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New nystatin polymeric complexes and their *in vitro* antifungal evaluation in a model study with *Fusarium oxysporum*

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Abstract

Six water-soluble nystatin-polyvilnylpyrrolidone complexes with respective MW of 10 kDa (NC1), 25 kDa (NC2), 30 kDa (NC3), 40 kda (NC4), 90 kDa (NC5), 360 kDa (NC6) were synthesized. The activity of the complexes was compared with that of nystatin against growth and spore germination of *Fusarium oxysporum* f.sp. *radicis-cucumerinum*. The ED_{50} value (effective dose) of free nystatin in aqueous solution on growth inhibition on solid medium was determined at 35.7 ppm. The ED_{50} of the complexes NC3, NC4, NC5, and NC6 ranged from 2.2 to 4 times lower than that of nystatin. The NC6 complex exhibited the highest activity, followed by NC5, NC4, and NC3. The activities of NC1 and NC2 were about 3 and 1.7 times higher than nystatin respectively in the same *in vitro* model. The complexes NC6, NC1 and NC4 were 25.4, 13.6 and 6.9 times more active respectively than nystatin against spore germination of *F. oxysporum*. The activity of the nystatin complexes was dependent on the molecular weight of the polymeric carrier.

Key words: nystatin, polyvinylpyrrolidone, controlled release formulations, *Fusarium oxysporum*

Introduction

Nystatin ($C_{47}H_{75}NO_{17}$) is a polyene antibiotic, derived from *Streptomyces noursei* and *Streptomyces aureus*. Nystatin is well known to be active against a broad spectrum of fungi in vitro and *in vivo* [1] including *Candida* spp., *Aspergillus* spp. and *Cryptococcus* spp., [1–7]. Nystatin has exhibited antifungal activity in humans following administration by different routes [8–10]. It is also used in veterinary and agricultural practices [4–6, 11–14]. However, the selective toxicity of nystatin on mammalian cells and natural membranes by different mechanisms is well known [15–18]. Nystatin administration thus is limited, especially by its dose-dependent nephrotoxicity [19, 20].

The last few decades have witnessed concerted efforts to enhance the effectiveness of drugs used in therapeutic and preventive medicine by improving their selectivity and solubility and lowering their toxicity and biodegradation. Bioactive substances in controlled release forms are used for elimination of these disadvantages. Diffusion systems, liposomes, systems with hydrolysable bonds between bioactive ligand and polymer carrier are such formulations. The incorporation of nystatin into liposomes has allowed the preparation of new parenteral nystatin formulations with improved *in vitro* distribution in human plasma [21]. They can be safely administrated to rabbits at doses much higher than free nystatin [22]. Promising new formulations of nystatin, amphotericin B and the azoles are still under development [23].

The aim of the present study was to investigate the *in vitro* activity of new polymeric complexes of nystatin against growth inhibiton and spore germination of *F. oxysporum* f.sp. *radicis-cucumerinum*.

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Materials and methods

Reagents and chemicals

Methanol was a trade product of analytical grade. Nystatin and polyvinylpyrrolidone (PVP) with Mw = 10 kDa, 40 kDa and 360 kDa were provided by the Sigma Chemical Co. Polyvinylpyrrolidone with Mw = 25 kDa, 30 kDa and 90 kDa, dextrose and agar were provided by Merck. Nystatin dihydrate (MW = 962.10), a gift from Bioprepare (Papanicolaou, N. Pchyhiko, Athens, Greece), was from Sigma-Aldrich.

Synthesis of nystatin-PVP complexes

Complexes were prepared in methanol as follows. Two and a half mg of nystatin and 1000 mg of PVP with Mw = 10 kDa, 25 kDa, 30 kDa, 40 kDa or 500 mg of PVP with Mw = 90 kDa and 360 kDa were added to 50 ml of methanol. Temperature was adjusted to 45 °C for 1 h and to 70 °C for 30 min and the mixture was continually agitated in a magnetic stirrer. The methanol was removed at low temperature (<20 °C) in a vacuum and the residue was redissolved in 10 ml of distilled water. The prepared nystatin complexes contained nystatin attached to the polymer via a hydrogen bond.

Fungus

Isolate AFu-68 of *F. oxysporum* f.sp. *radicis-cucumerinum* was used in this study as a model for biological materials. It is a cucumber pathogen [24].

Stock solutions

Nystatin was suspended in methanol (C = 40 mg/ml) and diluted subsequently with distilled water. For growth inhibition essays, nystatin was suspended directly in the culture medium. All solutions were stored in the dark at −20 °C.

