Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 275 (2004) 61–72
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Leucinostatin-A loaded nanospheres: characterization and in vivo toxicity and efficacy evaluation

Maurizio Ricci, Paolo Blasi, Stefano Giovagnoli, Luana Perioli, Claudia Vescovi, Carlo Rossi*

Dept. of Chimica e Tecnologia del Farmaco, Università degli Studi di Perugia, Via del Liceo 1, 06123 Perugia, Italy

Received 6 January 2004; accepted 19 January 2004

Abstract

Leucinostatin A (Leu-A) is a nonapeptide exerting a remarkable activity especially against *Candida albicans* and *Cryptococcus neoformans*; nevertheless, its employment is limited due its toxicity. Therefore, we recently developed liposomal formulations, as suitable delivery systems, in order to increase its therapeutic index. However, liposomes present disadvantages related to their long-term instability. For this reason poly(lactic-co-glycolic) nanospheres (NS) were chosen as alternative colloidal carriers for Leu-A delivery. NS were formulated by spontaneous emulsification solvent diffusion method. This study investigates the effects of different parameters on drug encapsulation efficiency and particle size as well. The best preparation obtained was also characterized for its in vitro release, in vivo acute toxicity (LD_{50}), and effectiveness against *C. albicans* in mice. In vitro release was performed over 100 h and resulted sufficiently sustained with more than 93% of the peptide released. Acute toxicity showed that the LD_{50} was increased more than 18-fold and the study on systemic candidiasis models revealed high effectiveness of the NS in reducing either the growth of fungal colonies in infected mice liver or in the mortality index. In conclusion, we can propose that Leu-A loaded NS could represent a new promising therapeutic system against *Candida* infection.

Keywords: Leucinostatin A; Nanospheres; *Candida albicans* infection

1. Introduction

The incidence of morbidity and mortality for systemic fungal infection has been constantly growing during the last 20 years. This phenomenon is closely related to the increasing immunocompromised patients, including those undergoing bone marrow and solid organ transplantation, hospitalized cancer and intensive care patients receiving chemotherapy and broad-spectrum antimicrobial treatment, patients with primary immunodeficiencies, with AIDS and neutropenia, premature babies and the "super-old" people (Bodey et al., 1992; Andriole, 1993; Walsh and Groll, 1999; Ellis, 2002).

Candida species constitute the most common causes of disseminated nosocomial fungal infection (Verduyn Lunel et al., 1999), while *Aspergillus* species have emerged as one of the main species responsible for pneumonic mortality in bone marrow transplant patients (Andriole, 1993) and *Cryptococcus neoformans* is the major cause for CNS mycosis in HIV-infected patients (Brummer, 1999; Walsh and Groll, 1999).

* Corresponding author. Tel.: +39-075-5855127; fax: +39-075-5855163.
E-mail address: cfrossi@unipg.it (C. Rossi).

0378-5173/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2004.01.030

Moreover, less common fungal pathogens, such as azole-resistant *Candida albicans* and non-*albicans Candida* species (i.e. *Candida glabrata* and *Candida krusei*) (Milan et al., 1998; Petrocheilou-Paschou et al., 2002), have assumed increasing importance as the causes of infections in hospitalized immunocompromised patients (Wingard, 1995) as well as in those who make use of central venous catheters for ambulatory antimicrobial therapy and parenteral nutrition.

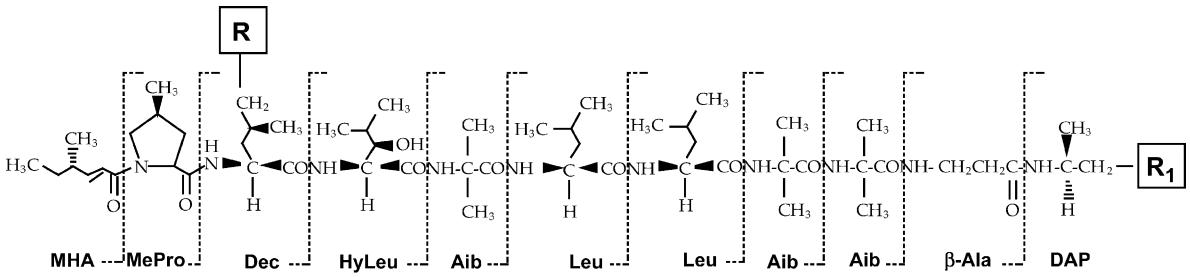
Although Amphotericin B (AmB) remains the drug of choice for the treatment of most life-threatening systemic fungal infections (Sheehan et al., 1999; Andriole, 2000), especially as liposomal formulation, azole derivatives, as Fluconazole, Itraconazole and Ketoconazole, have been playing a big role in the last 10 years.

Unfortunately, several non-*albicans Candida* species, most notably *C. krusei* and numerous strains of *C. glabrata*, have shown strong resistance to treatments with AmB and azoles as well (Nguyen et al., 1996; Walsh and Groll, 1999). Moreover, new resistant strains of *Aspergillus* and *C. neoformans* have appeared (Warnock et al., 1999; Canuto and Rodero, 2002). Although, in some cases, combination of AmB and Fluconazole has given good results, the use of AmB with azoles is controversial because of the potential antagonism based on their mechanisms of action (Ghannoum, 1997).

Therefore, the recent emergence of new fungal infections and resistant strains requires the development of more effective antifungal agents, possibly with different mechanism of action, in order to avoid the insurgence of classical mode of fungal resistance. This is the case of novel peptides that have been taken into account as potential new antifungal agents because of their particular interaction with fungal biological membranes (De Lucca and Walsh, 1999; Oh et al., 1999).

In this regard, we have focused our attention on Leu-A (Fig. 1), an interesting nonapeptide produced in submerged cultures by the phytotoxic microorganism *Paecilomyces marquandii* (Massee) Hughes (Casinovi et al., 1983) as well as by *Paecilomyces lilacinus* A257 (Mikami et al., 1984) and *Acremonium* sp. (Strobel et al., 1997). The peptide is characterized by a high activity against Gram-positive bacteria and some strains of *Candida* (Casinovi et al., 1986). Furthermore, the importance played by this molecule is increased by its activity against the emerging *C. krusei* and *C. neoformans* strains (MIC = 5 and 1 μg/ml, respectively) (Casinovi et al., 1986). This activity is important especially if considering that, as already mentioned, *C. neoformans* infections are often associated with HIV insurgences.

