Mechanism of activity and toxicity of Nystatin-Intralipid

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A novel lipid formulation of Nystatin (NYT), Nystatin-Intralipid (NYT-IL), which was found to be more active and less toxic in vitro and in vivo, was developed in our laboratory. The aim of the present study was to explore the possible mechanisms underlying its biological activity. To assess mechanisms affecting fungal cells we conducted the following experiments: killing kinetics, scanning and transmission electron microscopy (EM), measurements of potassium ion leakage and susceptibility in the presence of ergosterol. To study mechanisms affecting mammalian cells, we evaluated the effect of NYT-IL on a kidney cell line, with respect to viability, metabolic activity, potassium leakage and internalization of FITC-labeled human transferrin. NYT-IL exhibited killing kinetics patterns against Candida albicans similar to those of NYT and caused disruption of fungal cells and potassium ion leakage. Susceptibility tests showed that NYT-IL had lower antifungal activity in the presence of ergosterol. Thus, NYT-IL acts apparently by damaging fungal membrane, possibly through interaction with ergosterol, and maybe by additional modes of action. NYT-IL did not cause potassium leakage from mammalian kidney cells at any tested concentration and was not cytotoxic, whereas NYT, at high concentrations, led to K⁺ leakage and was cytotoxic. Furthermore, the high NYT concentration interfered in the internalization process of human transferrin receptor (hTfnR) while NYT-IL did not. In summary, the Intralipid formulation of NYT diminishes the mechanisms responsible for toxicity to mammalian cells but preserves mechanisms of action against fungi, thereby suggesting superiority of NYT-IL as compared to NYT as an antifungal agent.

Keywords Nystatin-Intralipid, activity, toxicity

Introduction

Although much progress has been made in the development of antifungals for the treatment of systemic mycoses [1], there is still a need for enlarging the arsenal of drugs with broad antifungal spectra, low toxicity and reduced cost. We have previously reported [2–7] on the development of a low-cost Intralipid formulation of amphotericin B (AMB-IL) which we found to be stable, standardized, effective in vitro and in vivo and less toxic than conventional AMB. This formulation has thus far not reached the stage of clinical use.

We followed this line of research focusing on another polyene – Nystatin (NYT), a broad spectrum antifungal drug limited to topical use due to poor water solubility and toxicity upon parenteral administration. NYT exhibits activity even against some AMB-resistant strains [8] and thus may be superior to AMB in certain clinical situations. This encourages attempts to develop a formulation of NYT suitable for systemic administration, which may lead to enlargement of the arsenal of antifungal drugs that may be used against invasive mycoses.

Thus, we developed a novel, stable, standardized, Nystatin formulation, i.e., Nystatin-Intralipid (NYT-IL) [9], by combining NYT with the clinically used, non-toxic, food supplement Intralipid™ (IL) – a lipid emulsion administered intravenously [10,11].

The NYT-IL preparation was characterized as to particles’ size, since this property may have an impact on...
toxicity and stability of the emulsion [11]. In addition, the association of nystatin with the emulsion particles was assessed, as high level may be crucial for achieving potential advantages of colloidal carrier formulation [12]. Optimal time and conditions of storage were determined as well [9]. The antifungal activity was assessed in vitro against major pathogenic Candida, Aspergillus and Fusarium species. The data showed that the mean minimal inhibitory concentration (MIC) and mean minimal fungicidal concentration (MFC) of NYT-IL were, in most cases, lower than those of NYT, indicating a better in vitro antifungal activity. In addition, NYT-IL exhibited significantly lower in vitro toxicity than NYT [9]. In vivo experiments showed that NYT-IL can be administered systemically and is effective against experimental systemic murine candidiasis in both naïve and immunocompromised mice [13].

Since NYT-IL differs from the conventional NYT formulation in its antifungal and toxic effects, it seems significant to explore its mode of activity, to determine whether NYT-IL acts in a similar mode as NYT or possibly in a different way.

Hence, we initiated a study to explore possible mechanisms of activity of NYT-IL in comparison to NYT. We report here the data obtained in this study, which provides information on the mechanism(s) of the antifungal activity of the NYT-IL preparation and its toxicity to mammalian cells.

**Materials and methods**

*Candida albicans* culture and NYT-IL preparation

*Candida albicans* CBS 562 [14] was used throughout the study and grown and maintained as described previously [8]. NYT-IL was prepared from NYT and IL as reported in previous studies [9,13].

