Mechanism of Action of Nikkomycin and the Peptide Transport System of *Candida albicans*

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Nikkomycin was found to be a potent growth inhibitor of *Candida albicans* through competitive inhibition of chitin synthase [$K_i = 0.16 \mu\text{M}$ ($0.1 \mu\text{g ml}^{-1}$)]. The activity of the peptide-nucleoside drug was antagonized by both peptone and defined peptides. Transported dipeptides were effective antagonists while transported oligopeptides were not. A mutant of *C. albicans* resistant to the effects of nikkomycin through a transport defect was unable to transport dipeptides, while oligopeptide uptake was apparently unaffected. At least two peptide permeases are operational in this organism.

INTRODUCTION

The nikkomycins are peptide-nucleosides isolated from culture supernatants of *Streptomyces tendae* Tu 901 (Dahn *et al.*, 1976). In both structure and activity they are closely related to the polyoxins, being inhibitors of the cell wall synthetic enzyme chitin synthase. As peptide derivatives, the nikkomycins are of potential use in the study of peptide permeases: peptide drugs such as L-ethionyl-L-alanine (Marder *et al.*, 1978) and bacilysin (Nisbet & Payne, 1979a) have been used to show the presence of a single peptide transport system in *Saccharomyces cerevisiae*. Despite studies by several groups of workers, the number of peptide permeases in the pathogenic fungus *Candida albicans* has not been fully elucidated. Logan *et al.* (1979) suggested that multiple permeases may be present; however, Davies (1980) produced evidence for a single system. Recently, further evidence for the presence of multiple permeases has been derived from kinetic analyses (Sarhou *et al.*, 1983). In this communication we report results concerning the uptake of nikkomycin by *C. albicans* and demonstrate its value for the study of peptide permeases.

METHODS

Organism. *Candida albicans* 124, obtained from the Sandoz Research Institute, Vienna, was used in all experiments. The organism was maintained on 2% (w/v) malt extract plates at 37 °C and subcultured weekly.

Growth conditions. Unless otherwise stated, two liquid media were used for the cultivation of *C. albicans*: (a) yeast nitrogen base containing 0.5% (w/v) ammonium sulphate (YNB, Difco) supplemented with 1% (w/v) glucose, and (b) YNB without ammonium sulphate or amino acids (Difco) supplemented with 2% (w/v) bacto-peptone (Oxoid) and 2% (w/v) glucose (YNB/peptone). For batch growth, cells were taken from plates and inoculated into liquid medium to give 10^5 cells ml^{-1} . Growth, at 30 °C and 120 r.p.m., was followed using an EEL colorimeter with green filter.

Measurement of peptide transport. Cells were grown to mid-exponential phase in YNB/peptone, harvested by centrifugation at 1500 g and washed three times with PCG (10 mM- Na_2HPO_4 -citric acid pH 5.0, supplemented with 0.8% glucose). The pellet was finally resuspended in a small volume of PCG, stored at 4 °C and used within 2 h.

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(a) Fluorescamine method (Nisbet & Payne 1979b). *C. albicans* blastospores were diluted and incubated at 37 °C for 10 min. Peptide stocks, prepared in PCG and preincubated at 37 °C, were added to the cells to give a working volume of 10 ml and a cell density equivalent to 1.0 mg dry wt ml⁻¹. Incubation was at 37 °C with shaking (80 strokes min⁻¹). Samples (0.5 ml) were removed at timed intervals and filtered through Whatman GF/A filters using a 10-place filtration unit (Hoefer Scientific Instruments, California, USA). The filtrate was collected and its peptide concentration assayed as follows. Samples of peptide solution (50 µl) containing up to 250 nmol peptide were diluted with 2.5 ml 0.1 M-sodium tetraborate (adjusted to pH 6.2 with HCl). Fluorescamine solution (0.15 mg ml⁻¹ in acetone; 0.5 ml) was added with vortexing. The fluorescence of the solution was measured within 30 min using a Perkin Elmer MPF-3 spectrofluorimeter (excitation 390 nm; emission 480 nm). Calibration curves were determined for each peptide.