Leu-A biological activity has been ascribed to the inhibition of mitochondria ATP synthesis and, at higher concentration, to the uncoupling of oxidative phosphorylation (Shima et al., 1990). Afterwards, we showed the weak ionophoric activity of the peptide which is probably correlated to its self-assembling property within phospholipid membranes (Csermely et al., 1994; Fresta et al., 2000). These effects on membranes, as shown for other antifungal peptides (Subbalakshmi and Sitaram, 1998; Sokolov et al.,



R = $CH(OH)CH_2COCH_2CH_3$

$R_1 = N(CH_3)_2$

Fig. 1. Chemical structure of Leu-A.

1999), seems to have an important role in Leu-A biological activity.

Hence, in this scenario, Leu-A may represent a new potential drug for therapies involving HIV-infected patients for three main reasons: (i) its activity against emerging new fungal infections, such as *C. neoformans* as well as *C. krusei*, (ii) its comparable activity with respect to AmB against *C. albicans*, (iii) the limited risk of presence of Leu-A resistant strains, since the molecule has never been used in therapy and because of its mode of action that, in theory, is not affected by the common antibiotic-resistance mechanism (Canuto and Rodero, 2002).

However, in spite of its antimicrobial activity, the potential use of Leu-A is unfortunately severely limited by its low LD_{50} ranging from 0.8 mg/kg (intravenously, i.v.) to 1.2 mg/kg (intraperitoneally, i.p.) (Mikami et al., 1984). Therefore, for a therapeutic application of this molecule, it is necessary to find a suitable formulation in order to maintain and/or enhance, if possible, its effectiveness and to limit its dangerous side effects.

In the last decades, colloidal carriers, such as liposomes and biodegradable polymeric nanoparticles, provided outstanding outcomes in reducing the toxicity of important drugs without significantly compromising their efficacy.

In this regard, recently, we developed different Leu-A liposome formulations (MLV and LUV) (Ricci et al., 2000), but, in spite of their efficacy, liposomes showed drawbacks because of their physical and chemical long-term instability (data not shown).

Therefore, we focused our attention on the preparation of PLGA NS containing Leu-A, because of their well-known stability in biological fluids and during storage, biocompatibility, biodegradability, easy preparation, as well as drug targeting properties.

With this purposes we developed and characterized Leu-A loaded nanospheres (Leu-A NS) in order to obtain particles with suitable size distribution and loading. To reach this goal, some parameters of the preparation process were investigated. The most interesting formulation was assessed for: (i) in vitro drug release, (ii) in vivo evaluation of Leu-A loaded NS acute toxicity (LD_{50}), (iii) in vivo activity on systemic candidiasis model.

2. Materials and methods

2.1. Materials

Leu-A was obtained from cultural broth benzene extracts of *P. marquandii* (Massee) Hughes and purified by extensive flash chromatography on silica gel column as reported elsewhere (Rossi et al., 1987, 1990). PLGA (85:15, MW 90,100) and polyvinyl alcohol (PVA, MW 13,000–23,000, 87–89% hydrolyzed) were supplied by Sigma Chemical (Milan, Italy); triethylammonium phosphate buffer (TEAP) was purchased from Fluka (Milan, Italy). High-performance liquid chromatography (HPLC) reagents were supplied by J.T. Baker (Milan, Italy). Ultra-pure water was obtained by reverse osmosis through a Milli-Q System (Millipore, Rome, Italy). All other reagents and solvents were of the highest purity available.

2.2. Leu-A solubility determination

Leu-A solubility in different buffer solutions (pH 2, 7.4, 8, 8.5, 9) was determined in a constant temperature bath ($25 \pm 0.1\,^{\circ}C$) by adding an excess of the solid to 5 ml of aqueous buffers in a series of 10 ml vials. The mixtures were kept under constant stirring (70 rpm). After equilibration (48 h) an aliquot was filtered through a 0.45 μm membrane filter. The first 1 ml of the filtrate was discarded to avoid any adsorptive effect by the membrane. An aliquot of the remaining filtrate was diluted with appropriate buffer solution and analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) analysis as described in Section 2.3. All experiments were performed in triplicate and errors were expressed as ±S.D.

2.3. HPLC analyses of Leu-A

All Leu-A determinations were performed by RP-HPLC with an HP1050 chromatograph (Hewlett Packard, Waldbronn, Germany) and a Delta-Pak (Waters, Milford, MA, USA) column (C18 100 Å; 300 mm × 3.9 mm; 15 μm). Elution was performed in isocratic manner using acetonitrile/isopropanol/1% TEAP water solution (50:40:10) as mobile phase. Leu-A was monitored at 254 nm. Under these conditions the retention time was 5.9 min. Calibration curve for the validated RP-HPLC assay of Leu-A in the

elution medium (concentration range 6–340 μg/ml) had a correlation coefficient of >0.99.

2.4. Preparation of Leu-A loaded nanospheres (Leu-A NS)

Leu-A NS were prepared by spontaneous emulsification solvent diffusion (SESD) method (Niwa et al., 1994). PLGA (60 mg) and Leu-A (5 and 10% (w/w) of the polymer) were dissolved in different volume ratio mixtures of acetone and dichloromethane. The resultant organic phases were emulsified in 25 ml of PVA solution (0.1–1% (w/v)) under moderate stirring at room temperature (4 or 8 h). Finally, organic phases were evaporated under reduced pressure in a rotary evaporator at 38 °C and the colloidal aqueous suspensions were adjusted to the desired final volume (15 ml). The whole dispersed systems was filtered through 1.2 μm pore size Isopore™ membrane filter (Millipore, Milan, Italy) to separate microparticles eventually formed, and then ultracentrifuged by an Optima™ TL ultracentrifuge equipped with a TLA-100.4 rotor (Beckman, Palo Alto, CA, USA) at 50,000 rpm at 4 °C for 40 min. This step was repeated three times to wash out the free peptide and the exciding PVA. The recovered NS were lyophilized and stored under nitrogen atmosphere at 4 °C.

2.5. Nanosphere size distribution and morphology

Particle size analyses were performed using two different methods. In order to evaluate the presence of microparticles eventually formed during the preparation, freshly prepared NS suspensions were analyzed by Accusizer 770 granulometer (PSS Inc., Santa Barbara, CA, USA). On the contrary, filtered NS were characterized for the mean particle size and polydispersity by photon correlation spectroscopy (PCS) using a Nicomp 370 autocorrelator (PSS Inc.) equipped with a Coherent Innova 70-3 Argon Ion Laser System (Laser Innovations, Moorpark, CA, USA) operating at 514.5 nm. Analyses were performed at a scattering angle of 90° and at 20 ± 0.2 °C. Samples were prepared by diluting 10 μl of Leu-A NS suspension with 2 ml of deionized water previously filtered through a 0.2 μm Acrodisc LC 13 PVDF filter (Pall-Gelman Laboratory, Ann Harbor, MI, USA). During the experiment, samples were maintained in a refractive index matching liquid (toluene) to avoid stray light. Data reported represent the average of three measurements and the error was calculated as ±S.D.