**Killing kinetics**

Time-kill curve studies were performed based on Klepser et al. [15]. Briefly, a 2.5 × 10^5/ml *C. albicans* suspension prepared from a 24 h culture was exposed to 0.5, 1, 2 and 4 × MIC of NYT-IL or NYT (mean MIC of NYT and NYT-IL = 2.35 and 1.4 mg/l, respectively [9]) for 24 or 48 h. Controls included *C. albicans* in IL or in medium. The treated *C. albicans* were fixed overnight with 2.5% gluteraldehyde, after which, for SEM analysis (Joel 840 SEM), the samples were post-fixed with 1% OsO₄ solution and dehydrated in an alcohol series. For TEM analysis (Joel TEM 1200EX), following the alcohol dehydration, the samples were subjected to propylene-oxide and to glycid ether for polymerization. Thin sections were stained with uranyl-acetate and lead-citrate for examination.

**K⁺ leakage experiments in yeasts**

K⁺ leakage experiments were based on the method described previously [18]. *C. albicans* cells (2.5–5 × 10⁶/ml) in RPMI 1640 medium were exposed to 0.5, 1, 2 or 4 × MIC of NYT-IL or NYT for 5, 15, 30 and 60 min. At each time point, washed *C. albicans* cells were killed and disrupted by incubation in double distilled water (DDW) for 1 h at 70°C. Controls included *C. albicans* cells in medium or in IL. K⁺ concentration was determined in the supernatant of the disrupted *C. albicans* suspension using the atomic absorbance spectrophotometer Perkin Elmer 403 at 383 nm in reference to a calibration curve of KCl.

**In vitro susceptibility testing in presence of ergosterol**

The effect of ergosterol on antifungal activity of NYT-IL was tested based on the method described by Kotler-Brajtburg et al. [19]. In vitro antifungal activity of NYT-IL against *C. albicans* was determined by broth microdilution test [9], based on the Clinical and Laboratory Standards Institute (CLSI) protocol [20]. Ergosterol (Sigma, Germany), at concentrations ranging from 2.5–20 mg/l, was added to the test wells, and MICs and MFCs were determined.

**Cell culture**

Madin-Darby canine kidney (MDCK)-PTR9 epithelial cells [21], which are transfected cells expressing the human transferrin receptor (hTfnR), were used. The cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 1% L-glutamine, 200 μg of streptomycin and 200 IU of penicillin per ml and 10% heat-inactivated fetal calf serum. The cell cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

**Viability and metabolic activity assays**

The effect of NYT-IL on cell viability was assessed by exclusion of Trypan blue dye test [22] and metabolic activity was measured by the XTT assay [16]. The Trypan blue exclusion test [22] was used to determine the number of viable cells present in a cell suspension and was based on
the principle that live cells exclude Trypan blue dye, whereas dead cells do not. Twenty-four hour cultures of MDCK cells were exposed for 24 h to 15–250 mg/l of NYT-IL or NYT. Controls included untreated cells. Thereafter, the cells were stained with 0.2% Trypan blue (Fluka, Switzerland) solution in saline, with stained and unstained cells counted.

Tetrazolium salt, 2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydrazide (XTT), can be reduced into a colored formazan compound by mitochondrial enzymes, which are inactivated shortly after cell death. Twenty-four hour cultures of MDCK cells were exposed for 24 h to 15–250 mg/l of NYT-IL or NYT and non-treated cells were used as a control. Thereafter, the medium was removed, XTT reagent (Biological Industries, Israel) was added and the microplates were incubated until development of a formazan color. The color intensity was measured by an ELISA reader (Spectra MAX 340) at a wavelength of 490 nm.

K⁺ leakage experiments in MDCK-PT9R cells

MDCK cells were treated for 2 h with 1 MIC of NYT-IL or NYT (1.4 and 2.35 mg/l, respectively) or with 125 mg/l of each drug. Non-treated cells were used as controls. After incubation the cells were disrupted by adding DDW followed by 1 h incubation at 70°C and the disrupted cell mass was sedimented by centrifugation. Potassium concentration was determined in the supernatant by inductively coupled plasma mass spectrometry method using a 7500cx ICP-MS device (Agilent technologies, US), according to a KCl calibration curve.