Culture conditions

Fusarium oxysporum f.sp. *radicis-cucumerinum* was grown on autoclaved potato dextrose agar (PDA) for 72 h at 25 °C, unless otherwise stated. The medium was prepared by adding 200 g boiled potato extract to 600 ml of distilled water, that contained 20 g of dextrose and 18 g of agar at a final volume of 1 L. The pH of the growth medium was adjusted to pH = 6.4 by adding phosphate buffer 1 M Na_2HPO_4/KH_2PO_4.

Growth inhibition essay

For growth inhibition measurements on a solid medium, the fungal inhibitors were added to PDA in various quantities. The final concentrations in PDA were 0, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0, 30.0 ppm for nystatin and 0, 2.5, 5.0, 10.0, 15.0, 20.0 ppm for each nystatin complex respectively. The substrate was transferred into 9 cm in diameter sterile petri-dishes and a 5 mm mycelium plug was placed at the center of each dish. The dishes were incubated in the dark at 27 °C for 6 days. Readings were taken daily by measuring the diameter of each colony. Growth inhibition for each concentration was calculated from the diameter measurements as a mean of five replicate dishes compared with the control.

Measurement of inhibition of Fusarium oxysporum spore germination

The effect of the complexes NC1, NC4 and NC6 on *F. oxysporum* f.sp. *radicis-cucumerinum*'s spore germination was also assessed. The fungus was grown in autoclaved potato dextrose broth (PDB), which was prepared as described previously but without agar. PDB was dispersed into several 100 ml Erlenmeyer flasks, (50 ml PDB/flask). Five mm mycelium plugs of the test organism were added to the flasks that were incubated on a rotary shaker for 3 days at 19 °C in the dark. Conidia were removed from the PDB, filtered through a double layer of cheesecloth and the mycelial mat was washed with sterile distilled water. The suspension was then centrifuged at 3000 gfor 10 min. Spores were resuspended in sterile distilled water and the concentration was adjusted to 0.5×10^7 pores/ml. An aliquot of 0.1 ml of the above spore suspension, 0.1 ml of 0.1% w/v glucose solution, 0.1 ml of 1 M Tris-buffer, pH = 7.0, 0.2 ml of sterile distilled water and 0.5 ml solution of each one of the complexes were transferred to 1 ml Eppendorf tubes. The final concentrations in the tubes were 0, 0.25, 0.5, 1, 2, 5, 10 ppm for nystatin and 0, 0.125, 0.5, 1, 2, 5 ppm for each nystatin complex respectively. Potato dextrose broth (PDB) without growth inhibitors but with a suitable amount of methanol was used as a control.

The tubes containing the mixtures were incubated for 10 h at 28 °C. The numbers of germinated conidia were counted with a hematocytometer (Newbauer plate).

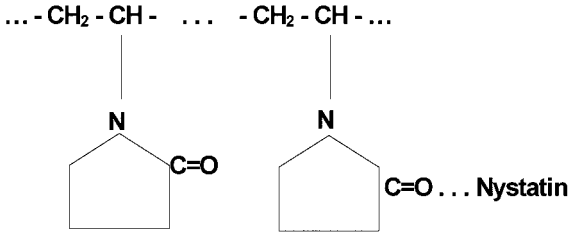

Figure 1. The suggested structure of a hydrogen bonding complex of nystatin with PVP.

Statistical analysis

The statistical analysis of the results for both, inhibition of growth and spore germination, was performed by one way ANOVA (Excel).

Results

Nystatin-polyvinylpyrrolidone (PVP) complexes

Figure 1 shows the expected molecular structure of a basic unit of a nystatin-PVP complexe molecule. Polyvinylpyrrolidone is a polymeric molecule from one repeated unit; in the newly synthesized nystatin-PVP complexes, nystatin is attached by a hydrogen bond at a specific position of the repeated polymeric unit as shown in Figure 1.

Six new polymeric formulations of nystatin with PVP with molecular weights of 10 kDa (NC1), 25 kDa (NC2), 30 kDa (NC3), 40 kDa (NC4), 90 kDa (NC5) and 360 kDa (NC6) were prepared. The nystatin content of complexes 1–4 with molecular weights of 10 kDa, 25 kDa, 30 kDa and 40 kDa respectively was 0.25% mass. For complexes 5 and 6 with molecular weights of 90 kDa and 360 kDa respectively, the nystatin content was 0.50% mass.