Morphological evaluation was carried out by scanning electron microscopy (SEM) using a Philips XL30 microscope. A drop of nanoparticle suspension was placed on an aluminum specimen stub. After freeze-drying, the sample was coated with gold using an EMITECH K-550X sputter coater (Ashford, Kent, UK). Coating was done at 20 mA for 4 min.

2.6. Determination of Leu-A loading

Leu-A entrapped in NS was determined as follows. A weighed amount of lyophilized NS was dissolved in dichloromethane. Afterwards, methanol was added to the clear solution to selectively precipitate the polymer. RP-HPLC analyses were performed on the clear supernatant, in triplicate, after centrifugation at 3800 rpm for 20 min by using an ALC 4218-D centrifuge (ALC International s.r.l., Milan, Italy). Leu-A incorporation efficiency was expressed both as drug recovery and drug content, represented as follows:

$$\text{Drug recovery (\%)} = \frac{\text{Mass of Leu-A in NS}}{\text{Mass of Leu-A fed to the system}}$$

$$\text{Drug content (\%)} = \frac{\text{Mass of Leu-A in NS}}{\text{Mass of NS recovered}}$$

2.7. In vitro release study

Several batches of the best Leu-A NS formulation were mixed in order to perform the in vitro release study. Ten samples (18 mg each) were placed in test tubes containing 4.5 ml phosphate-buffered solution (pH 7.4) to ensure “sink” conditions. These test tubes were shaken (100 rpm) in a Gallenkanp orbital incubator (Fisons Instruments, Crawley, UK) at 37 °C for 100 h.

Samples were withdrawn and ultracentrifuged (70,000 rpm for 10 min at 4 °C) at given time intervals. Supernatants were analyzed by RP-HPLC in triplicate. The cumulative amount of Leu-A released is plotted versus incubating time. Errors were expressed as ±S.D.

2.8. In vivo studies

Charles River (Calco, Milan, Italy) inbred CD2F1 female mice (20–25 g in weight) were used for in vivo experiments.

2.8.1. Acute toxicity

Groups of 10 mice were injected i.p. with 0.5 ml of scalar doses (0.6, 1.2, 1.8, 2.4, 4.8, 9.6, 19.2 mg/kg) of Leu-A alone and entrapped in NS. Mice were under observation for toxic effects or death, immediately after injection, then, daily for 7 days. A linear graph of survival as function of dose was plotted, and the approximate LD_{50} was extrapolated from the graph. The confidence interval was also reported at $n = 10$ and $\alpha = 0.05$ significance level.

2.8.2. Treatment of systemic candidosis

Groups of seven mice were infected i.v. with 2×10^5 *C. albicans* (CA-6) blastoconidia (0.5 ml per mouse) via the lateral tail vein. The mice were treated i.p. 24 h after infection with 0.5 ml of single doses (0.7, 0.35, 0.175, 0.12, 0.08 mg/kg of body weight) of free Leu-A or Leu-A entrapped in NS. Mice were observed daily for 30 days and deaths were recorded. Nine days after infection, three mice per group were killed by CO_2 asphyxiation, kidneys were aseptically excised, weighed, homogenized, diluted, and cultured in duplicate to evaluate the yeast colony clearance.

The value of colony forming units (CFU) was determined by a plate dilution method. Colonies of *C. albicans* cells were counted after 48 h of incubation at room temperature and results were expressed as the number of CFU per organ.

The results were expressed as % reduction of CFU recovered from the kidneys of three mice. This procedure was performed on three groups of mice each. The confidence interval was also calculated at $\alpha = 0.05$ significance level and $n = 3$.

3. Results and discussion

3.1. Preparation and characterization of Leu-A NS

Leu-A NS were prepared as previously reported (Niwa et al., 1994), by using PLGA with high molecular weight (MW = 90,100) in order to obtain particles with suitable peptide loading. In fact, generally, the faster deposition of higher molecular weight polymers at the interface between the droplets and the aqueous medium can partially prevent leakage of slightly water-soluble drugs and hence improve NS trapping efficiency. This effect along with the high lipophilicity of such polymers is supposed to enhance the carrier entrapment efficiency of amphiphilic low water-soluble compounds, such as Leu-A.

The high acetone/CH_2Cl_2 volume (ml) ratio (12.5:0.25) was used to ensure the production of particles of 200–300 nm in size with a standard deviation of ±10–24 nm, indicating a quite narrow population distribution (Table 1).

Table 1
Preparation process parameters influencing Leu-A encapsulation in biodegradable polymeric nanospheres

Sample	Leu-A/PLGA (%, w/w)	Evaporation times (h)	PVA (%)	pH	Acetone/CH_2Cl_2 (ml)	Drug content (%) ± S.D.	Drug recovery (%) ± S.D.	Encapsulation efficiency (%) ± S.D.	Mean diameter (nm) ± S.D.
1	5	8	1	7.4	12.5–0.25	0.18 ± 0.04	2.9 ± 0.7	3.6 ± 0.8	198 ± 18
2	5	8	0.3	7.4	12.5–0.25	0.2 ± 0.06	3.0 ± 0.9	4.0 ± 0.8	250 ± 23
3	5	8	0.1	7.4	12.5–0.25	0.22 ± 0.04	3.3 ± 0.4	6.6 ± 0.7	310 ± 20
4	5	8	0	7.4	12.5–0.25	–[a]	–[a]	–[a]	322 ± 22
5	5	4	0.3	7.4	12.5–0.25	0.4 ± 0.03	6 ± 2	8.6 ± 0.9	283 ± 21
6	5	4	0.3	8	12.5–0.25	0.3 ± 0.03	5 ± 1	6 ± 1	254 ± 10
7	5	4	0.3	8.5	12.5–0.25	0.2 ± 0.02	3 ± 1	4.2 ± 0.8	309 ± 24
8	10	4	0.3	7.4	12.5–0.25	0.5 ± 0.03	4.2 ± 0.9	5 ± 1	227 ± 12
9	10	4	0.1	7.4	12.5–0.25	0.6 ± 0.04	4.8 ± 0.5	6.1 ± 0.9	338 ± 24
10	10	4	0.3	7.4	10–2.75	1.1 ± 0.09	7.2 ± 0.2	11 ± 1	228 ± 20
11	10	4	0.1	7.4	12.5–0.25	1.7 ± 0.08	8.1 ± 0.8	17 ± 1	263 ± 24
12	10	4	0.1	7.4	10–2.75	2.8 ± 0.10	24.6 ± 0.4	28.3 ± 2	201 ± 14

[a] Nanospheres not formed.