Internalization of FITC-labeled hTfnR in MDCK-PT9R cells

We based our experiments for studying the process of internalization on investigations reported in literature [21,23,24]. Briefly, MDCK cells grown in Minimal Essential Medium (MEM)/BSA, on glass cover slips were exposed for 24 h to 125 mg/L NYT-IL or NYT. Then, 60 mg/l of Fluorescein isothiocyanate (FITC) labeled-hTfn (FITC-hTfn) was added to the cells on ice for 60 min to allow binding of FITC-hTfn to its receptors on the cells’ membrane. Afterward the cells were incubated for 5 and 30 min at 37°C in MEM/BSA with or without drugs for occurrence of endocytosis. At the specified times the cells were fixed with 4% paraformaldehyde in PBS and analyzed by confocal microscopy (LSM710 confocal microscope, Zeiss, Germany).

Statistical analysis

Statistical analysis of data was performed using the unpaired two-tailed Student’s t-test for single comparisons or by one-way analysis of variance (ANOVA) for multiple comparisons. Significance level was defined as p < 0.05.

Results

Killing kinetics

During the first hour after adding NYT or NYT-IL at concentrations equivalent to 0.5 and 1 MIC, the number of live C. albicans cells remained at the same level as in the control tubes (saline or IL; Fig. 1). However, 2 and 4 MIC equivalent concentrations of NYT or NYT-IL caused a substantial decrease in CFU counts within 1 h, with killing effect of NYT being more prominent than at lower concentrations.

Follow-up tests after 24 h demonstrated an increase in cell number in the control and in the 0.5 MIC NYT and NYT-IL treated groups, as a result of normal growth at the logarithmic stage, but there was a less marked increase at the 0.5 MIC. No change in the concentration of live cells during 24 h was noted in 1 MIC NYT or NYT-IL treated cells, whereas decrease in CFU numbers was found following treatment with 2 and 4 MIC of NYT or NYT-IL. Thus, it can be noted in Figure 1 that NYT and NYT-IL have similar time- and concentration-dependent effects on killing of C. albicans cells.

Scanning and transmission electron microscopy (SEM and TEM)

The SEM and TEM observations (Fig. 2) revealed that both NYT and NYT-IL affect the structure of C. albicans cells. Examination of SEM samples demonstrated the disruption of cells and masses of cellular material, with the effects more marked with NYT-IL than NYT. These destructive effects are concentration- and exposure time-dependent. TEM analysis demonstrated, as well, the damaging effects of both drugs, which were also concentration- and exposure time-dependent.

K⁺ leakage experiments in yeast cells

K⁺ leakage experiments (Table 1) showed that both NYT and NYT-IL preparations caused leakage of K⁺ from C. albicans cells in a similar manner. At a concentration of 0.5 MIC, both preparations caused a decrease in intracellular K⁺ concentration of nearly 50% within 5 min incubation, but no further decrease occurred with longer incubation or at higher drug concentrations. Medium or IL alone did not cause K⁺ leakage from C. albicans cells.

The effect of ergosterol on in-vitro NYT-IL antifungal activity

Fig. 3 shows the effect of ergosterol supplementation on MIC and MFC values of NYT-IL against C. albicans. It
can be noted that the addition of ergosterol resulted in increased MIC and MFC values, with the effect being dose dependent (20 mg/l caused a significant effect, i.e., $p < 0.01$ on MFC value).

**Cytotoxicity to MDCK-PTR9 cells**

Fig. 4A (Trypan blue exclusion test) demonstrates that with NYT, at a concentration of 250 mg/l, the percent of viable cells is close to zero, but NYT-IL has no noticeable effect on cell viability at the same level. Measurements of the metabolic activity of the kidney cells (XTT test; Fig. 4B) revealed that at concentrations up to 125 mg/l, NYT-IL and NYT presented a similar behavior in that they had no noticeable effect on the cells’ metabolic activity. However, NYT at the concentration of 250 mg/l caused a significant ($p < 0.05$) decrease in the percentage of metabolically active cells, compatible with the Trypan blue results. However, the same concentration of NYT-IL has only a moderate effect on the metabolic activity of the cells, suggesting a lower toxicity of NYT-IL.

**K⁺ leakage experiments in MDCK-PTR9 cells**

K⁺ leakage experiments in MDCK-PTR9 cells were conducted at NYT and NYT-IL concentrations of 2.35 mg/l and 1.4 mg/l, respectively (equivalent to MIC) and at 125 mg/l, a concentration at which toxic effects can be noted in XTT and Trypan blue exclusion tests. The data in Fig. 5 show that 2 h treatment with NYT, at a concentration of 125 mg/l, caused significant ($p < 0.05$) decrease in intracellular potassium content of the kidney cells. Treatment with NYT and NYT-IL at the concentrations equivalent to 1 MIC, as well as with NYT-IL at the high concentration of 125 mg/l, did not affect intracellular K⁺ concentrations. Treatment with IL alone resulted in similar intracellular potassium concentrations as in the untreated cells. Taking these results all together, the data suggest lower toxicity of NYT-IL on kidney cells.