(b) Uptake of [U-¹⁴C]alanyl-alanine. The method used was essentially the same as that described in (a) above. Radiolabelled peptide stocks (37 µCi mmol⁻¹; 1.37 MBq mmol⁻¹) and unlabelled competitors were prepared in PCG and preincubated at 37 °C. These were added to a cell suspension, also preincubated at 37 °C, to give a working volume of 10 ml and a cell density equivalent to 1 mg dry wt ml⁻¹. Incubation was at 37 °C with shaking (80 strokes min⁻¹). Samples of the cell suspension (1 ml) containing the radiolabelled peptide were filtered through Whatman GF/A filters which were then washed with 10 ml PCG at 25 °C. The filters were dried under infrared illumination, placed in small scintillation vials with 3 ml PCS scintillation cocktail (Amersham) and counted with a Packard Tricarb 3375 Scintillation Counter.

Drug activity assays (a) Minimum inhibitory concentration. A 15 h YNB culture was used as inoculum for serial dilutions of nikkomycin in YNB giving a final density of 10³ cells ml⁻¹. After 48 h at 37 °C total cell numbers were determined by light microscopy and viable cell numbers by growth of a diluted cell suspension on YNB plates.

(b) Disc diffusion assay. A 15 h culture was diluted to 10⁶ cells ml⁻¹ and 500 µl of this suspension was spread over the surface of a 20 ml malt extract plate (2%, w/v, agar) which was then incubated at 37 °C for 30 min. The required drug was absorbed onto a disc (6 mm diameter) of chromatography paper (Whatman 17 Chroma) as a solution in either water or dimethylformamide. The discs were dried and then placed onto the prepared plates in conjunction with solvent controls, to allow residual effects of the solvents to be observed. Diffusion of the drug occurred and, after incubation at 37 °C for 48 h, the diameter of the zone of growth inhibition was determined in comparison with solvent control plates.

Preparation of chitin synthase. The method used was based upon that of Ryder & Peberdy (1977). A 15 h culture grown on brain heart infusion (Difco) at 37 °C was harvested by centrifugation at 1500 g and washed twice with 0.1 M-sodium phosphate buffer pH 6.5 containing 10 mM-MgCl₂ and 1 mM-EDTA (PME buffer). Cells were resuspended in PME (1 ml packed cell volume per 0.5 ml buffer) and were broken with an equal volume of 0.45 mm glass beads in a Braun homogenizer (Melsungen AG, West Germany) with liquid CO₂ cooling. The beads were removed by filtration and the whole cells and cell walls sedimented by centrifugation at 1500 g. The supernatant was centrifuged at 125000 g for 40 min and the pellet resuspended in 3 ml PME. The membrane fraction was incubated with trypsin (bovine pancreas; 50 µg ml⁻¹) at 25 °C for 10 min and a twofold excess of soybean trypsin inhibitor was then added to terminate proteolysis. This suspension was used as a crude chitin synthase preparation.

Chitin synthase assay. The assay medium contained the required concentration of UDP-*N*-acetyl-D-glucosamine; UDP-*N*-acetyl-D-[U-¹⁴C]glucosamine (1 µCi ml⁻¹; 37 kBq ml⁻¹); 400 mM-*N*-acetyl-D-glucosamine; PME; competitors in PME; and the membrane suspension in a total volume of 100 µl. Chitin synthesis was allowed to proceed at 25 °C and was terminated by the addition of 400 µl 10% (w/v) trichloroacetic acid. The resulting pellet was washed by two cycles of centrifugation and sonication with 1% (v/v) acetic acid and finally resuspended in 1% acetic acid. Incorporated radiolabel was assayed using Instagel scintillant (Packard) and a Searle Mark III 6880 scintillation counter. Radioactivity was related to *N*-acetyl-D-[U-¹⁴C]glucosamine incorporated into chitin. The protein concentrations of the membrane fraction were determined by the Lowry method using bovine serum albumin as standard.