Unfortunately, in these conditions Leu-A entrapment was low, irrespective to PLGA MW (sample 1, Table 1). Therefore, other preparation parameters, such as PVA%, evaporation time, pH, dichloromethane/acetone, and Leu-A/PLGA ratios, were modified in order to obtain a formulation with the desired characteristics.

Lowering PVA% in the aqueous medium led to an improvement in encapsulation efficiency from 3.6 to 6.6%. This result is in agreement with the fact that high surfactant concentrations favor amphipathic molecule partition, such as Leu-A, in the continuous phase (Feng and Huang, 2001). Constant NS size increase was observed when PVA concentration was reduced and no nanospheres were formed when PVA was absent even though a certain peptide emulsifying effect was expected.

The Leu-A amount entrapped in the NS was more than doubled when evaporation time was reduced from 8 to 4 h. From this result, we can infer that long evaporation times, although ensuring complete elimination of organic solvent residues, may induce passive Leu-A diffusion from the polymeric matrix.

In order to assess the best process conditions for NS preparation, pH effect on Leu-A encapsulation was investigated as well. In this respect, preliminary peptide solubility versus pH studies were necessary to determine the pH value that causes the lower peptide solubility without inducing a rapid polymer hydrolysis. The low water solubility of the molecule in fact may limit its diffusion in the continuous phase during the preparation process.

Solubility tests accomplished in various buffers solutions, at different pH values, showed that, in the range between pH 2 and pH 7.4, there is a constant solubility decrease that suddenly dropped just above pH 7.4 (Fig. 2).

On the basis of these results, three different pH values (7.4, 8, 8.5) were used to carry out NS preparation. The pH effect on the solubility of the molecule was expected to modify the partition of the peptide between the organic and aqueous phase, favoring Leu-A distribution mainly in the acetone/dichloromethane medium. Surprisingly, we observed practically no effect on the peptide encapsulation on pH increase (Table 1). This is in contrast with the fact that a lower water solubility should favor the entrapment of the peptide into the polymeric matrix during the organic solvent evaporation process. Therefore, additional factors need to be considered in order to explain such behavior: (i) the fast acetone diffusion into the aqueous phase may drag Leu-A in the external phase at a much higher rate than PLGA film deposition at the interface between water and organic phase, (ii) Leu-A O/W distribution coefficient that opposes acetone effect, especially at highest pH value, (iii) the possible ionic interaction between the peptide positive charge and polymer negative charge. We can hypothesize that increasing the amount of peptide free base with pH, the ionic interaction with the polymer is reduced and the acetone effect is limited only by the high solubility of the peptide in dichloromethane. On the contrary, when pH $<$ pK_a the effect of Leu-A/polymer interactions coupled to the high solubility of the peptide in dichloromethane (even at pH $=$ 7.4), oppose the acetone effect, determining a higher entrapment in the polymeric matrix.

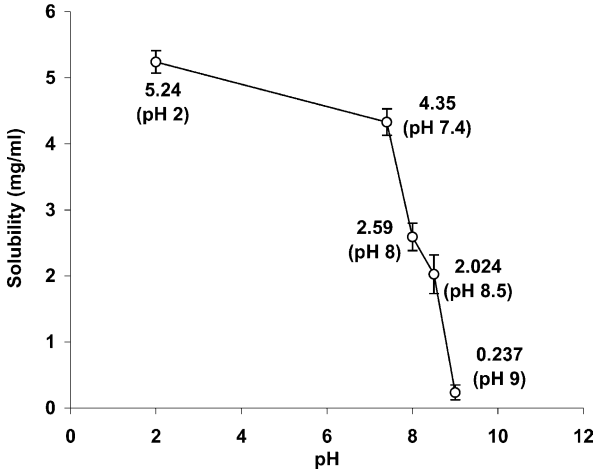


Fig. 2. Leu-A solubility as function of pH.

Prevalence of acetone diffusion over other factors may be the reason for such a low Leu-A entrapment obtained so far.

In order to achieve a higher peptide entrapment efficiency, the effect of additional parameters, such as Leu-A/polymer (w/w) and acetone/dichloromethane ratios, were investigated.

As expected, higher theoretical loading from 5 to 10% (w/w) corresponded to higher drug contents that were further increased by reducing the amount of PVA.

However, among the parameters investigated, the acetone/CH_2Cl_2 ratio resulted the most important to achieve higher encapsulation efficiencies.

In fact, the best result was obtained by fixing: (a) the theoretical loading of Leu-A in the formulation at 10% (w/w), (b) reducing the PVA amount in the aqueous phase from 1 to 0.1% (w/w), (c) changing the acetone/CH_2Cl_2 ratio from 12.5–0.25 to 10–2.75, and (d) maintaining the evaporation time for 4 h under low vacuum. Under these conditions, the highest peptide recovery (Table 1) (24.6%), drug content (2.83%), and encapsulation efficiency (28.3%) were obtained, with a process yield of 81.1%.

Moreover, it is important to point out that, when using the 10–2.75 acetone/CH_2Cl_2 ratio instead of the 12.5–0.25 ratio, the increase of PVA concentration (from 0.1 to 0.3%), although preventing fine droplets coalescence during stirring, determines a remarkable encapsulation efficiency drop from 28.3 to 11% (Table 1). This effect can be addressed to the reduced contribution of acetone diffusion to the peptide leakage when the 10–2.75 acetone/CH_2Cl_2 ratio is employed and, in this condition, the surfactant concentration may become the main factor affecting peptide entrapment.

The Leu-A entrapment obtained suggests that the molecule always tends to move in the bulk aqueous phase in spite of its low solubility and that this process is favored by either the acetone spontaneous diffusion or surfactant concentration that are the main parameters affecting NS preparation optimization.

Granulometric assays (Fig. 3A) as well as SEM analysis (Fig. 3B) highlighted the formation of a less homogeneous particle size distribution with higher polydispersity and a small microparticle population at around 5 μm. The presence of microparticles was due to the increased interface tension, generated by a higher concentration of dichloromethane, that caused larger droplet sizes (Niwa et al., 1993). However, proper filtration allowed the complete removing of the microparticle population (Fig. 4A). In addition, PCS analyses, performed on the filtered batches, showed a colloidal size distribution ranging between 98 and 889 nm (201.0 nm mean diameter) (Fig. 4B). Scanning electron micrographs of filtered NS (Fig. 4C) showed the presence of discrete spherical NS having diameters around 300 nm in agreement with PCS analyses and the absence of particle aggregates as well as Leu-A precipitates.

