

## Pharmacokinetics, tissue distribution and immunomodulatory effect of intralipid formulation of nystatin in mice

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**Objectives:** We developed a novel lipid formulation of nystatin suitable for parenteral administration, nystatin-intralipid (NYT-IL), with antifungal activity and reduced toxicity in mice. We investigated the pharmacokinetics, tissue distribution and immunomodulatory effect of NYT-IL in mice.

**Methods:** Nystatin levels in serum and organs were determined using HPLC after NYT-IL or nystatin administration in mice. The levels of the pro-inflammatory cytokines tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) and the anti-inflammatory cytokine interleukin 10 (IL-10) produced by splenocytes from mice injected with NYT-IL or nystatin were evaluated by an ELISA assay.

**Results:** Injection of NYT-IL resulted in similar levels and similar kinetics of nystatin in serum, higher concentrations in the liver and lower concentrations in the kidneys, in comparison with nystatin injection. Injection of mice with NYT-IL yielded higher levels of IL-10 than that of nystatin, whereas the levels of TNF- $\alpha$  and IFN- $\gamma$  induced by NYT-IL were lower than those elicited by nystatin.

**Conclusions:** Since polyene treatment is associated with nephrotoxicity, lower levels of nystatin in the kidneys following NYT-IL injection suggest the possibility of reduced toxicity. As the acute infusion-related adverse effects associated with polyene treatment are considered to be induced by pro-inflammatory cytokines, a higher level of anti-inflammatory and lower levels of pro-inflammatory cytokines elicited by NYT-IL administration suggest the possibility of amelioration of such effects. In summary, the altered pharmacokinetics, tissue distribution and immune response due to the use of this intralipid formulation of nystatin merit further research towards the development of a therapeutic agent against invasive mycoses.

**Keywords:** nystatin-intralipid preparation, pharmacological characteristics, cytokines

### Introduction

Nystatin is a highly potent and broad-spectrum polyene antifungal drug<sup>1–4</sup> that has been used successfully for many years as a topical treatment for mucocutaneous mycoses.<sup>5</sup> Despite attempts to develop preparations of nystatin for systemic administration,<sup>6,7</sup> there is no such preparation thus far. Nystatin, like other polyenes, cannot be given orally for treatment of systemic mycoses, since it is not absorbed from the gastrointestinal tract. Intravenous administration of polyenes in general and of nystatin specifically is hindered by acute, infusion-related adverse effects, such as fever, chills, headache, nausea, vomiting, hypertension/hypotension and hypoxia, and by chronic toxic effects, of which nephrotoxicity is the most

important.<sup>8–13</sup> Liposomal and lipid formulations of amphotericin B, the major polyene drug used systemically in the therapy of invasive mycoses, have been developed and are associated with reduced side effects. Although they are commercially available, their high cost prohibits in many instances their use as first-line treatment.

We have previously reported the development of a novel, standardized, affordable lipid formulation of nystatin, nystatin-intralipid (NYT-IL). This preparation exhibited *in vitro* and *in vivo* activity against pathogenic yeasts and moulds.<sup>14</sup> Furthermore, the intralipid formulation reduced the toxicity of nystatin, enabling its systemic administration, as demonstrated in an experimental murine model.<sup>15</sup> NYT-IL is well tolerated by mice in therapeutic doses up to 8 mg/kg.

The availability of a drug in the bloodstream and its distribution to visceral organs are necessary properties of a new formulation for systemic use. Experiments conducted by other researchers showed that lipid formulations may alter the pharmacokinetics of polyenes, with implications for their toxicity.<sup>16,17</sup>

In this study we explored the tissue distribution of nystatin following parenteral administration of NYT-IL in mice in comparison with nystatin administration and determined its pharmacokinetic parameters. It is also of interest to investigate the possible immunomodulatory effects of NYT-IL, as it is known that polyenes induce the production of pro-inflammatory cytokines.<sup>18–22</sup> Moreover, it is believed that the induction of pro-inflammatory cytokines by polyenes is responsible for many of the acute adverse effects associated with systemic treatment with polyenes.<sup>23–29</sup> In addition, amphotericin B has the potential to induce apoptosis in the kidney, in association with generalized up-regulation of pro-inflammatory cytokines.<sup>30</sup> In this study the immunomodulatory effect of NYT-IL compared with that of nystatin in mice was tested by determination of levels of three kinds of cytokines produced by splenocytes: tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ), which are promoters of the inflammatory response, and interleukin 10 (IL-10), which is an anti-inflammatory cytokine.