Production of mutants. Cells were grown for 12 h on YNB, harvested by centrifugation (1500 g), washed and resuspended in 65 mM-KH₂PO₄ (0.5 ml packed cell volume in 10 ml KH₂PO₄). A portion (700 µl) of this suspension was distributed on a 20 ml YNB plate and incubated at 37 °C for 24 h. This process produced singlet blastospores of *C. albicans* which were diluted to 10⁶ ml⁻¹ in YNB. This suspension (15 ml) was irradiated for 50 s with a bactericidal UV lamp (Englehard-Hanovia, Cinderford, UK) from a distance of 30 cm producing a 0.5% survival rate. Drug resistant mutants were selected by plating irradiated cells onto 2% (w/v) malt extract plates containing 50 µg nikkomycin ml⁻¹. After incubation at 37 °C any viable colonies were picked out and again plated onto malt extract agar containing nikkomycin.

Chemicals. Nikkomycin (80% nikkomycin containing X and Z as major components) was a gift from Bayer AG; bacilysin was a generous gift of Professor C. P. Abraham, University of Oxford; UDP-*N*-acetyl-D-[U-¹⁴C]glucosamine was purchased from Amersham; [U-¹⁴C]alanyl-alanine (37 µCi mmol⁻¹) was prepared by P. J. McCarthy at the Sandoz Research Institute, Vienna. *m*-F-Phe-Ala-Ala was synthesized by Dr W. D. Kingsbury of Smith Kline and French Laboratories. Fluorescamine (Fluram) was from Roche; remaining chemicals were from Sigma. All amino acid residues were in the L-configuration unless otherwise stated.

Fig. 1. Effect of nikkomycin on *C. albicans* 124 (wild-type) chitin synthase. Synthesis of chitin from UDP-*N*-acetyl-D-[U-¹⁴C]glucosamine was measured in the presence of various concentrations of nikkomycin and at two concentrations of unlabelled UDP-*N*-acetyl-glucosamine (□, 0.3 mM; ○, 0.6 mM). Initial rates of incorporation of radiolabel into chitin were determined in triplicate and plotted according to the method of Dixon (1953).

Table 1. Rate of uptake of specific peptides into wild-type *C. albicans* and the *NIK5* mutant and the potency of these peptides as antagonists of nikkomycin activity against wild-type *C. albicans*

Peptide	Antagonism of nikkomycin action*	Rate of uptake†	
		Wild-type	NIK5
Ala ₂	+	38.0	7.4
Gly-Tyr	—	0	0
D-Leu-Gly	—	0	0
L-Leu-Gly	+	32.0	0
Gly ₂	—	0	0
Ala ₃	—	30.3	41.6
Ala ₄	—	52.6	90.9

* At concentrations of 10 mM-peptide and 50 μg nikkomycin ml⁻¹.

† As determined by the fluorescamine method from an initial peptide concentration of 0.2 mM. Results are expressed as nmol min⁻¹ (mg dry wt cells)⁻¹.

RESULTS

Inhibition of chitin synthase. Nikkomycin was a potent inhibitor of growth of *Candida albicans*. Partial inhibition of growth was observed at 3 μg ml⁻¹ with the MIC occurring at 25 μg ml⁻¹. It was also found to be a competitive inhibitor of the membrane fraction chitin synthase (Fig. 1) with a *K_i* of 0.16 μM (based on the average molecular weights of the X and Z components of the nikkomycin sample).

Effect of growth medium upon nikkomycin activity. The activity of nikkomycin against intact cells was highly medium dependent. In contrast to the MIC of 25 μg ml⁻¹ in YNB, the inclusion of 2% (w/v) Bacto-peptone in the medium abolished the activity of the drug even at 50 μg ml⁻¹ with neither a reduction in the growth rate nor an alteration in morphology. Microscopic examination revealed that the small increase in turbidity observed during incubation of cells in YNB containing nikkomycin was due to the clumping of cells associated with cell enlargement and lysis.

The inclusion of certain defined peptides (10 mM) in YNB was also found to antagonize the action of nikkomycin (Table 1). This was not the case for all peptides assayed. Ala₂ and L-Leu-Gly were antagonistic whereas D-Leu-Gly, Gly₂, Gly-Tyr, Ala₃ and Ala₄ showed no such effect.