3.2. In vitro release

Leu-A release profile from the best NS preparation (sample 12, Table 1) was investigated over a period of 100 h (Fig. 5). The results indicated a biphasic pattern with an initial burst effect with about 45% of the peptide released within the first 10 h followed by a linear release profile.

The initial burst can be explained considering the mechanism of NS production and consequently Leu-A distribution into the polymer matrix.

In fact, Leu-A has a good solubility in acetone which, diffusing toward the aqueous phase, not only enhances significantly the peptide leakage from NS but also promotes its distribution in proximity of the oil–water interface. This determines an immediate Leu-A release that is enhanced by the fast hydration process as well. Hydration occurs very rapidly because of particles nanometric size. Additional contribution to the initial burst can be ascribed to the surface-bound drug desorption as well.

After 10 h the rate of release is mainly controlled by the slow degradation of the high molecular weight polymer used, resulting in a pattern matching closely a zero order profile kinetics. The total amount of drug released was more than 93% of the entrapped peptide. These findings showed that such formulation is able to provide a suitable release profile and exerts an adequate control on the drug diffusion rate.

3.3. In vivo toxicity and effectiveness against *Candida albicans*

Remarkable results were obtained from in vivo acute toxicity experiments (Fig. 6). In fact, dose–survival curves pointed out the great effectiveness of nanoparticle system on reducing the toxicity of the peptide compared to Leu-A aqueous solution. In fact, the Leu-A LD_{50} extrapolated from the graph was about 1.2 mg/kg when Leu-A was employed as free drug, whereas it showed an increase of more than 18-fold when the peptide was entrapped in the NS. This result was confirmed by the fact that no decrease resulted in the concentration range investigated. These data can be correlated with the decrease of Leu-A

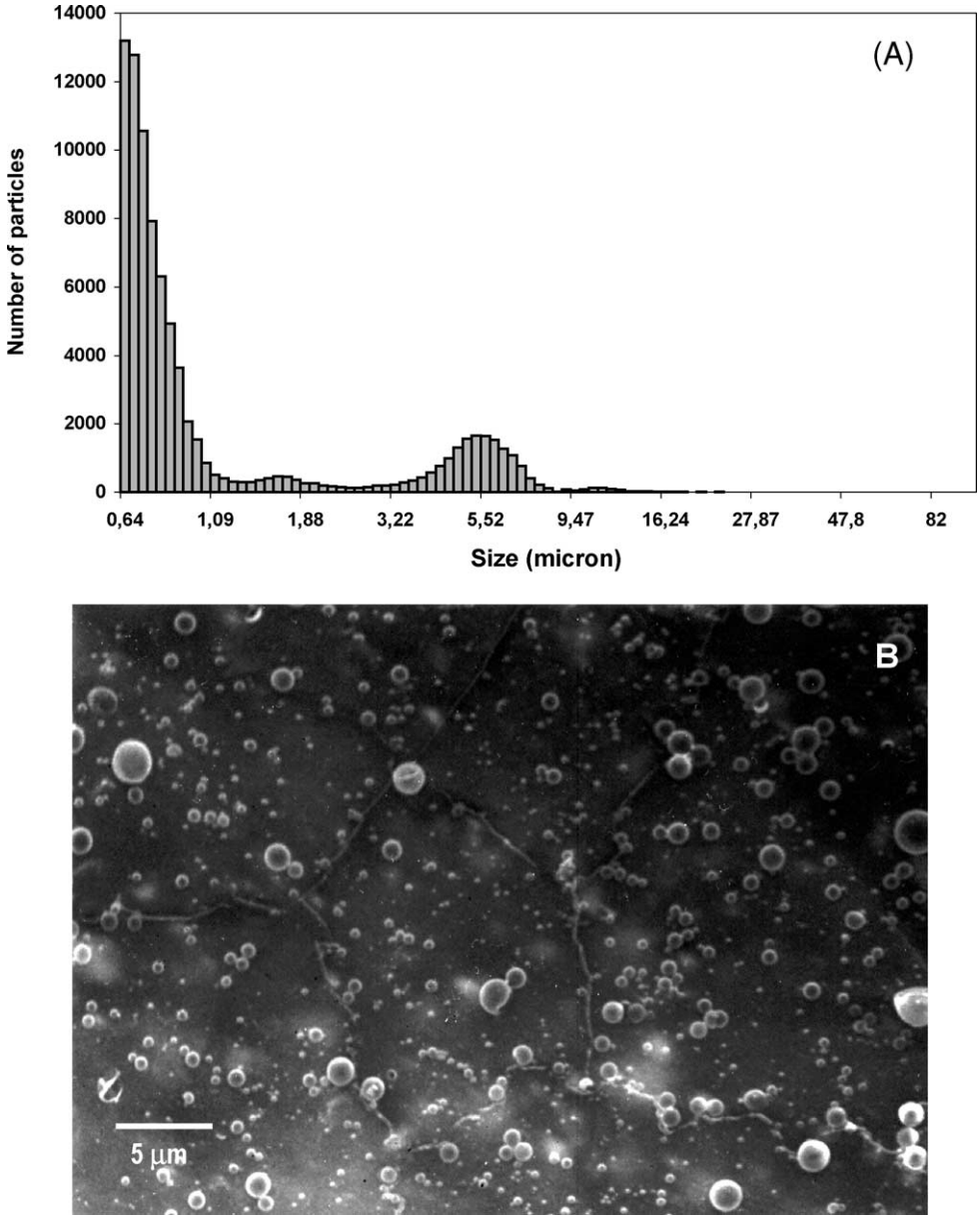


Fig. 3. Freshly Leu-A loaded NS: (A) particle size distribution, (B) SEM micrograph.

acute toxicity that confirm the control exerted by the carrier on the peptide release rate. It was not possible to determine the exact LD_{50} value for the NS because of the excessively large injection volume required that could modify the physiological mice conditions.

