Synergistic action of nikkomycin X/Z with azole antifungals on *Candida albicans*

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Fluconazole, ketoconazole and tioconazole were shown to act synergistically *in vitro* with the antibiotic nikkomycin X/Z on the pathogenic fungus *Candida albicans*. The phenomenon was demonstrated using a checkerboard technique and growth inhibition experiments. The azole antifungal agents, even at concentrations not affecting growth, decreased the incorporation of the ¹⁴C-label from [¹⁴C]glucose into chitin of the candidal cell wall. After 3 h incubation with tioconazole, 1 µg ml⁻¹, the incorporation of the radiolabelled glucose into chitin of intact cells and regenerating spheroplasts of *C. albicans* was inhibited by 43% and 30%, respectively. Moreover, the relative chitin content was approximately 45% lower than that of control cells. The chitin content increased after prolonged incubation with azoles, thus confirming the known phenomenon of azole-induced uncoordinated chitin synthesis and deposition. On the other hand, azole derivatives had very little effect on the rate of nikkomycin transport into *C. albicans* cells. A sequential blockade mechanism of synergism is proposed.

Introduction

Nikkomycins, peptidyl nucleoside antibiotics produced by *Streptomyces* spp. (Dahn *et al.*, 1976), are strong inhibitors of chitin synthase (UDP-2-acetamido-2-deoxy-D-glucose: chitin 4-β-acetamidodeoxy-D-glucosyl-transferase, EC 2.4.1.16) in the pathogenic fungus *Candida albicans* (McCarthy *et al.*, 1985). Since this enzyme does not exist in mammalian cells, nikkomycins are considered as promising potential antifungals of low toxicity for mammals. Unfortunately, their antifungal efficacy, studied *in vivo* in an animal model, was rather low (Becker *et al.*, 1988). Chitin synthase is localized in the cytoplasmic membrane, which in fungi contains a large amount of ergosterol. Several effective antifungal drugs, including synthetic azole derivatives, are inhibitors of ergosterol biosynthesis (Saag & Dismukes, 1988). It has been reported that chitin synthesis is affected by polyene macrolides (Rast & Bartnicki-Garcia, 1981), cerulenin and sodium butyrate (Braun *et al.*, 1987), i.e. by compounds altering membrane fluidity. Previously azole antifungals were shown to cause the uncoordinated synthesis and irregular distribution of chitin in *C. albicans* (Vanden Bossche, 1985) and some other fungi (Marichal *et al.*, 1984; Kerkenaar & Barug, 1984). On the

other hand, an inhibitory effect of these compounds on some membrane enzymes, including chitin synthase, was reported (Surarit & Shepherd, 1987). Therefore, we decided to investigate whether inhibitors of Δ¹⁴-lanosterol demethylase, such as ketoconazole, tioconazole and fluconazole, can enhance the *in vitro* antifungal efficacy of nikkomycin.

Methods

Organisms and culture conditions. Five *C. albicans* strains were used, ATCC 26278 and four clinical isolates, numbers 2043, 886, 4477 and 16, obtained from the Institute of Medicinal Microbiology, Medical Academy of Gdańsk. The fungi were maintained on Sabouraud Dextrose Agar. Inocula were grown overnight in Sabouraud Dextrose Broth and prepared by suspending the yeast cells in sterile water. Concentration of yeast cells was determined by haemocytometer counts.

Antifungal compounds and other chemicals. Nikkomycin X/Z was a generous gift of Professor Hans Zähler from the University of Tübingen, Germany. Fluconazole and tioconazole were obtained from Pfizer. Ketoconazole, lyticase and chitinase were from Sigma. [U-¹⁴C]Glucose solution, 8 MBq ml⁻¹, was from UVVVR, Prague, Czechoslovakia.

Susceptibility testing. Nikkomycin and azole antifungals were tested for possible synergism by a checkerboard technique in modified Yeast Nitrogen Base-glucose (YNBG) solid medium (Milewski *et al.*, 1988). Serial twofold dilutions of nikkomycin and azole antifungals were prepared so that each plate contained a unique combination of two

Abbreviations: FIC, fractional inhibitory concentration; TNBS, 2,4,6-trinitrobenzene sulphonate.

antifungal agents. The concentrations were chosen to encompass the MIC for the strains to be tested. Plates were inoculated with 10^4 cells ml^{-1} from overnight cultures. After 24 h incubation at 37 °C, plates were examined and fractional inhibitory concentration (FIC) indexes were calculated as follows: FIC index = (MIC of nikkomycin in combination)/(MIC of nikkomycin alone) + (MIC of the azole antifungal in combination)/(MIC of the azole antifungal alone). The FIC index for each row of dilutions was calculated and the greatest deviation from 1.0 was used to characterize the interaction of the drugs (Ellion *et al.*, 1954).