Rates of peptide uptake. There was a correlation between transported dipeptides (Ala₂ and L-Leu-Gly) and those peptides able to compete with nikkomycin activity (Table 1). A likely explanation of this phenomenon is that nikkomycin was being transported through a system which also transported peptides. Confirmation that competition was at the level of transport

Fig. 2. Effect of nikkomycin on $[U-^{14}C]$ Ala-Ala incorporation by *C. albicans* 124 (wild-type). Cells were grown in YNB/peptone, harvested, washed and resuspended in PCG. The initial rates of incorporation of $[U-^{14}C]$ Ala-Ala were determined in the presence of various concentrations of nikkomycin. Cells were diluted to $1.0 \text{ mg dry wt ml}^{-1}$, and $[U-^{14}C]$ Ala-Ala added to a concentration of either 0.1 mM (○) or 0.2 mM (□). The suspension was incubated at 37°C with shaking. Samples (1 ml) were removed at timed intervals and the radioactivity incorporated into cells was determined. Initial rates of incorporation were calculated in triplicate. Mean values are plotted according to the method of Dixon (1953).

Table 2. *Effects of various inhibitors on growth of wild-type C. albicans and the NIK5 mutant*

Zones of inhibition were determined by the disc diffusion assay described in the methods section. The quantity of inhibitor applied to the disc is indicated in parentheses.

Drug (μg)	Diameter of zone of inhibition (mm)	
	Wild-type	NIK5
Acriflavin (500)	15.0	15.0
Ethidium bromide (500)	25.0	25.0
5-Fluorocytosine (500)	37.0	37.0
Actinomycin D (500)	10.5	10.5
Cycloheximide (500)	0.0	0.0
<i>m</i> -F-Phenylalanine (500)	0.0	0.0
Nikkomycin (50)	30.0	0.0
Polyoxin (crude) (500)	8.5	0.0
Bacilysin (100)	27.0	0.0
5-Fluorouracil (50)	10.5	10.5
Miconazole (50)	13.5	13.5
Nystatin (20)	24.0	24.0
<i>m</i> -F-Phe-Ala-Ala (30)	20.0	11.0

rather than at the target enzyme, chitin synthase, came from the observation that nikkomycin was a competitive inhibitor of $[U-^{14}C]$ alanyl-alanine uptake by cells ($K_i = 4.85 \mu\text{g ml}^{-1}$; Fig. 2). The oligopeptides Ala₃ and Ala₄ did not show antagonism of nikkomycin activity even though they were transported into *C. albicans* (Table 1).

Resistance to nikkomycin. UV irradiation of *C. albicans* 124 resulted in the production of six mutants resistant to the effects of nikkomycin in YNB. Each mutant was assayed for its ability to transport Ala₂ since this peptide had been shown to utilize the same peptide permease as nikkomycin. One mutant, designated NIK5, was found to have a defect in peptide transport with no apparent alteration of its chitin synthase which remained competitively inhibited by nikkomycin with a K_i of $0.17 \mu\text{M}$. This value was comparable with that of the enzyme prepared from the wild-type.

The susceptibility of *C. albicans* 124 NIK5 to most antifungal agents was found to be equivalent with that of the wild-type (Table 2). However, cross resistance was observed with

other peptide antibiotics. NIK5 was not susceptible to either the dipeptide antibiotic bacilysin or a crude sample of polyoxin containing 10% active material. Partial cross resistance was observed with the toxic tripeptide *m*-F-Phe-Ala-Ala although the susceptibility of *C. albicans* 124 to this peptide was also low.

Peptide uptake by C. albicans 124 NIK5. The mutant was assayed for its ability to transport the peptides previously used (Table 1). Although its capacity to transport Ala₂ and L-Leu-Gly was greatly reduced or abolished, the rate of transport of Ala₃ and Ala₄ was increased when compared to those rates obtained with the wild-type.