Studies performed on *C. albicans*-infected mice models showed significant results as well. Comparing the data reported in Table 2, we can infer that free Leu-A induces a dramatic reduction in fungal colonies demonstrating that the peptide, at these concentrations, reduces the infection without causing tissue damages. This behavior could be explained with the fact that the peptide distributes, to a large extent, in the kidney, where it interacts mainly with fungal

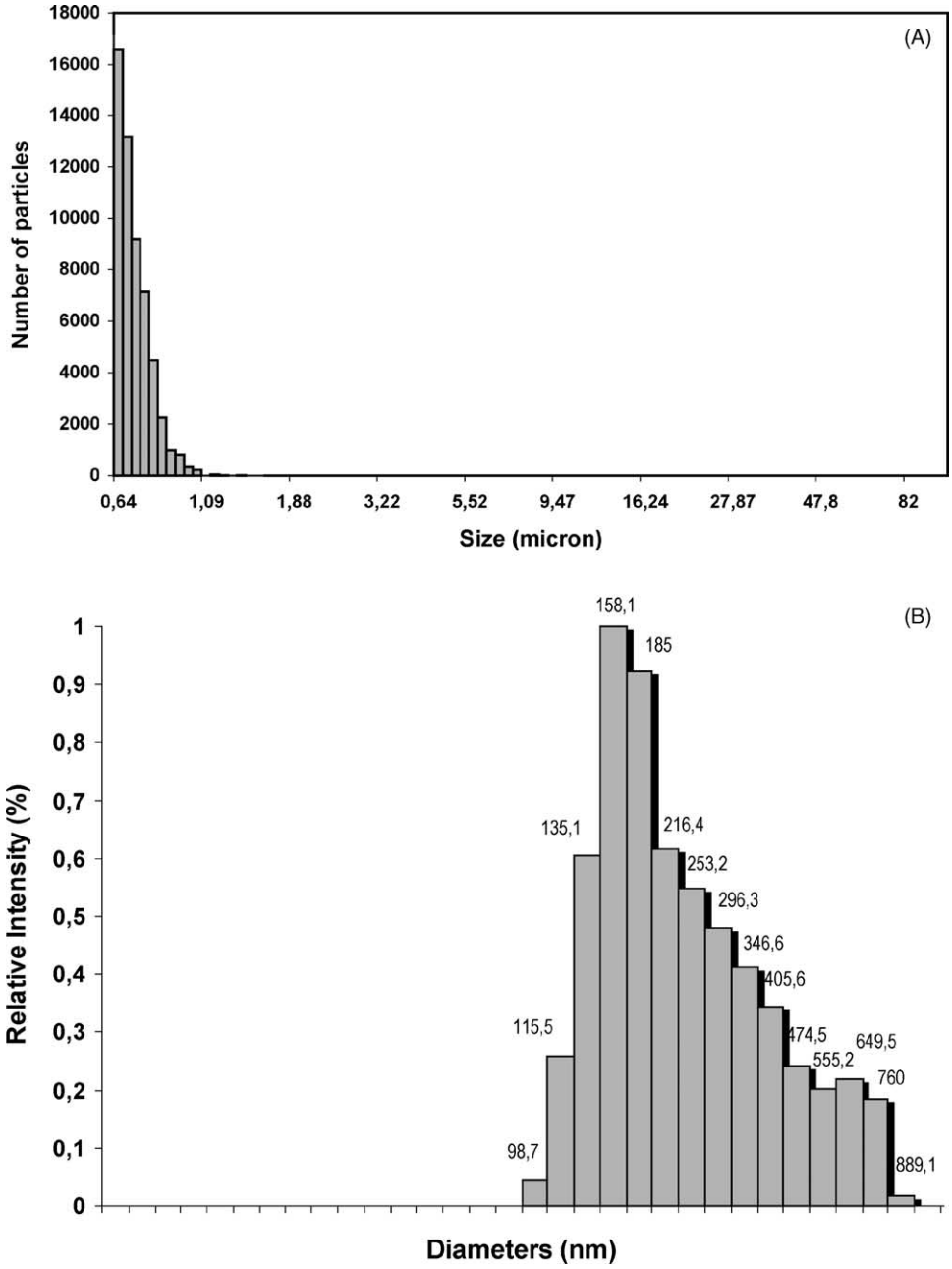


Fig. 4. Leu-A loaded NS after filtration: (A) particle size distribution, (B) PCS analysis, (C) SEM micrograph.

rather than mammalian membranes. Unfortunately, high doses of free Leu-A were very toxic to the mice as shown by mortality percent. On the contrary, when Leu-A is entrapped in NS a high effectiveness was shown even at high doses, because this formulation reduces either growth of fungal colonies or mortality. The mechanism by which NS improve the therapeutic index of Leu-A in the treatment of disseminated

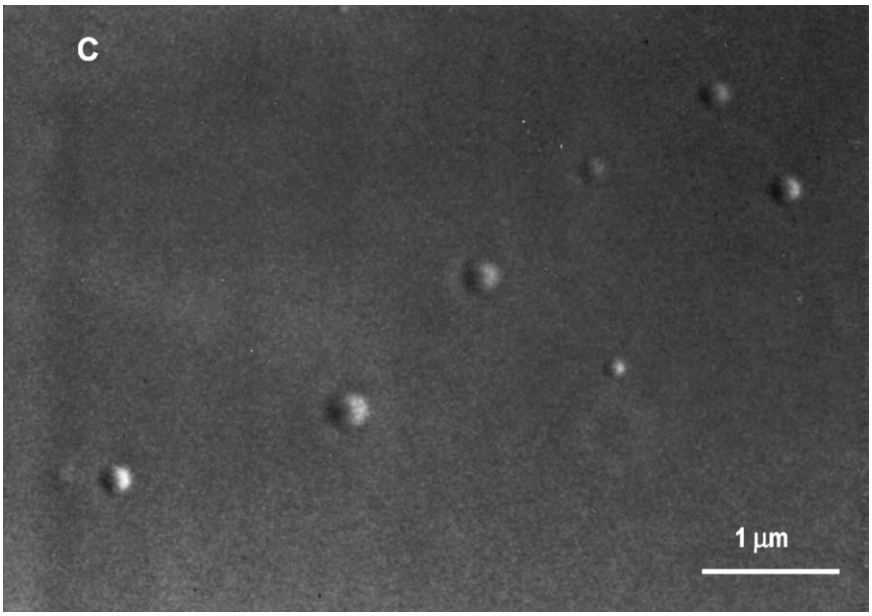


Fig. 4. (*Continued*).

