars. Cells are also swollen and have unusual protuberances at bud scar sites (see arrow). (C) Micrograph of cells treated with 0.2 μg of PB per ml. Cells are demonstrably brighter than untreated controls, but are not abnormally shaped. (D) Micrograph of cells treated with 0.0156 mM NX and 0.39 μg of PB per ml. Cells are chained, and have multiple bud sites and high levels of fluorescence. Magnification bars, 10 μm.

with NX or NZ singly resulted in a significant decrease in fluorescence with readings at 0.002 mM, only 53 and 44% of the control fluorescence values, respectively (Fig. 2A; data shown for NX only). The greater inhibition seen with NX compared to NZ is consistent with the known properties of the compounds. PB, however, caused increased fluorescence up to the point of the MIC (Fig. 2B). Combinations of the two compounds not resulting in decreased growth generally resulted in levels of fluorescence intermediate to that seen with both agents used singly (data not shown). Cells from strain C-215 also were examined by light and epifluorescence microscopy. Untreated controls are shown in Fig. 3A. With concentrations as low as 0.001 mM NX, the treatment had a demonstrable effect, with unusual protuberances evident in the area of bud scars (data not shown). These cells also were noticeably less fluorescent in comparison with the controls. At 0.0156 mM NX (Fig. 3B), the chitin content of the cells was apparently greatly reduced, since fluorescence was evident primarily in the region of the bud scars. Additionally, there was an increase in aberrant shapes. At 0.125 mM NX, cells were only faintly fluorescent, and the chaining of cells was common. At 1 mM NX, a

concentration greater than the MIC, proliferation had apparently been halted because only occasionally were cells seen.

In contrast to the reduction of staining intensity seen with NX, treatment with PB appeared to result in increased synthesis of chitin, as cells fluoresced with greater intensity at all concentrations up to the MIC. While the lower concentrations did not appear to have much effect on the morphology of the cells (Fig. 3C), a concentration of 0.78 μg/ml resulted in cells which were larger and more spherical than the untreated controls (data not shown).

Those cells treated with combinations of the two compounds had morphological characteristics of each compound singly, but the characteristics varied with each concentration. Generally, increasing concentrations of NX at constant concentrations of PB resulted in smaller forms that fluoresced less, while increasing the concentrations of PB caused larger, brighter forms. Figure 3D shows cells treated with higher concentrations of both compounds, and is representative of the chains of cells with multiple buds seen in this group.

Cells treated with combinations of NZ and PB showed similar effects to those described above except that the level
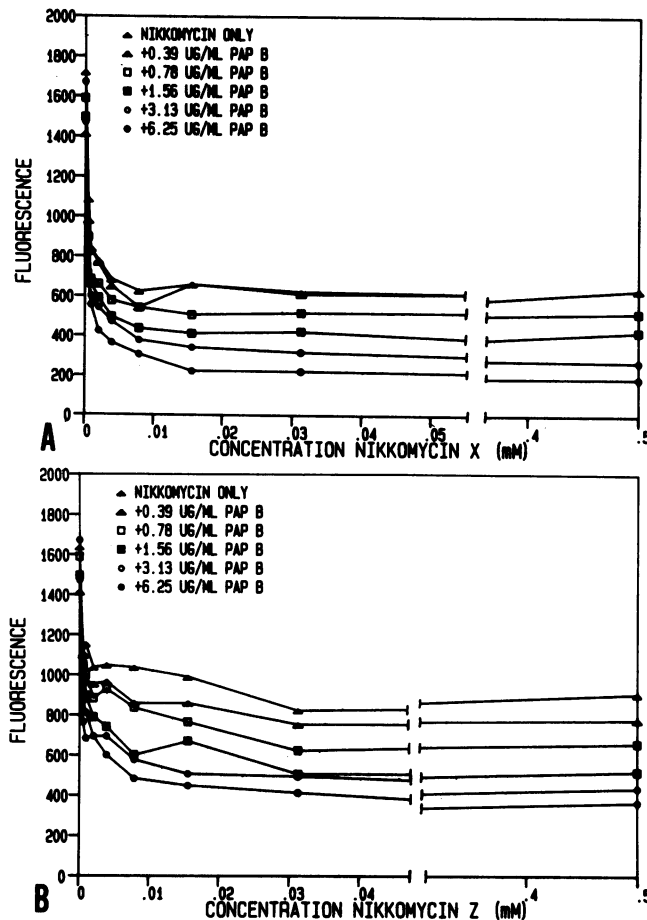
FIG. 4. Effects of NX and PB (A), or NZ and PB (B), on regenerating protoplasts of *C. albicans* C-215. Fluorescence levels were determined by microfluorimetry after staining with calcofluor white. Fluorescence is expressed as arbitrary units of methylumbelliferone.