Growth experiments. YNBG liquid medium (Milewski *et al.*, 1988) was inoculated with 10^5 yeast cells ml^{-1} from overnight culture. After 3 h preincubation at 37 °C with shaking (100 r.p.m.), antifungal agents were added and incubation was continued under the same conditions. Growth was determined spectrophotometrically at 660 nm.

Transport studies. Yeast cells from overnight cultures were washed with sterile water and suspended in YNBG medium at 10^6 cells ml^{-1} . In some experiments antifungal azoles were added at this stage. Cell suspensions were incubated for 3 h at 37 °C with shaking (100 r.p.m.). Then the cells were harvested, washed with 50 mM-potassium phosphate buffer, pH 7.2, and suspended in the same buffer containing 1% (w/v) glucose, in 50 ml flasks. Antifungal agents and nikkomycin or model peptides were added and flasks were incubated with shaking (100 r.p.m.), at 37 °C. Peptide uptake was determined using 2,4,6-trinitrobenzene sulphonate (TNBS), as described previously (Milewski *et al.*, 1988). In some experiments, cells were preincubated with azole antifungals before the determination of nikkomycin uptake.

Formation of spheroplasts. Cells from the early exponential growth phase were harvested and washed twice with saline. Wet cells (200 mg) were suspended in 5 ml 50 mM-Tris buffer, pH 8.9, containing 5 mM-EDTA and 50 mM-dithiothreitol, and incubated for 30 min at 30 °C with gentle shaking. The cells were then harvested, washed with 50 mM-potassium phosphate buffer, pH 6.0, and suspended in 5 ml of the same buffer containing 0.6 M-KCl as an osmotic stabilizer. Lyticase (1 mg) dissolved in a minimal amount of phosphate buffer was added and the suspension was incubated at 37 °C with occasional shaking. Formation of spheroplasts was assessed microscopically by the observation of lysis in hypotonic solution. Usually 99% conversion was achieved after 0.5 h incubation. Spheroplasts were washed twice with stabilizing buffer and suspended in a minimal amount of the same buffer.

Incorporation of ^{14}C -label from ^{14}C glucose into cell wall polysaccharides of intact cells or regenerating spheroplasts. Intact cells from the exponential phase of growth or freshly prepared spheroplasts were suspended in 0.1 M-Tris/HCl buffer, pH 7.2, containing 0.5 M- MgSO_4 and 5 mM-glucose. Each flask contained a known concentration of cells (about 10^8 cells ml^{-1}), 0.2 MBq ^{14}C glucose, and azole antifungals at appropriate concentrations, in 40 ml medium. Incubation was carried out at 37 °C for 3 h. At hourly intervals, 10 ml samples of cell suspensions were withdrawn. Cells were centrifuged (1000 g, 10 min) and the pellet was washed twice with 20 ml portions of unlabelled medium and once with Tris/HCl buffer. The distribution of ^{14}C -label into the various polysaccharide fractions was studied using a slight modification of the method of Gopal *et al.* (1984). Pellets were extracted with 1 M-NaOH at 90 °C for 1 h. Mannoproteins were precipitated with Fehling's solution (0 °C, 18 h), and then dissolved in 1 ml 0.1 M-potassium phosphate buffer, pH 6.0. Alkali-insoluble fractions were suspended in 2 ml portions of 0.1 M-potassium phosphate buffer, pH 6.0, and divided into two equal parts. One part was treated with 1 mg lyticase, the other with 1 mg chitinase. After incubation at 37 °C for 12 h, the insoluble residues were removed by centrifuging and the supernatants were analysed for radioactivity.

Analysis of soluble fractions. Samples of soluble fractions obtained after cell wall fractionation and enzyme treatment were deionized and subjected to paper chromatography as described by Gopal *et al.* (1984).