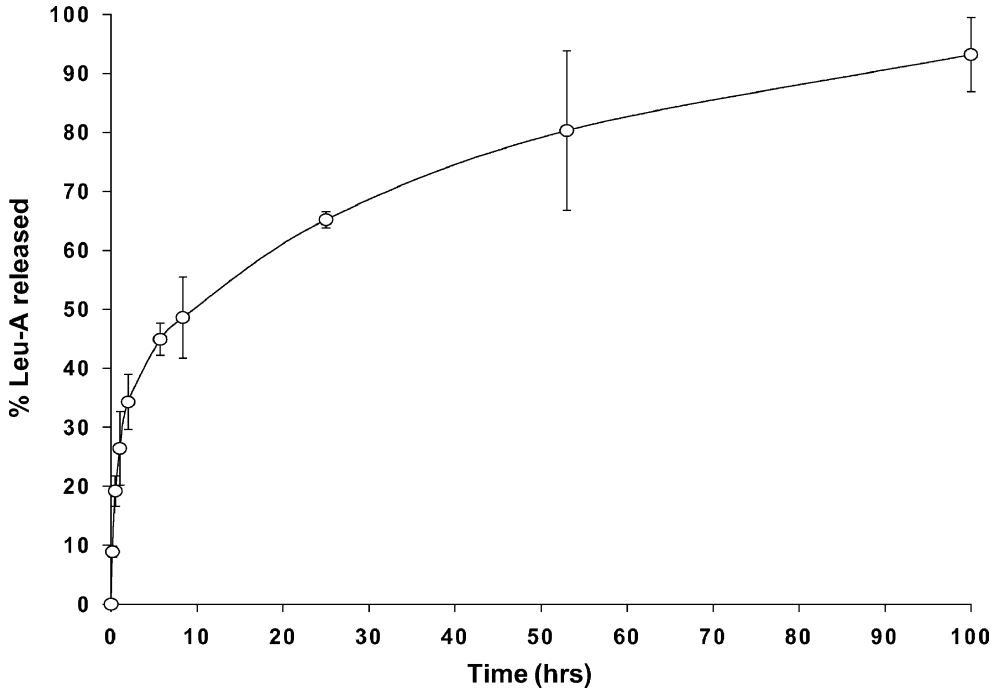


Fig. 5. Leu-A release from PLGA NS incubated at 37 °C for 100 h under mechanical shaking (160 rpm).

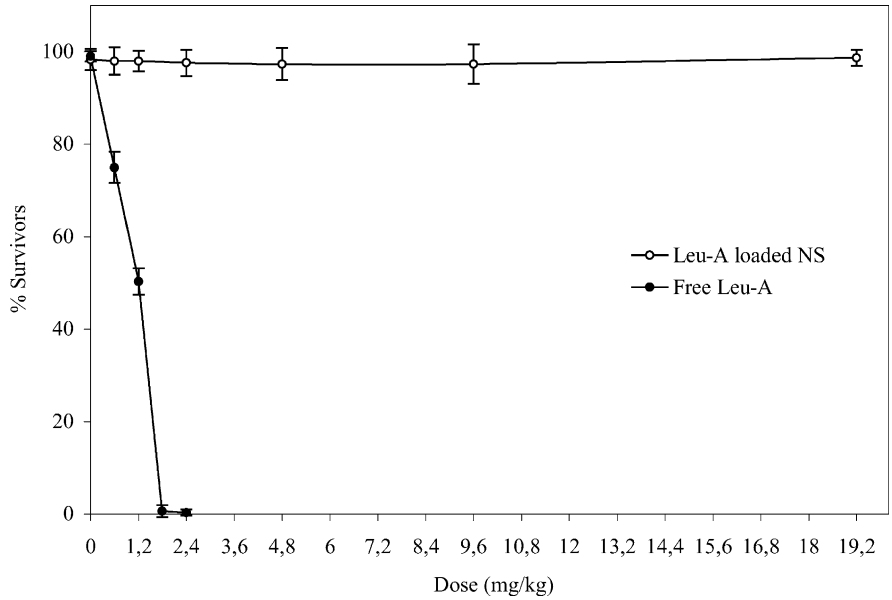


Fig. 6. Dose–mortality curves for free Leu-A and Leu-A loaded NS injected via i.p. in CD2F1 female mice. Error bars are reported as confidence interval at 0.05 significance level and $n = 10$.

Table 2
Leu-A loaded NS efficacy in *Candida*-infected mice model: data relative to mortality and inhibition of *Candida albicans* colonies growth

Dose (mg/kg)	% Reduction of colonies number ± confidence ($\alpha = 0.05$, $n = 3$)	MST[a] (days)	D/T[b]
Free Leu-A			
Control[c]	0	10	7/7
0.7	81 ± 2	13	4/7
0.35	73 ± 3	19	3/7
0.175	61 ± 2	–	0/7
0.12	50 ± 2	–	0/7
0.08	0.1 ± 0.6	10	7/7
Leu-A in NS			
Control[c]	0	10	7/7
0.7	90 ± 2	–	0/7
0.35	80 ± 1	–	0/7
0.175	55 ± 3	–	1/7
0.12	40 ± 2	12	3/7
0.08	0.2 ± 0.6	10	7/7

Confidence is also reported at 0.05 significance level and $n = 3$.
[a] Median survival time.
[b] Dead mice over total animals tested after 30 days.
[c] Mice treated with phosphate-buffered saline.

candidiasis in this model is not completely understood. We can hypothesize that nanospheres release the peptide very slowly, thus maintaining a Leu-A concentration in the organism under the toxic level. It is noteworthy to point out that a dose of 0.175 mg/kg is enough to exert a therapeutic action on this type of infection. Lower doses of the peptide (0.12 and 0.08 mg/kg) produce a high mortality that can be explained by the fact that the peptide released is not enough to restrain *Candida* growth.

4. Conclusions

In conclusion, we can suppose that Leu-A loaded NS at 0.35 mg/kg could represent a new effective therapeutic system for *Candida* infection. In fact, we demonstrated that by selecting proper conditions it is possible to obtain Leu-A loaded NS with suitable amount of entrapped peptide, shape, size, and good in vitro sustained release pattern.

In vivo experiments showed a drastic reduction of Leu-A toxicity (therapeutic index increase) along with an increased efficacy of the compound when entrapped in NS. Therefore, this formulation is able to maintain a drug hematic level sufficiently high to ensure activity

against *Candida* without determining lethal side effects. These encouraging results represent a good base to further develop such carriers as vectors for this interesting peptide. In particular, NS surface coating is needed to reduce the uptake by the immune system in order to prolong NS blood half-life and make possible their intravenous administration.

References

Andriole, V.T., 1993. Infections with *Aspergillus* species. Clin. Infect. Dis. 17, S841–S846.

Andriole, V.T., 2000. Current and future antifungal therapy: new targets for antifungal therapy. Int. J. Antimicrob. Agents 16, 317–321.