## Materials and methods

### Animals

Female ICR mice, 4–6 weeks old and weighing 23–28 g, were used in all experiments. Animals were kept under conventional conditions and were given food and water *ad libitum*. The Ethics Committee of the Faculty of Medicine of Tel-Aviv University granted permission for the animal experiments described in this study.

### NYT-IL preparation

Stock solution of nystatin 25 mg/mL was prepared by dissolving nystatin (Sigma Chemicals, USA) in DMSO. NYT-IL was prepared by diluting stock solution of nystatin with intralipid 20% (Kabi Pharmacia, Stockholm, Sweden) to a final concentration of 1 mg/mL and mixed for 18 h at 24°C with shaking at 280 rpm. The NYT-IL was kept at 2–8°C for up to 1 month. Nystatin 1 mg/mL was obtained by dilution of stock solution with saline.

### Injection of mice

Mice were injected intravenously (*iv*) with 8 mg/kg of NYT-IL or nystatin. Control mice received saline or intralipid, diluted to the same degree as in the NYT-IL treatment. Blood was drawn, mice were sacrificed at different timepoints after injection, and organs were collected and kept frozen (–20°C). The liver, kidneys, spleen and lungs were harvested.

### HPLC analysis

Determination of nystatin in serum and organs was based on the method described previously by Groll *et al.*<sup>31</sup> HPLC was used to determine the nystatin concentration. Nystatin was extracted from serum with 1:2 (*v/v*) HPLC grade methanol and from organs by homogenization with 1:2 (*w/w*) HPLC grade methanol. The AUC, which represents the extent of nystatin absorption, was determined at 305 nm and retention time of 8–11 min. The AUC values obtained were converted to nystatin

concentrations using serum and organ calibration curves. Nystatin concentrations were expressed in mg/L for serum and  $\mu\text{g/g}$  for organs. At least three repetitions were performed for each timepoint.

## Cytokine detection from splenocytes and in serum

### Splenocyte culture and supernatants

Production of cytokines from splenocytes was measured based on a method described by Caraher *et al.*<sup>32</sup> Spleen populations from each mouse were cultured separately. ICR female mice, 5–6 weeks old, were injected *iv* with 8 mg/kg of NYT-IL or nystatin. Control mice received saline or intralipid, diluted to the same degree as in NYT-IL. Spleens were removed from sacrificed mice 48 h after drug administration and disrupted by passage through a sterile 70  $\mu\text{m}$  cell strainer using RPMI culture medium (Sigma) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL of penicillin and 100 mg/L of streptomycin. The spleen cell suspension obtained was centrifuged (1500 rpm at 4°C for 5 min) and erythrocytes were lysed by exposure to double-distilled water for 15 s. The pelleted cells were resuspended in culture medium and the suspension was adjusted to  $2 \times 10^6$  cells/mL of culture medium. Splenocytes ( $2 \times 10^6$  cells/well) were incubated in 12-well flat-bottom culture plates (Greiner Bio-One North America) in the presence of phorbol 12-myristate 13-acetate (5  $\mu\text{g/L}$ ) and ionomycin (250  $\mu\text{g/L}$ ) for splenocyte activation for 24 h at 37°C with 5% CO<sub>2</sub>. The well contents were then centrifuged and the supernatants were collected for detection of cytokines using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, as detailed below.

### Serum sampling

Mice were injected with NYT-IL, nystatin, intralipid or saline. After 0.5 and 2 h blood was drawn and serum was collected for cytokine detection.

### Determination of cytokines by ELISA

Ninety-six-well flat-bottom half-area microplates were coated with 25  $\mu\text{L}$  per well of capture antibody and incubated overnight at 4°C. After washing and blocking with PBS containing 10% FBS, samples of serum, supernatants of splenocyte cultures or the standards were added to each well. The plates were incubated for 2 h at room temperature (RT). The plates were washed and detecting antibody was added to each well. The plates were incubated for 1 h at RT before addition of streptavidin–horseradish peroxidase. After incubation for 30 min the plates were washed and incubated with tetramethylbenzidine at RT for up to 20 min. The reaction was stopped by addition of 25  $\mu\text{L}$  stop solution of 1 M H<sub>2</sub>SO<sub>4</sub> and optical density was measured at 450 nm using an ELISA reader. The amounts of cytokines were calculated from the linear portion of the standard curve.