Determination of chitin content. Cells were incubated with nikkomycin or/and azole antifungals in YNBG liquid medium at 37 °C with shaking (100 r.p.m.). Samples of 3 ml were withdrawn from the incubation mixtures, and cells were harvested and washed with phosphate buffered saline (PBS). Pellets were suspended in 1 ml PBS containing Calcofluor white, 100 $\mu\text{g ml}^{-1}$. The suspensions were incubated at 37 °C for 5 min. Then cells were harvested, washed twice with PBS and suspended in PBS to $\text{OD}_{660} = 0.1$. Fluorescence associated with cell suspensions was measured with a Perkin Elmer luminescence spectrometer LS 5B. Excitation was at 365 nm and band pass was at 420 nm. Fluorescence of each sample was corrected by the reference measurement of the ground fluorescence associated with samples treated as above excluding staining with Calcofluor white.

Glucose uptake into intact cells and spheroplasts was determined essentially as described by Gopal *et al.* (1984).

Results

Synergistic action of nikkomycin with azole antifungals

Two representative isobolograms, shown in Fig. 1, demonstrate a synergistic action between nikkomycin X/Z and azole antifungals on *C. albicans* strains. Isobolograms for other strains and combinations were similar (data not shown). Nevertheless, some differences were observed. FIC indexes, calculated for each strain and each combination, are summarized in Table 1. Only in two cases was an additive effect observed (FIC = 1.0). The combination of nikkomycin and tioconazole seemed to be more effective than the other two tested.

The synergistic action of nikkomycin with azole derivatives was confirmed in growth-inhibition studies (Fig. 2). In the presence of tioconazole, 0.1 $\mu\text{g ml}^{-1}$, in combination with nikkomycin X/Z, 0.1 $\mu\text{g ml}^{-1}$, growth of *C. albicans* was inhibited by 50% after 6 h incubation. Alone, these agents affected fungal growth by no more than 10%.

Influence of azole antifungals on nikkomycin transport

In order to find an explanation for the observed synergism, the effect of azoles on nikkomycin transport was studied. Table 2 contains results of transport rate determinations performed for nikkomycin and some model peptides. None of the three azoles tested, when added simultaneously with nikkomycin or model peptides, affected their transport rates (data not shown). A slight effect was observed only when cells were preincubated with azoles before transport determination. It may be noteworthy that whereas nikkomycin transport rate was slightly enhanced, the model peptides were transported with lower rates.

Fig. 1. Isobologram representations of combinations of nikkomycin X/Z and azole antifungals against two candidal strains: (a) nikkomycin and fluconazole against *C. albicans* 16, (b) nikkomycin and tioconazole against *C. albicans* ATCC 26278. —, Additive line.

Table 1. Fractional inhibitory concentration (FIC) indexes determined for combinations of nikkomycin with azole antifungals for *C. albicans*

Strain	Nikkomycin + ketoconazole	Nikkomycin + tioconazole	Nikkomycin + fluconazole
ATCC 26278	0.14	0.14	0.5
2043	0.65	0.5	0.85
886	0.85	0.24	1.0
4477	0.14	0.24	0.625
16	1.0	0.625	0.38

Table 2. Effect of azole antifungals on the transport of nikkomycin and model peptides by *C. albicans* ATCC 26278

Cells from overnight culture were washed and suspended in YNBG modified medium (10^6 cells ml^{-1}). Cells were preincubated in this medium in the presence of azole antifungals (4 h, 37°C). Then cells were harvested and suspended in potassium phosphate buffer containing 1% glucose (10^8 cells ml^{-1}). Nikkomycin or model peptides were added simultaneously with a new portion of azole antifungals and the rate of peptide transport was determined using the TNBS method. Transport rates are expressed as nmol peptide taken up by 1 mg cells (dry weight) per minute. Values are the means of three independent experiments $\pm$ SD.