Growth inhibition of Fusarium oxysporum

Table 1, shows the ED_{50} values of the nystatin complexes against the growth of he test fungus. The ED_{50}value of micromolecular nystatin in aqueous solution was determined at 35.7 ppm. The complexes NC6, NC5, NC4, NC3 showed activities about 4 to 2.2 times lower than nystatin. Activity was decreased by increasing the molecular weight. The complexes NC1 and NC2 exhibited ED_{50}. 3 and 1.7 times lower respectively than NC6.

Table 1. Effective doses (ED_{50} values, in ppm) for nystatin and its complexes for growth inhibition of *Fusarium oxysporum* f.sp. *radicis-cucumerinum*

Antifungal	ED_{50} (ppm)	SD*	CV(%)**	P***
Nystatin	35.7	±5.214	14.605	0.000131
Complex 1	11.6	±2.422	17.877	
Complex 2	20.8	±4.154	19.947	
Complex 3	16.3	±1.962	12.057	
Complex 4	13.7	±2.585	18.812	
Complex 5	11.7	±0.151	1.288	
Complex 6	9.1	±2.437	18.655	

*SD = Standard Deviation.
**CV = Coefficient of variation.
***P=probability.

Table 2. Effective doses (ED_{50} values, in ppm) for nystatin and its complexes NC1, NC4 and NC6 with molecular weights of 10 kDa, 40 kDa and 360 kDa respectively against spore germination of *Fusarium oxysporum* f.sp. *radicis-cucumerinum*

Antifungal	ED_{50} (ppm)	SD*	CV(%)**	P***
NC6	0.1239	±0.0160	12.89	0.0362
NC4	0.4553	±0.0364	15.734	
NC1	0.2314	±0.0851	18.694	
Nystatin	3.1427	±0.1390	4.423	

*SD = Standard Deviation.
**CV = Coefficient of variation.
***P = probability.

Inhibition of spore germination

Table 2 shows the ED_{50} for inhibition of spore germination. The effect of methanol used for preparing the nystatin stock solution on spore germination was evaluated and was subtracted from the total ED_{50} values of nystatin. The effect of the organic solvent was measured by the ED_{50} value of nystatin against spore germination. It was 3.1427 ppm (Table 2). The most effective complex on the germination of *F. oxysporum* sp. was found to be NC6 (ED_{50} = 0.1239 ppm) followed by NC1 and NC4 (Table 2).

Statistical parameters

The ED_{50} values obtained by all inhibitors were significantly different ($P < 0.05$), while the % values of the coefficient of variation (CV%) were small (<20%), thus confirming the reproducibility of the experiments (Tables 1 and 2).

Discussion

Nystatin is classified among the most efficient antifungal agents, widely used since the 1950s but is insoluble in water and it is very toxic. New forms or new ways of transporting nystatin to mycelial cells and areas of infection are currently under development with respect to decreasing cytotoxicity and increasing efficacy. Experiments with new formulations, such as liposomal nystatin *in vitro* and *in vivo*, are encouraging and represent potential antifungal candidates [5, 22].

In a recent study, Johnson *et al.* [25] compared the *in vitro* activity of four liposomal formulations of amphotericin B with those of free nystatin and a liposomal formulation of nystatin against 200 isolates of *Aspergillus* spp., *Candida* spp. and *Cryprococcus neoformans* by a broth microdilution method. She reported that liposomal nystatin is more active than free nystatin against most species but it is less active than DMPC-DMPG amphotericin (Abelcet (ABLC) or deoxycholate amphotericin B (Fungizone). However, the development of new carrier systems for the aqueous solubilisation and transporation of antifungal agents represents still one of the challenges.

In our *in vitro* model, the activity of nystatin in aqueous solution was relatively low compared to the new water-soluble nystatin complexes. The most effective against the growth of *F. oxysporum* was complex 6 with a molecular weight of 360 kDa. With the exception of complex 1 with a molecular weight of 10 kDa, the activity of the complexes increased with the increase of molecular weight.

Nystatin complexes have shown better activity than micromolecular nystatin on the germination of *F. oxysporum* spores. In particular, the activity of complex 6 was 25.4 times higher than that of nystatin, followed by NC1 13.6 times and lastly NC4 6.9 times. In both, growth inhibition and spore germination essays, the above-mentioned complexes showed molecular weight dependent activities. This result was consistent with the controlled release characteristics of the complexes.

In conclusion, the polymeric complexes of nystatin, in which the bio-active ligand is ttached to the polymeric carrier by hydrogen bonds, are effective inhibitors of mycelial growth and function much better than micromolecular nystatin against spore germination of *F. oxysporum*.

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