**Effect of NYT-IL on FITC-hTfn internalization in MDCK-PTR9 cells**

It can be seen in all samples (Fig. 6) that after 5 min of internalization, FITC-hTfn is localized in punctuate peripheral endosomal compartments known as early endosomes [24], whereas after 30 min of internalization it is found in perinuclear compartments. The morphology of the later compartment in the NYT-treated cells differs from that observed in the control. While the morphology of this compartment in the control cells looks like the recycling endosomes (REs) described by Sheff *et al*. [24], in
Fig. 2 Scanning and transmission electron microscopy (SEM and TEM) of Candida albicans treated with NYT-IL or NYT. (A) Scanning electron microscopy of C. albicans treated with NYT-IL or NYT. (B) Transmission electron microscopy of C. albicans treated with NYT-IL or NYT. C. albicans was treated with NYT-IL or NYT at concentrations equivalent to 0.5 or 1 MIC and analyzed by electron microscopy 24 or 48 h after treatment initiation. 1 MIC of NYT-IL and NYT is equivalent to 1.4 mg/l and 2.35 mg/l, respectively. Magnifications are indicated on the photos.
(B) Transmission electron microscopy of *Candida albicans* treated with NYT-IL or NYT.

**Fig. 2 (Continued)**
NYT-treated cells the FITC-hTfn compartment resembles the apical recycling endosomes (AREs) described by Apodaca et al. [25]. Therefore, NYT treatment causes either missorting of the FITC-hTfn, or changes the morphology of the REs. In contrast, the localization of FITC–hTfn after 30 min of internalization in cells treated with an equal dose of NYT-IL (125 mg/l) is similar to that found in the control. Treatment with IL does not change significantly the FITC-hTfn localization.

Discussion

It is known that modifications in formulation or delivery system of a drug may affect its biological activity and toxicity [26]. This was shown in our previous studies with AMB-IL [2], where much higher concentrations were required to cause hemolysis of RBC and potassium leakage than with conventional AMB. However, AMB-IL’s better tolerance in vivo allowed for the use of higher doses [3,4]. In the present study, the new formulation NYT-IL permitted the systemic use of NYT with reduced toxicity, along with preserving its antifungal properties [9,13].

We explored the possible mechanism underlying the changes in NYT-IL’s biological activity in comparison to

| Time (min) Control IL 0.5 MIC IL 1 MIC IL 2 MIC IL 4 MIC NYT 0.5 MIC NYT 1 MIC NYT 2 MIC NYT 4 MIC |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 100 ± 0 (3.63 mg/l) | 76 ± 4 | 59 ± 24 | 45 ± 21 | 56 ± 17 | 59 ± 20 | 58 ± 25 | 46 ± 24 | 57 ± 19 | 57 ± 19 |
| 5 | 115 ± 40 | 52 ± 15 | 54 ± 13 | 58 ± 20 | 58 ± 19 | 55 ± 20 | 47 ± 24 | 58 ± 20 | 58 ± 20 |
| 15 | 151 ± 65 | 187 ± 86 | 51 ± 13 | 50 ± 15 | 59 ± 20 | 59 ± 20 | 54 ± 18 | 46 ± 25 | 58 ± 21 | 58 ± 21 |
| 30 | 229 ± 59 | 202 ± 35 | 51 ± 13 | 61 ± 14 | 56 ± 18 | 55 ± 20 | 55 ± 19 | 47 ± 25 | 57 ± 19 | 57 ± 20 |

The data are expressed as a percentage of intracellular potassium after different treatments at various time points in comparison to control (100%; 100% is equivalent to a concentration of 3.63 mg/l). NYT-IL, Nystatin-Intralipid; MIC, Minimal inhibitory concentration (NYT-IL = 1.4 mg/l; NYT = 2.35 mg/l). The test was performed in two repetitions at separate occasions.

![Fig. 3](https://example.com/f3.png) The effect of ergosterol on NYT-IL in vitro antifungal activity. MIC and MFC of NYT-IL against Candida albicans were determined by broth microdilution technique in presence of various ergosterol concentrations. Two-four repetitions were performed for each ergosterol concentration. Wells without ergosterol were used as control.