using epifluorescence. Figure 5A shows untreated controls after the 2-h period of incubation. After regenerating the cell wall, the cell produces a protuberance similar to a germ tube, but much more irregular in shape. The germ tube may or may not show the same level of fluorescence after staining as the mother cell, and a small percentage are osmotically fragile and lyse at the terminus during the staining procedure. Cells treated with concentrations of NX as low as 0.001 mM clearly were affected by the compound (Fig. 5B). The cells had reduced levels of fluorescence in comparison with the controls, the protuberances were shorter; and the majority of cells had lysed at the terminus of the protuberance. Those cells treated with increasing concentrations of NX showed reduced levels of fluorescence and cell wall synthesis. Cells treated with 0.125 mM NX were unable to synthesize protuberances (data not shown).

Despite the fact that the fluorimeter measured decreased levels of fluorescence in the wells treated with PB singly, an examination of the cells revealed that individual cells appeared to stain as intensely as the controls (Fig. 5C). The cells treated with higher concentrations of PB, however, did not have protuberances as long as those seen in the controls, which would account for a lower figure for total fluorescence. The cells treated with higher concentrations of PB also had a "floppy" and nearly transparent appearance that was not apparent in the light micrographs.

Combinations of NX and PB resulted in cells with a reduced capacity to regenerate their cell wall, and virtually all cells showed evidence of lysis (Fig. 5D). The combination of the two compounds clearly had more potent effects than either agent had singly. At 0.0039 mM NX and 0.39 µg of PB per ml, many cells had spots of intense fluorescence, indicating a nonuniform synthesis of chitin in the protoplasts. At high concentrations of both compounds, virtually all protoplasts showed evidence of lysis and minimal regeneration (Fig. 5E).

An examination of cells treated with NZ either singly or together with PB revealed that the severity of effects was reduced in comparison with that seen with equivalent concentrations of NX (data not shown).

of inhibition of chitin synthesis by NZ was not as great as that seen with NX (data not shown).

Protoplast regeneration assay. By using similar concentrations as those employed in the susceptibility experiment, the effects of NX, NZ, and PB were tested singly or in combinations against regenerating protoplasts of the same four strains of *C. albicans*. After a 2-h incubation period, previously determined to be optimal (Hector and Braun, submitted), the cells were stained with calcofluor white, and total fluorescence was measured. Since the results were essentially identical for all four strains, only data from those tests using strain C-215 are presented. The effects of NX and NZ singly, as measured by levels of fluorescence, were similar to those seen with whole cells since increasing concentrations resulted in decreased fluorescence (Fig. 4A and B). The effects of PB singly, however, were different from the effects seen with whole cells in that levels of fluorescence apparently decreased with increasing concentrations, as measured by the fluorimeter. Combinations of the two classes of compounds caused an additive effect so that fluorescence for a given concentration of NX or NZ dropped in a linear fashion with increasing concentrations of PB above 0.2 µg/ml.