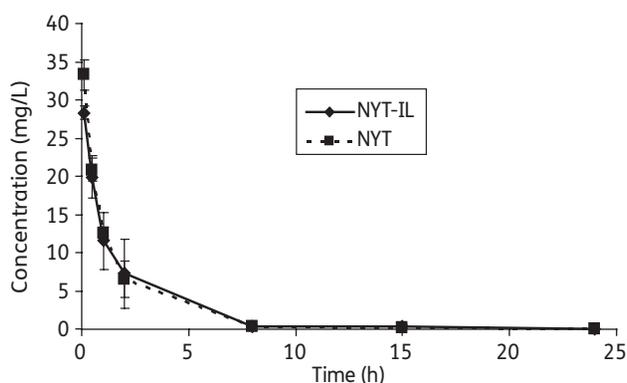
### Statistical analysis

All the data are presented as means  $\pm$  standard deviation. Statistical evaluation of the results was performed using the unpaired Student's *t*-test for single comparisons or by one-way analysis of variance for multiple comparisons. The significance level was defined as  $P \leq 0.05$ .

## Results

### Pharmacokinetics and tissue distribution of NYT-IL and nystatin

Figure 1 demonstrates nystatin concentrations in serum samples at different timepoints after NYT-IL or nystatin injection. Table 1 shows the pharmacokinetic parameters of NYT-IL and nystatin that were calculated according to the data presented in Figure 1. The data show that iv administration of NYT-IL or nystatin to mice resulted in similar levels of nystatin in serum with similar kinetics (Figure 1 and Table 1). The nystatin serum concentration versus time curve (Figure 1) exhibited immediate high peak serum levels, which decreased rapidly during the first 2 h after NYT-IL or nystatin administration. Thereafter, the drug concentration profile was characterized by a moderate decrease throughout the time course.



**Figure 1.** Nystatin concentration in serum after NYT-IL or nystatin (NYT) injection. ICR female mice were injected intravenously with 8 mg/kg of NYT-IL or NYT. Serum concentrations (mg/L) of NYT were measured by HPLC at different intervals after administration. Each point on the curves presents an average value obtained from at least three experiments  $\pm$ SD.

**Table 1.** Pharmacokinetic parameters following administration of nystatin (NYT) and NYT-IL (8 mg/kg) by intravenous injection in mice

Parameter	NYT	NYT-IL
$k$ (1/h) <sup>a</sup>	0.100	0.093
$t_{1/2}$ (h) <sup>b</sup>	6.934	7.422
$V_D$ (L) <sup>c</sup>	0.010	0.010
CL (L/h) <sup>d</sup>	0.001	0.001
AUC (mg·h/L) <sup>e</sup>	186.811	208.443
$C_{max}$ (mg/L) <sup>f</sup>	33.289	28.330

<sup>a</sup> $k$ , elimination rate constant ( $\ln C_1 - \ln C_2 / t_1 - t_2$ ).

<sup>b</sup> $t_{1/2}$ , elimination half-life (0.693/ $k$ ).

<sup>c</sup> $V_D$ , volume of distribution (dose/ $C_0$ ).

<sup>d</sup>CL, clearance ( $k \times V_D$ ).

<sup>e</sup>AUC, area under the serum drug concentration–time curve (dose/CL).