![Fig. 4](https://example.com/f4.png) Viability and metabolic activity assays in MDCK-PTR9 cells. (A) Trypan blue exclusion test; (B) XTT test. Viability of MDCK PTR9 cells was assessed by Trypan blue exclusion test (A) and by XTT test (B). The percentage of viable cells in the well was calculated by dividing the total number of viable cells in the well by the total number of cells multiplied by 100. The tests were performed in 2–3 repetitions at separate occasions.

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the conventional formulation of NYT. Similarity of the killing kinetics against *C. albicans* of both formulations suggests that a common mechanism of action may be considered. Electron microscopy studies support this assumption demonstrating that both preparations have a destructive effect on yeast cells under the same conditions, causing changes in the ultrastructure of the cells. Interestingly, NYT-IL is even more damaging to *C. albicans* cells, as noted by SEM analysis. Consequently, NYT-IL not only has lower MIC and MFC values [9] but also its antifungal activity is more potent than NYT when the same MIC equivalents are compared. Based on this data, it could be possible that an additional mechanism of activity is involved.

The potassium ion leakage experiments provide evidence that the target of NYT-IL, as well as NYT is the fungal cell membrane. The effect is immediate even in sub-MIC concentrations of both preparations, whereas the killing effect becomes evident at higher concentrations and after a longer period of exposure. The difference between the two effects, ion leakage and killing effect, may point to additional mechanisms involved in the drugs’ activity. It is of interest that other investigators have also reported dissociation between ion permeability and the lethal action of polyene antibiotics [27].

Our susceptibility assays of NYT-IL in the presence of ergosterol revealed lower antifungal activity, which may suggest that NYT-IL interacts with ergosterol as is the case with NYT, under these experimental conditions, albeit not constituting a direct evidence of binding [28,29].

All these data taken together allow us to assume that NYT-IL acts against fungi by damaging the fungal cell membrane. However, the possibility of additional modes of action that can explain the antifungal activity of NYT-IL cannot be excluded.

Polynes can cause damage to mammalian cells by similar mechanisms as they have on fungal cells. Indeed, NYT causes K⁺ leakage from kidney cells, but at much higher concentration than from *C. albicans* cells (125 vs. 1.175 mg/L, respectively). This difference can be explained by better affinity of polynes to fungal ergosterol than to mammalian cholesterol [19,30–32]. However, NYT-IL does not cause K⁺ leakage from kidney cells even at the high concentration of 125 mg/L and is not cytotoxic. It should be emphasized that significantly lower concentrations of NYT-IL (0.7 mg/l) are sufficient to cause K⁺ leakage from *C. albicans* cells. This suggests that the Intralipid formulation prevents the damage that may be caused by NYT to the mammalian cells, while maintaining its effects fungal cells, thus constituting a safe antifungal preparation.

Polynes appear to injure mammalian cells by an additional mechanism, i.e., interfering in the intracellular endocytic and post-endocytic processes [33,34]. NYT, at a concentration of 125 mg/L, interrupted the normal post-endocytic pathway, whereas NYT-IL, at the same concentration, did not. This further corroborates the safety of the new formulation of NYT.

In conclusion, a novel Intralipid formulation of NYT reduces NYT’s toxicity to mammalian cells but preserves mechanisms of activity against fungal cells,

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**Fig. 5** K⁺ leakage experiments in MDCK-PTR9 cells. MDCK-PTR9 cells were exposed to NYT-IL (1.4 mg/L (equivalent to 1 MIC) and 125 mg/L) or NYT (2.35 mg/L (equivalent to 1 MIC) and 125 mg/L) for 2 h. Cells in medium or in IL were used as controls. The intracellular potassium ion concentration was measured and expressed in %; untreated control was considered as 100%. Obtained values represent percentage of intracellular potassium remaining after different treatments. The experiments were performed in duplicates at separate occasions.
**Fig. 6** Effect of NYT-IL on FITC-hTfn internalization to MDCK-PTR9 cells. (A) FITC-hTfn distribution after 5 min of internalization. (B) FITC-hTfn distribution after 30 min of internalization. The cells were treated with NYT (125 mg/l) or NYT-IL (125 mg/l) for 24 h and hTfn-FITC was added. FITC-hTfn internalization into the cells was enabled for 5 min (A) or for 30 min (B), followed by fixation with 4% PFA in PBS and analysis by confocal microscopy. The experiments included two controls: cells in medium or in IL.
resulting in a safe and effective antifungal preparation for systemic use.

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