The protoplasts were also examined microscopically by

DISCUSSION

The role of chitin in medically important fungi has been the subject of numerous publications recently (1, 2, 8, 10, 19, 20, 23). In *Coccidioides immitis* and *Wangiella dermatitidis*, two fungi with unusual parasitic phases, chitin was found to play an essential role in maintaining normal cell wall architecture, with treatment by the polyoxins resulting in aberrant forms or lysis (4, 11). Chitin also plays an integral role in *C. albicans*. The mycelial phase, which is assumed to be the dominant form in invasive disease, has both more chitin (3) and more active synthetic capabilities for the polymer than the yeast phase (2). Despite these facts, the mycelial form still has a much smaller percentage of chitin in its cell wall than many dimorphic fungi. Thus, while specific chitin synthase inhibitors such as polyoxin D have had demonstrable effects on *C. albicans*, the concentrations necessary to kill the fungus would probably preclude the use of such compounds as chemotherapeutic agents. Several factors complicate this situation. While the K_i of these compounds is in the micromolar range, MICs against *C. albicans* are millimolar (1). There is good evidence that this discrepancy is due to the poor transport of these nucleoside-peptide agents across the cytoplasmic membrane (19). Attempts to improve the uptake of polyoxin by altering the peptide
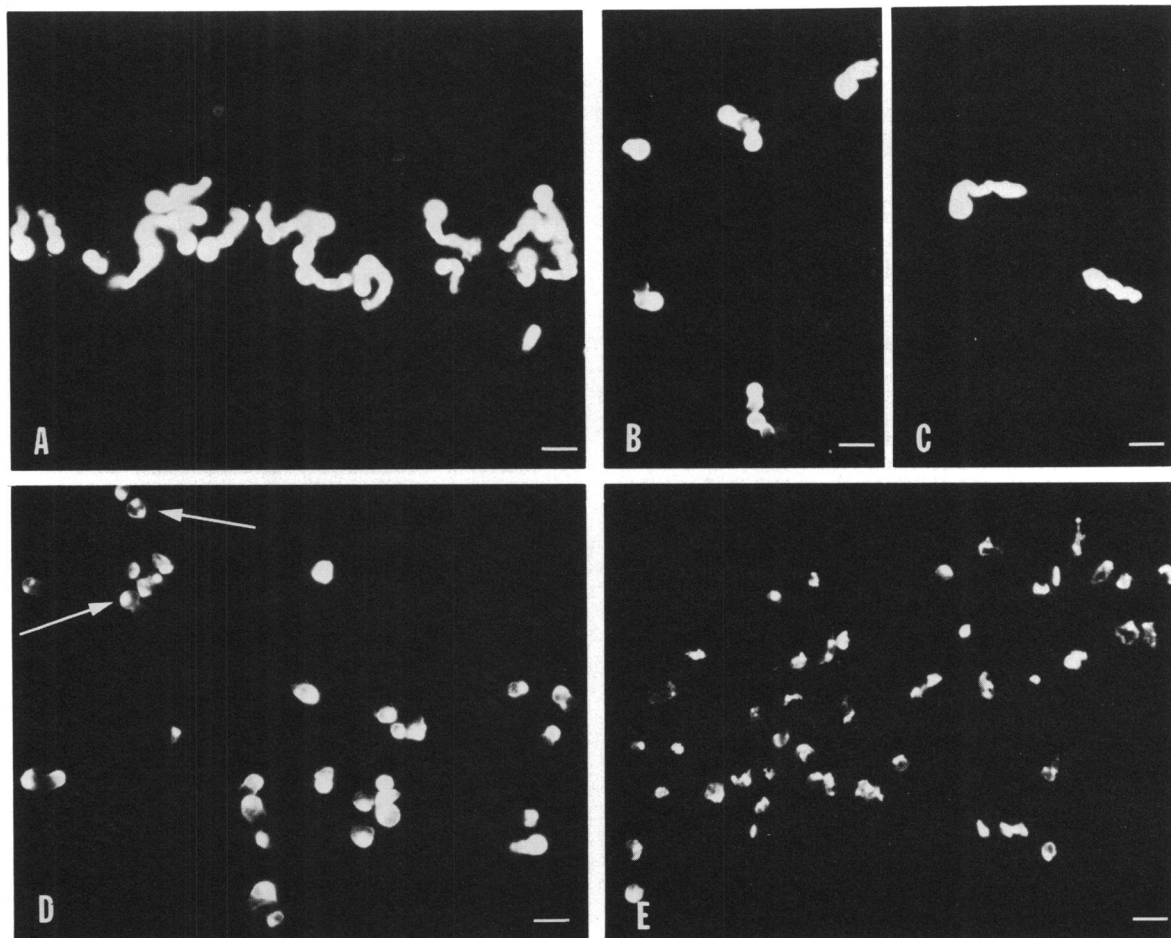
FIG. 5. Epifluorescnece micrographs of stained regenerating protoplasts. (A) Untreated controls. Cells typically had an irregularly shaped protuberance emerging from the mother cell. (B) Protoplasts treated with 0.001 mM NZ showing shorter protuberances and less fluorescence than controls. Cells were typically lysed at the terminus of the protuberance. (C) Cells treated with 1.56 μg of PB per ml. Cells had an appearance similar to that of the controls, but cells walls seemed less rigid and had a transparent quality. (D) Cells treated with 0.0039 mM NX and 0.39 μg of PB per ml. Majority of cells failed to synthesize a protuberance. Note also discrete areas of high fluorescence within some protoplasts (see arrows) suggesting nonuniform synthesis of chitin. (E) Cells treated with 0.125 mM NX and 6.25 μg of PB per ml, demonstrating significant destruction. Magnification bars, 10 μm.