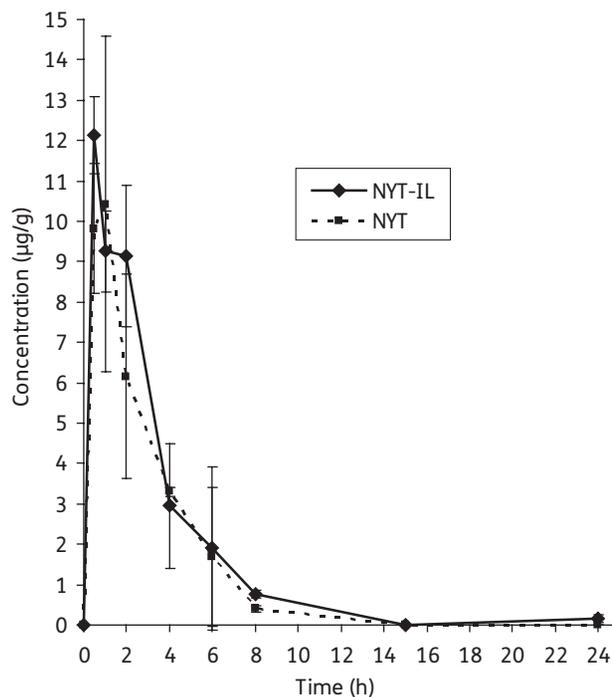
<sup>f</sup> $C_{max}$ , highest drug concentration observed in serum.

Nystatin levels in organs were measured at 0.5, 1, 2, 4, 6, 8, 15 and 24 h after NYT-IL or nystatin injection. Nystatin was distributed predominantly in the liver and kidneys following NYT-IL or nystatin parenteral administration, as can be deduced from the AUC values (Table 2). Figures 2 and 3 show nystatin concentrations in liver and kidneys, respectively, as a function of time after NYT-IL or nystatin injection. Nystatin levels in other organs, such as the spleen and lungs, were much lower, as may be seen from their AUC values (Table 2), and close to the detection threshold; therefore, they are not shown here in graph form.

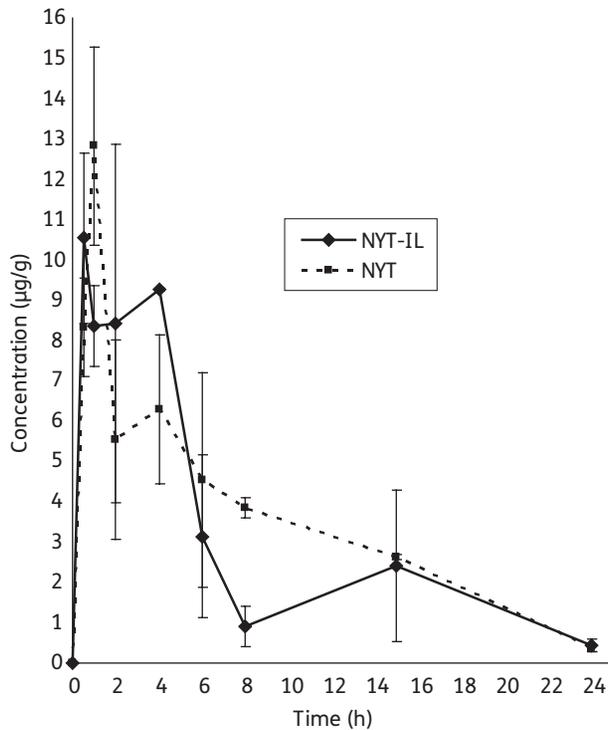
**Table 2.** Comparison of AUC values of NYT-IL compared with nystatin in serum and organs

Organ	AUC (mg·h/L)		Difference (%) <sup>a</sup>
	NYT	NYT-IL	
Serum	186.811	208.443	111.58
Liver	33.744	42.497	125.94
Kidneys	105.168	90.844	86.38
Spleen	3.846	3.694	96.05
Lungs	8.717	9.825	112.71

<sup>a</sup> $100 \times \text{AUC (NYT-IL)} / \text{AUC (NYT)}$ .



**Figure 2.** Nystatin concentration in liver after NYT-IL or nystatin (NYT) injection. ICR female mice were injected intravenously with 8 mg/kg of NYT-IL or NYT. Concentrations ( $\mu\text{g/g}$ ) of NYT in liver extracts were measured by HPLC at different intervals after administration. Each point on the curves presents an average value obtained from at least three experiments  $\pm$ SD.