Azole	Transport rate (nmol min^{-1} mg^{-1} , $\pm$ SD) of:		
	Nikkomycin	Ala-Ala	Gly-Gly-Phe
None	1.15 ± 0.24	5.45 ± 0.38	1.27 ± 0.08
Fluconazole			
100 $\mu\text{g ml}^{-1}$	1.32 ± 0.17	4.24 ± 0.21	1.08 ± 0.08
5 $\mu\text{g ml}^{-1}$	1.23 ± 0.15	4.82 ± 0.32	1.15 ± 0.22
Tioconazole			
20 $\mu\text{g ml}^{-1}$	1.28 ± 0.09	4.35 ± 0.27	1.12 ± 0.13
1 $\mu\text{g ml}^{-1}$	1.25 ± 0.31	4.92 ± 0.17	1.32 ± 0.07
Ketoconazole			
20 $\mu\text{g ml}^{-1}$	1.38 ± 0.22	4.35 ± 0.27	0.96 ± 0.20
1 $\mu\text{g ml}^{-1}$	1.22 ± 0.15	4.67 ± 0.45	1.21 ± 0.14

Fig. 2. Growth-inhibitory effect of nikkomycin, tioconazole and their combination against *C. albicans* ATCC 26278. Cells from overnight cultures were suspended in YNBG modified medium (10^5 cells ml^{-1}), and preincubated for 3 h at 37°C with shaking before addition of the tested compounds. $\circ$, Control; $\blacksquare$, tioconazole $0.1 \mu\text{g ml}^{-1}$; $\bullet$, nikkomycin $0.1 \mu\text{g ml}^{-1}$; $\blacktriangle$, tioconazole $0.1 \mu\text{g ml}^{-1}$ and nikkomycin $0.1 \mu\text{g ml}^{-1}$.

Effect of azole antifungals on cell wall polysaccharide biosynthesis

C. albicans cells were incubated with antifungal azoles and labelled with $[^{14}\text{C}]$ glucose. Cell walls were isolated and fractionated as described in Methods. The identity of soluble fractions obtained after treatment with lyticase and chitinase was confirmed by paper chromatography. *N*-Acetyl-D-glucosamine and *N,N'*-diacetylchitobiose were the only products of degradation by chitinase. Lyticase released only D-glucose and short glucose oligomers. Therefore, the respective fractions were assigned as chitin and alkali-insoluble glucan.

Fig. 3. Time-course of incorporation of ^{14}C -label from $[^{14}\text{C}]$ glucose into (a) chitin, (b) mannan and (c) glucan of intact cells of *C. albicans* ATCC 26278. Suspensions (40 ml) of intact cells (10^8 cells ml^{-1}) were incubated with $[^{14}\text{C}]$ glucose (0.2 MBq) and antifungal azoles in 0.5 M- MgSO_4 containing 0.1 M-Tris/HCl, pH 7.2, and 5 mM-glucose. At hourly intervals, 10 ml samples were withdrawn, and cells were harvested and fractionated as described in Methods. The radioactivity of each fraction is expressed as total c.p.m. per mg dry weight of cells. ●, Control; ▼, tioconazole, 10 $\mu\text{g ml}^{-1}$; ■, tioconazole 1 $\mu\text{g ml}^{-1}$; ▲, tioconazole, 0.1 $\mu\text{g ml}^{-1}$; ○, fluconazole, 1000 $\mu\text{g ml}^{-1}$; □, fluconazole, 100 $\mu\text{g ml}^{-1}$; △, fluconazole, 10 $\mu\text{g ml}^{-1}$.

Fluconazole, tioconazole and ketoconazole strongly inhibited incorporation of ^{14}C -label from $[^{14}\text{C}]$ glucose into chitin of intact *C. albicans* cells. The time-course of incorporation of radioactivity from the labelled glucose in the presence of fluconazole or tioconazole is shown in Fig. 3(a–c). Results for ketoconazole were practically the same as those for tioconazole. Incorporation of the labelled precursor into glucan and mannoproteins was markedly affected only at high azole concentrations, whereas at the lowest concentrations, the only effect observed was a partial inhibition of incorporation into chitin. The time-course of incorporation into chitin in the presence of fluconazole at 1000 $\mu\text{g ml}^{-1}$ or 100 $\mu\text{g ml}^{-1}$ was exactly the same. It should be also pointed out that because of the high cell density, growth was practically not affected (data not shown), in spite of the reduced incorporation of the radioactive label into chitin. A similar effect was observed when incorporation of labelled glucose into cell wall polysaccharides of regenerating spheroplasts was studied (Table 3). However, in this case, high concentrations of fluconazole and tioconazole caused practically complete inhibition of incorporation into chitin and glucan. At lower concentrations, only incorporation of radioactive label into chitin was still strongly reduced.