DISCUSSION

In common with its mode of action against other fungi such as *Mucor rouxii* (Dahn *et al.*, 1976) nikkomycin prevented growth of *C. albicans* through inhibition of the enzyme chitin synthase. However, its activity was antagonized by either bacto-peptone or certain defined peptides, an effect that has also been observed with polyoxin A activity against *Alternaria kikuchiana* (Hori *et al.*, 1977). Two explanations of peptide antagonism appeared likely: firstly, competition at the target enzyme, chitin synthase, and secondly an interaction at the level of transport into the cell. Competition at the level of transport would not be observed if the peptide/drug interaction was occurring solely at the target site. Studies of interactions between polyoxin and a chitin synthase preparation from *A. kikuchiana* (Hori *et al.*, 1977) have shown no competition with peptides. As a peptide-specific permease had previously been demonstrated in *C. albicans* (Logan *et al.*, 1979; Davies, 1980) it was possible that nikkomycin was entering the cells via such a system. The competitive inhibition of [U-¹⁴C]Ala-Ala incorporation exerted by nikkomycin demonstrates that transport of peptides and peptide-nucleosides occurs through a common permease in this organism.

Studies were undertaken to compare the ability of peptides to antagonize nikkomycin by examining their relative uptake rates. As would be expected, non-transported peptides were unable to inhibit nikkomycin activity while the transported dipeptides Ala₂ and L-Leu-Gly were effective competitors. Stereospecificity was evident in comparing the antagonistic activity of D-Leu-Gly and L-Leu-Gly. This again indicates a specific site of competition rather than a non-specific effect and agrees with previous studies which indicated that dipeptide uptake is highly stereospecific (Davies, 1980). Interestingly the oligopeptides Ala₃ and Ala₄ were transported at a significant rate and yet did not antagonize nikkomycin activity. Sarthou *et al.* (1983) have reported preliminary kinetic evidence which suggests the presence of multiple peptide permeases in this organism. It is therefore possible that Ala₃ and Ala₄ were transported via a system separate from that through which nikkomycin entered.

Resistance to nikkomycin may arise through a number of mechanisms including alterations in the target enzyme or the peptide permease. Only a single report of an altered chitin synthase leading to drug resistance exists involving polyoxin D resistance in *Schizophyllum commune* (MacGruder, 1979). There have been no previous reports of mutations leading to alteration of the *C. albicans* peptide permease. However, such instances in *S. cerevisiae* lead to a total loss of peptide transport capacity (Marder *et al.*, 1978; Nisbet & Payne, 1979a). It was felt that a mutation in the peptide permease leading to nikkomycin resistance would produce further information concerning the nature of this system. However, of the six drug-resistant mutants produced only one (NIK5) showed such a defect. The nature of the mutation in the remaining variants is being elucidated, however, it is possible that these have alterations in chitin synthase or in the hydrolytic system which could produce nikkomycin resistance.

Initial studies of peptide uptake in NIK5 showed only a defect in dipeptide transport while the rate of oligopeptide uptake was increased. Such an effect appears to be due to an overall increase in the activity of a second (oligopeptide) permease in response to the peptone-containing growth medium employed in transport studies. The loss of dipeptide transport was confirmed by the cross resistance observed between nikkomycin and both bacilysin and a crude sample of polyoxin. That tripeptide uptake was, to some extent, separate from dipeptide uptake was confirmed by the susceptibility of NIK5 to the toxic tripeptide *m*-F-Phe-Ala-Ala. MIC

values for the wild-type and variant could not be determined due to the apparently low susceptibility of this strain when compared with others (Kingsbury *et al.*, 1983).

The present study indicates that nikkomycin is able to enter the cell via a system with high affinity for dipeptides. The nikkomycin used consisted of dipeptide nikkomycins X and Z and is therefore consistent with the argument that dipeptides share a common transport system. Inhibition of growth arises from competition at the target enzyme, chitin synthase, as nikkomycin is an analogue of the natural substrates. The fact that nikkomycin resistance has apparently arisen through a defect in peptide transport indicates the presence of at least two peptide permeases in this organism, system I having high affinity for dipeptides including the dipeptide nikkomycins, and system II having high affinity for oligopeptides. Studies are currently in progress to define further the substrate specificities of the two systems.

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