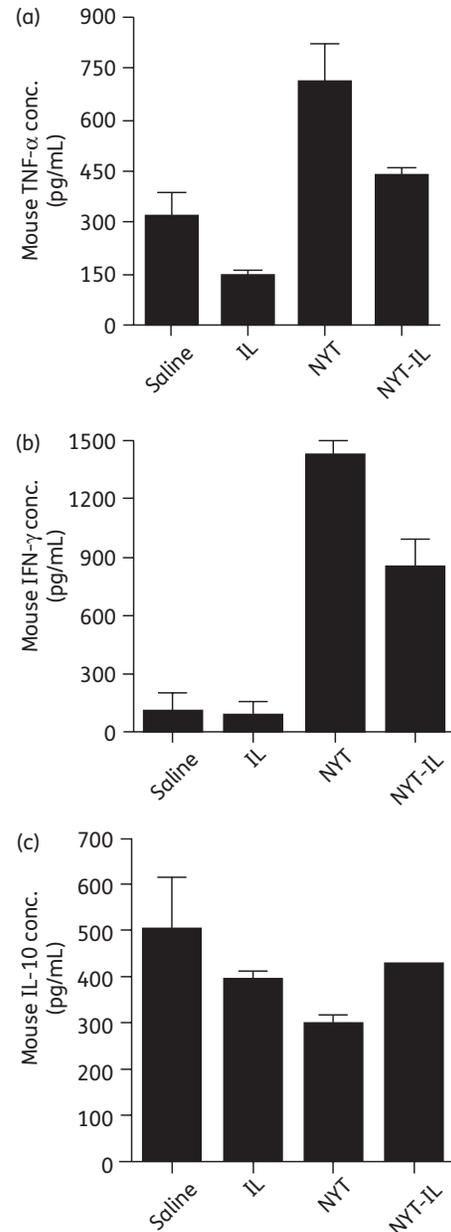
**Figure 3.** Nystatin concentration in kidneys after NYT-IL or nystatin (NYT) injection. ICR female mice were injected intravenously with 8 mg/kg of NYT-IL or nystatin. Concentrations ( $\mu\text{g/g}$ ) of nystatin in extracted kidneys were measured by HPLC at different intervals after administration. Each point on the curves presents an average value obtained from at least three experiments  $\pm$ SD.

NYT-IL or nystatin administration resulted in different nystatin pharmacokinetics in liver and kidneys. Following NYT-IL injection the peak of nystatin in the kidneys (Figure 3) was observed after only 0.5 h, with swift clearance. Following nystatin administration a higher peak was noticed after 1 h, with a moderate decline and with significant differences in concentrations of nystatin following the two treatments at timepoints of 1 and 8 h ( $P < 0.05$ ). In the liver (Figure 2) the situation was reversed: the peak of nystatin following NYT-IL injection was higher than that after nystatin inoculation, with significant differences at the timepoints of 0.5, 8 and 24 h ( $P < 0.05$ ).

Comparison of the AUC values (Table 2) shows that after NYT-IL injection  $\sim 26\%$  more nystatin was found in the liver than after nystatin injection. In the kidneys, injection of NYT-IL resulted in  $\sim 14\%$  less nystatin than after nystatin injection.

### Immunomodulatory effect of NYT-IL compared with nystatin

Figure 4 shows cytokine concentrations of TNF- $\alpha$ , IFN- $\gamma$  and IL-10 produced by splenocytes from mice inoculated with nystatin or NYT-IL. Administration of either nystatin or NYT-IL yielded elevated levels of TNF- $\alpha$  (Figure 4a) and of IFN- $\gamma$  (Figure 4b) compared with controls, with different profiles. Injection of NYT-IL induced lower concentrations of the pro-inflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  ( $P < 0.05$ ) than the concentrations found



**Figure 4.** Levels of cytokines produced in mice injected with NYT-IL, nystatin (NYT) or intralipid (IL). Concentrations of TNF- $\alpha$  (a), IFN- $\gamma$  (b) and IL-10 (c) in splenocyte culture supernatants were determined by ELISA. The results are presented as the mean of at least two repetitions  $\pm$ SD. Significant differences between IFN- $\gamma$  levels ( $P < 0.05$ ) and IL-10 levels ( $P < 0.01$ ) induced by NYT-IL and nystatin were noted.

following nystatin inoculation. On the other hand, as shown in Figure 4(c), higher levels ( $P < 0.01$ ) of the anti-inflammatory cytokine IL-10 were induced by injection of NYT-IL than by injection of nystatin.

Assays to detect the three cytokines in serum at the timepoints of 0.5 and 2 h after injection of either NYT-IL or nystatin and the relevant controls (intralipid and saline, respectively) were carried out. The data obtained (not shown) revealed that

the levels of cytokines in serum were below or at the detection limit.