Cells treated with antifungal azoles and/or nikkomycin X/Z were stained with Calcofluor white and the total fluorescence after excitation by UV light was measured in order to determine the relative chitin content (Table 4). A significant decrease of fluorescence was observed for tioconazole-treated cells as well as for the combination of tioconazole, 0.1 $\mu\text{g ml}^{-1}$, and nikkomycin, 0.1 $\mu\text{g ml}^{-1}$. The lowest level of fluorescence was observed after 3 h incubation with tioconazole. However, after 24 h

Table 3. Distribution of label from exogenous $[U-^{14}\text{C}]$ glucose into alkali-insoluble glucan and chitin of regenerating spheroplasts

Suspensions (10 ml) of spheroplasts (10^8 cells ml^{-1}) were regenerated in medium containing $[U-^{14}\text{C}]$ glucose (50 kBq) and azole antifungals. After 2 h incubation at 37 °C spheroplasts were isolated and fractionated as described in Methods. The radioactivity of each fraction is expressed as total c.p.m. and as a percentage relative to the counts associated with the control sample.

	Chitin		Glucan	
	C.p.m.	%	C.p.m.	%
Control	12100	100	12000	100
Fluconazole				
10 $\mu\text{g ml}^{-1}$	8550	71	9350	78
1 $\mu\text{g ml}^{-1}$	9250	77	11750	98
Tioconazole				
80 $\mu\text{g ml}^{-1}$	100	1	100	1
10 $\mu\text{g ml}^{-1}$	6900	57	9200	76
1 $\mu\text{g ml}^{-1}$	8350	70	10900	91

treatment with tioconazole, cells showed a substantial increase in fluorescence. On the other hand, the level of fluorescence associated with cells treated with tioconazole plus nikkomycin was still low. It should be noted that the results obtained for cells incubated for 24 h were poorly reproducible (high SD values).

Ketoconazole, fluconazole and tioconazole did not show any effect on glucose uptake into intact cells or regenerating spheroplasts (data not shown).

Table 4. *Effect of nikkomycin, tioconazole and the combination of these agents on the association of Calcofluor white with whole cells of C. albicans ATCC 26278*

Cells were incubated with antifungal agents. At the times indicated, samples of cell suspensions were withdrawn and cells were stained with Calcofluor white for 5 min. After harvesting and extensive washing with PBS, cells were suspended in PBS and the level of fluorescence associated with the cells was measured. Fluorescence is expressed as arbitrary units per mg dry weight of cells. Values are the means of three independent determinations $\pm$ SD.

Antifungal agent ($\mu\text{g ml}^{-1}$)	Fluorescence level ($\pm$ SD) after:			
	1 h	3 h	5 h	24 h
None	3240 $\pm$ 260	3450 $\pm$ 85	3350 $\pm$ 140	2750 $\pm$ 230
Nikkomycin				
10	2600 $\pm$ 155	2085 $\pm$ 215	1740 $\pm$ 135	1245 $\pm$ 120
0.1	2940 $\pm$ 190	3390 $\pm$ 375	3455 $\pm$ 265	2945 $\pm$ 310
Tioconazole				
10	2365 $\pm$ 120	1820 $\pm$ 130	1950 $\pm$ 125	4295 $\pm$ 1240
1	2585 $\pm$ 220	1905 $\pm$ 175	2245 $\pm$ 80	3850 $\pm$ 920
0.1	2640 $\pm$ 105	2350 $\pm$ 180	2830 $\pm$ 110	3445 $\pm$ 740
0.01	2880 $\pm$ 270	2785 $\pm$ 240	2950 $\pm$ 225	2785 $\pm$ 435
Nikkomycin (0.1) + tioconazole (0.1)	2225 $\pm$ 195	1870 $\pm$ 190	1740 $\pm$ 270	1950 $\pm$ 520

Discussion

The phenomenon of synergism often enables reduction of effective doses of chemotherapeutic agents. This possibility is especially noteworthy in chemotherapy of systemic fungal infections since some of the antifungal agents used in clinical practice are relatively toxic and show many adverse effects (Walsh & Pizzo, 1988).

In this paper we report that synthetic azole derivatives in combination with the antibiotic nikkomycin are stronger inhibitors of *C. albicans* growth than each of the agents alone. Although this phenomenon may be of some importance for possible chemotherapeutic applications, we were especially interested in explaining the biochemical basis of the observed synergism.