moiety have not resulted in an improvement in the lethal effects of the compound (19, 23). Additionally, since both the polyoxins and the nikkomycins are competitive inhibitors of fungal chitin synthase (9, 12, 18), it would be necessary to maintain sufficiently high blood levels of the compound in an infected host to constantly inhibit the enzyme.

More recently, compounds inhibitory to beta-glucan synthesis in fungi have been described (13, 16, 17, 21, 22). At present, the mechanism of action of these compounds is not known, but they do not appear to inhibit the glucan synthase in a competitive fashion (21). Since beta-glucan constitutes a major fraction of the cell wall mass in C. albicans, this polymer represents a possible target for a chemotherapeutic agent. Unfortunately, questions of toxicity with such compounds may preclude their use.

Because chitin and beta-glucan have been reported to be in close association in the cell walls of both saprophytic (24) and medically important fungi (5, 10), the possibility that combinations of agents active against chitin or glucan synthesis would have greater lethal effects than such agents acting singly seemed worth investigating. That results of the

susceptibility tests and the protoplast regeneration assay demonstrated pronounced synergistic effects for the two compounds demonstrates the importance of the two polymers in maintaining the integrity of the cell wall in C. albicans. Indeed, one of the more interesting findings of the present study was that the inhibition of beta-glucan synthesis by PB appeared to result in increased amounts of chitin being synthesized. While the nature of this increase is unknown. it underscores the importance of a balanced synthesis of the two polymers in the cell wall. It has been reported previously that chitin synthesis in regenerating protoplasts of both C. albicans (6) and Candida utilis (15) greatly exceeds that which is seen in intact cells, thereby suggesting that the regulation of the chitin synthase enzyme is altered in protoplasts.

In summary, results from both sets of experiments indicate that the combinations of NX and PB or NZ and PB are inhibitory to C. albicans and deserve further investigation. The present availability of several different compounds that depress either chitin or beta-glucan synthesis offers the possibility for additional in vitro testing of combinations of

those agents to determine if inhibitory concentrations can be achieved that would be without toxicity in an animal system.

ACKNOWLEDGMENT

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