## Discussion

The object of this study was the characterization of the pharmacokinetics and tissue distribution of the intralipid formulation of nystatin as well as investigation of its immunomodulatory effect in mice.

The single-dose pharmacokinetics of NYT-IL in mice were investigated by determination of nystatin concentrations in serum and selected organs (liver, kidneys, spleen and lungs) at different timepoints after NYT-IL injection, using HPLC technology. The results were compared with the data obtained from nystatin-treated mice.

NYT-IL or nystatin administration to mice yielded similar nystatin concentrations in serum. In addition, a similar profile over time was noted: a sharply descending nystatin concentration during the first 2 h after NYT-IL or nystatin injection followed by a much more moderate decline. The sharp nystatin descent in serum occurred in parallel to the escalating levels of nystatin in organs. This observation may be explained by a multi-compartment model of drug distribution in the body:<sup>33</sup> specifically, the initial rapid decline of serum drug concentration represents drug distribution from blood to organs. After the equilibrium between drug concentrations in blood and organs has been achieved, a slower rate of decline in drug concentration is observed, indicating drug elimination from the body.

Although inoculation of mice with NYT-IL or nystatin resulted in similar nystatin levels and kinetics in serum, differences were observed in patterns of tissue distribution of the two preparations. The pharmacokinetic data point to a trend in which higher levels of nystatin reached the liver after NYT-IL injections than after nystatin injections. This finding is compatible with conclusions from other studies showing that lipid formulations of polyenes route the drug preferentially to the organs of the reticulo-endothelial system, such as the liver.<sup>16,34,35</sup> In the case of the kidneys the trend was reversed. Since nystatin is mainly toxic to the kidneys, lower accumulation of nystatin in the kidneys following NYT-IL injection may suggest the possibility of a reduced toxic effect.

Experiments testing the immunomodulatory effect of NYT-IL and nystatin in mice revealed that injection of NYT-IL resulted in lower levels of pro-inflammatory cytokines than those seen after injection of nystatin. In addition, NYT-IL administration induced significantly higher levels of the anti-inflammatory cytokine IL-10 than did nystatin. However, since both drugs induced IL-10 levels lower than those seen in the controls, this actually represents less suppression.

Analysis of the data regarding the relationship between the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine TNF- $\alpha$  induced by nystatin versus that by NYT-IL showed a ratio of 0.38 versus 0.98, respectively. The ratio between these cytokines indicates a profile oriented towards the anti-inflammatory response of NYT-IL.

In view of these results, it may be concluded that combination of nystatin with intralipid is apparently able to diminish the extent of the inflammatory response induced by nystatin alone. As mentioned in the Introduction, infusion-related toxicity

of nystatin may be attributed to the capacity of this drug to accelerate the production of pro-inflammatory cytokines. Since the intralipid formulation of nystatin seems to attenuate this effect, NYT-IL is expected to have reduced infusion-related adverse effects. Further studies are necessary to demonstrate such effects in humans.

It is of interest that TNF- $\alpha$  and IFN- $\gamma$  may be involved in apoptosis,<sup>36</sup> a process believed to be associated with a mechanism through which amphotericin B can cause nephrotoxicity,<sup>37</sup> Thus, our data, showing reductions in the production of these pro-inflammatory cytokines, may indicate that both immediate and late adverse effects caused by polyenes are affected. Our findings are reinforced by results from the experiments with another polyene, amphotericin B. Amphotericin B lipid formulations induced lower levels of pro-inflammatory cytokines, as shown by Louie *et al.*<sup>29</sup> and by our studies,<sup>38</sup> causing a reduction in adverse effects.<sup>39</sup> Furthermore, a recent study by Simitopoulos *et al.*<sup>40</sup> described similar observations regarding immunomodulatory effects of amphotericin B lipid formulation (ABLCL), which were expressed in higher IL-10 levels than those elicited by the non-lipid amphotericin B.

In conclusion, alteration of the pharmacokinetics and immunomodulatory effects of nystatin by using an intralipid formulation may be responsible for the reduced toxicity of NYT-IL in comparison with that of nystatin.

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## Transparency declarations

None to declare. This study is part of the research project towards the PhD degree of R. S.

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