Bodey, G.B., Duguid, B., Gibbs, W., Hanak, D.H., Hotchi, M., Mall, G., Martino, P., Meunier, F., Milliken, S., et al., 1992. Fungal infection in cancer patients: an international autopsy survey. Eur. J. Clin. Microbiol. Infect. Dis. 11, 99–109.

Brummer, E., 1999. Human defenses against *Cryptococcus neoformans*: an update. Mycopathologia 143, 121–125.

Canuto, M.M., Rodero, F.G., 2002. Antifungal drug resistance to azoles and polyenes. Lancet Infect. Dis. 2, 550–563.

Casinovi, C.G., Tuttobello, L., Rossi, C., Benciari, Z., 1983. Two phytotoxic antibiotic peptides produced by submerged cultures of *Paecilomyces marquandii* (Massee) Hughes. Phytopathol. Medit. 22, 209–211.

Casinovi, C.G., Rossi, C., Tuttobello, L., Ricci, M., 1986. The structure of Leucinostatin C. A minor peptide from *Paecylomyces marquandii*. Eur. J. Med. Chem. Chim. Ther. 21, 527–528.

Csermely, P., Radics, L., Rossi, C., Szamel, M., Ricci, M., Mihaly, K., Somogyi, J., 1994. The nonapeptide leucinostatin A acts as a weak ionophore and as an immunosuppressant on T lymphocytes. Biochim. Biophys. Acta 1221, 125–132.

De Lucca, A.J., Walsh, T.J., 1999. Antifungal peptides: novel therapeutic compounds against emerging pathogens. Antimicrob. Agents Chemother. 43, 1–11.

Ellis, M., 2002. Invasive fungal infections: evolving challenges for diagnosis and therapeutics. Mol. Immunol. 38, 947–957.

Feng, S.-S., Huang, G., 2001. Effects of emulsifiers on the controlled release of paclitaxel (Taxol®) from nanospheres of biodegradable polymers. J. Control. Release 71, 53–69.

Fresta, M., Ricci, M., Rossi, C., Furieri, P.M., Puglisi, G., 2000. Antimicrobial nonapeptide Leucinostatin A dependent effects on physical properties of phospholipid model membranes. J. Colloid Interface Sci. 226, 222–230.

Ghannoum, M.A., 1997. Future of antimycotic therapy. Dermatol. Ther. 3, 104–111.

Mikami, Y., Fukushima, K., Arai, T., Abe, F., Shibuya, H., Ommura, Y., 1984. Leucinostatins, peptide mycotoxins produced by *Paecilomyces lilacinus* and their possible roles in fungal infection. Zbl. Bakt. Hyg. 257, 275–283.

Milan, E.P., Burattini, M.N., Kallás, E.G., Fishmann, O., Costa, P.R.de O., Colombo, A.L., 1998. Azole resistance among oral *Candida* species isolates from AIDS patients under ketoconazole exposure. Diagn. Microbiol. Infect. Dis. 32, 211–216.

Nguyen, M.H., Peacock, J.E., Morris, A., 1996. The changing face of candidemia: emergence of non-*Candida albicans* species and antifungal resistance. Am. J. Med. 100, 617–623.

Niwa, T., Takeuchi, H., Hino, T., Kunou, N., Kawashima, Y., 1993. Preparation of biodegradable nanospheres of water soluble and insoluble drugs with D,L-lactide/glycolide copolymer by a novel spontaneous emulsification solvent diffusion method, and the drug release behavior. J. Control. Release 25, 89–98.

Niwa, T., Takeuchi, H., Hino, T., Kunou, N., Kawashima, Y., 1994. L-Lactide/glycolide copolymer (PLGA) nanospheres with nafarelin acetate prepared by a novel spontaneous emulsification solvent diffusion method. J. Pharm. Sci. 83, 727–732.

Oh, J.E., Hong, S.Y., Lee, K., 1999. The comparison of characteristics between membrane-active antifungal peptide and its pseudopeptides. Bioorg. Med. Chem. 7, 2509–2515.

Petrocheilou-Paschou, V., Georgilis, K., Kontoyannis, D., Nanas, J., Prifti, H., Costopoulos, H., Stamatelopoulos, S., 2002. Pneumonia due to *Candida krusei*. Clin. Microbiol. Infect. 8, 806–809.

Ricci, M., Sassi, P., Nastruzzi, C., Rossi, C., 2000. Liposome-based formulations for the antibiotic nonapeptide Leucinostatin A. FTIR spectroscopic characterization and in vivo toxicological study. AAPS Pharm. Sci. Tech. 1 (article 2).

Rossi, C., Tuttobello, L., Ricci, M., 1987. Leucinostatin D, a novel peptide antibiotic from *Paecilomyces marquandii*. J. Antibiot. 40, 130–133.

Rossi, C., Ricci, M., Tuttobello, L., Cerrini, S., Scatturin, A., Vertuani, G., Ambrogi, V., Perioli, L., 1990. Leucinostatin F. A minor co-metabolite. Acta Technol. Legis Med. 1, 109–112.

Sheehan, D.J., Hitchcock, C.A., Sibley, C.M., 1999. Current and emerging azole antifungal agents. Clin. Microbiol. Rev. 12, 40–79.

Shima, A., Fukushima, K., Arai, T., Terada, H., 1990. Dual inhibitory effects of the peptide antibiotics leucinostatins on oxidative phosphorylation in mitochondria. Cell Struct. Funct. 15, 53–58.

Sokolov, Y., Mirzabekov, T., Martin, D.W., Lehrer, R.I., Kagan, B.L., 1999. Membrane channel formation by antimicrobial protegrins. Biochim. Biophys. Acta 1420, 23–29.

Strobel, G.A., Torczynski, R., Bollon, A., 1997. *Acremonium* sp.—a leucinostatin A producing endophyte of European yew (*Taxus baccata*). Plant Sci. 128, 97–108.

Subbalakshmi, C., Sitaram, N., 1998. Mechanism of antimicrobial action of indolicidin. FEMS Microbiol. Lett. 160, 91–96.

Verduyn Lunel, M.F., Meis, J.F.G.M., Voss, A., 1999. Nosocomial fungal infections: candidemia. Diagn. Microbiol. Infect. Dis. 34, 213–220.

Walsh, T.J., Groll, A.H., 1999. Emerging fungal pathogens: evolving challenges to immunocompromised patients for the twenty-first century. Transplant Infect. Dis. 1, 247–261.

Warnock, D.W., Arthington-Skaggs, B.A., Li, R., 1999. Antifungal drug susceptibility testing and resistance in *Aspergillus*. Drug Resist. Updates 2, 326–334.

Wingard, J.R., 1995. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patient. Clin. Infect. Dis. 20, 115–125.