There are three generally accepted basic mechanisms of synergistic activity (Neuman, 1980; Klastersky, 1984): increase by one agent of the permeability of the cell wall and membranes to the second agent; inhibition of enzymes able to degrade the second agent; and double blocking by the two components of successive steps in the metabolic sequence. The second mechanism seems to be unlikely in the present study, because none of the antifungal agents studied here inhibits any degrading enzyme. The potentiation of nikkomycin uptake by azoles was surprisingly low. This result was unexpected, because nikkomycin is transported into fungal cells by a peptide transport system (Yadan *et al.*, 1984). Since peptide permeases are localized in the cytoplasmic membrane, one might expect substantial alterations to

their activity as a secondary effect of the action of antifungal azoles. However, our results indicate that even long-term treatment of *C. albicans* cells with ergosterol biosynthesis inhibitors alters the activity and substrate specificity of peptide permeases only slightly.

Surarit & Shepherd (1987) reported that azole antifungals were able to inhibit chitin synthase in a plasma membrane fraction, but detailed data were not given. Since azole antifungals are Δ^{14} -lanosterol demethylase inhibitors, it seems likely that changes in membrane fluidity connected with the replacement of ergosterol with lanosterol may affect chitin synthase activity. This mechanism was suggested by other authors as an explanation for the effect of azoles on plasma membrane enzymes, especially those involved in the biosynthesis of the fungal cell wall (Odds *et al.*, 1985). However, in the present study, whereas the incorporation of ^{14}C label into chitin of intact cells was strongly inhibited by azoles, the respective incorporation into alkali-insoluble glucan and mannoprotein was only slightly affected. The azole-induced inhibition of chitin biosynthesis was confirmed by the fluorimetric determination of chitin content and studies on incorporation of ^{14}C label into cell wall of regenerating spheroplasts, which were previously reported to synthesize much more chitin than intact cells (Elorza *et al.*, 1983). The complete inhibition of incorporation into chitin and glucan of regenerating spheroplasts, observed for a high concentration of tioconazole (80 $\mu\text{g ml}^{-1}$), should have been connected with a known phenomenon of direct membrane damage

(Ansehn & Nilsson, 1984). The lack of the cell wall in spheroplasts makes the cell membrane easily accessible for azole derivatives and enables direct interactions. All the above results seem to be inconsistent with previous reports given by many authors who observed irregular distribution of chitin and unbalanced (possibly enhanced) biosynthesis of this polysaccharide as a result of the action of antifungal azoles. The phenomenon was demonstrated by fluorescence microscopy (Kerkenaar & Barug, 1984; Marichal *et al.*, 1984; Vanden Bossche, 1985) and by the determination of the chitin:total carbohydrate ratio (Vanden Bossche, 1985). It should be noted, however, that all these previous observations were made after 24 h treatment of fungal cells with azole derivatives. In the present study we also noted enhanced chitin content after such a long treatment. Nevertheless, other evidence presented in this work indicates the selective inhibition of chitin biosynthesis as an initial result of the action of antifungal azoles on *C. albicans* cells. This effect was also observed even when cell growth was not affected. Nicholas & Kerridge (1989) reported that azole antifungals could inhibit ergosterol biosynthesis without any growth inhibition. In this respect, although a direct interaction of azole antifungals with chitin synthase cannot be excluded, it is more likely that azole antifungals at subinhibitory concentrations prevent the formation of chitosomes (small vesicles delivering molecules of chitin synthase to the forming septum: Bartnicki-Garcia, 1981). The chitosomal membrane contains more ergosterol than the cytoplasmic membrane (Hernandez *et al.*, 1981), thus the lack of this sterol should affect chitosome formation. The postulated mechanism of the combined action of azole antifungals with nikkomycin might be considered as a sequential blockade of chitin biosynthesis. However, in cells surviving long-term treatment with low doses of azole antifungals the biosynthesis of chitin synthase molecules may be unaffected. In this case, irregular, uncoordinated chitin deposition would be an obvious consequence. This phenomenon may be also disadvantageous for candidal cells, as was shown previously in studies on the combined action of nikkomycin with papulacandin B, the inhibitor of $\beta(1\rightarrow3)$ glucan synthase. In that case the synergistic effect was observed although papulacandin B alone caused enhanced chitin deposition (Hector & Braun, 1986).

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