

## Tuftsins-bearing liposomes as drug vehicles in the treatment of experimental aspergillosis

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Encapsulation of amphotericin B in tuftsins-bearing liposomes greatly increased its efficacy in treatment of human aspergillosis in mice. Also, the drug efficacy was significantly increased by pretreating the animals with drug-free tuftsins-bearing liposomes. These results demonstrate that macrophage activation can considerably enhance the therapeutic efficacy of antifungal drugs, like amphotericin B.

Tuftsins; Macrophage activation; Fungal infection; Liposome; Amphotericin B

### 1. INTRODUCTION

Tuftsins is a natural killer activator of macrophages/monocytes [1] which can easily be grafted onto the surface of liposomes [2] by attachment of a fatty acyl chain through an ethylenediamine spacer arm (Thr-Lys-Pro-Arg-NH-(CH<sub>2</sub>)<sub>2</sub>-NH-COC<sub>15</sub>H<sub>31</sub>) to its C-terminus. The tuftsins-bearing liposomes (Tuft-Lip) thus formed have been shown to be quite effective in activating the host's macrophages [3] and also in increasing non-specific resistance against parasitic infections [4,5].

Elimination of fungi from the tissues of normal healthy subjects is often associated with stimulation of cell-mediated immune defences which, for antifungal activity, essentially involve activation of mononuclear phagocytes by sensitized T cells [6]. The main function of these T cells in this case is to release lymphokines which in turn arm the macrophages to exhibit the fungistatic or fungicidal activity [6]. In the case of immune compromised individuals, however, these mechanisms do not operate optimally due to impaired cellular immunity [6]. Since these individuals are the main target of systemic fungal infections, treatment of such patients with antifungal drugs along with some agent which can provoke macrophages/monocytes for their normal function should afford obvious advantages over conventional chemotherapy. Keeping this in view, we considered it of interest to evaluate the usefulness of Tuft-

Lip as amphotericin B (Amp B) vehicles in the treatment of experimental aspergillosis, as these liposomes, besides reducing drug toxicity [7–10], are also expected to activate the host's macrophages due to the presence of the tuftsins on their surface [3].

### 2. MATERIALS AND METHODS

#### 2.1. Materials

All the reagents used in the study were of the highest purity available. Cholesterol was bought from Centron Research Laboratory, Bombay, and used after crystallizing it three times from methanol. Egg phosphatidylcholine (egg PC) was isolated and purified according to the published procedure [11]. Tuftsins modified at the C-terminus was prepared as described earlier [2]. Amp B was purchased from Sigma Chemical Co.

#### 2.2. Liposomes

Liposomes were prepared from egg PC (49  $\mu$ mol) and cholesterol (21  $\mu$ mol) with or without modified tuftsins (7–8% by PC weight). All the ingredients, along with Amp B (1.0 mg), were dissolved in a round-bottomed flask in a minimum volume of chloroform/methanol (1:1, v/v). The solvents were carefully removed under reduced pressure so that a thin lipid film formed on the wall of the flask. Final traces of the solvent were removed by leaving the flask in vacuo overnight at 4°C. The dried lipid film was hydrated with 2.0 ml of 150 mM sterile saline under vigorous stirring for 1 h in a bath-type sonicator under N<sub>2</sub> atmosphere. The sonicated preparation was dialysed against saline for 24 h at 4°C in dark, and then centrifuged at 10,000  $\times g$  for 1 h at 4°C to remove traces of undispersed lipids. The supernatant was analysed for both Amp B and tuftsins.

#### 2.3. Estimation of Amp B and tuftsins

The amount of liposome-intercalated Amp B was determined by measuring its absorbance at 405 nm. The intercalation efficiencies of Amp B in tuftsins-bearing and tuftsins-free liposomes were about 95% and 85%, respectively.

The amount of tuftsins incorporated into liposomes was estimated as described earlier [2]. The incorporation efficiency was found to be about 98%.

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## 2.4. Animals

Male Balb/C mice weighing  $18 \pm 2$  g were used in the study. The animals were given a pellet diet (Hindustan Lever Ltd.) and water ad libitum.

## 2.5. Infection

*Aspergillus fumigatus* (strain VP<sub>236</sub>) originally isolated from an aspergillosis patient at V.P. Chest Institute, New Delhi, was maintained in vitro and also in animals. The animals were given a known number of spores intravenously 24 h prior to the drug treatment. For prophylactic studies, the infection was given after treating the animals with drug-free Tuft-Lip.

## 2.6. Treatment

Two sets of experiments were carried out under this study. In the first set, the efficacy of the various drug formulations was determined against the *A. fumigatus* infections by measuring both the fungal load (CFU) in various organs and survival time of the animals. In the second set, the effect of tuftsin-mediated macrophage activation on the therapeutic efficacy of Amp B was analysed. In this case, the antifungal activity of Amp B was evaluated in the animals that were treated with Tuft-Lip prior to infection.

### 2.6.1. First set

The infected animals were divided into 5 groups, and then treated by being given as follows: Group I, normal saline; Group II, drug-free Tuft-Lip; Group III, drug-free, Tuft-free liposomes; Group IV, Tuft-free Lip-Amp B; Group V, Tuft-Lip-Amp B.

### 2.6.2. Second set

The animals were first divided into two groups. The first group was pretreated for 3 consecutive days with Tuft-Lip (50  $\mu$ g Tuft/animal/day) on day 1–3 prior to infection, while the second group was left untreated. Each group, after being infected with *A. fumigatus* ( $\sim 1.8 \times 10^7$  spores/animal) was further divided into 3 groups; thus the animals were divided into a total of six groups. Group I, pretreated animals given no Amp B (PT(+),DT(-)); Group II, pretreated animals given Lip-Amp (PT(+),Lip-Amp B); Group III, pretreated animals given Tuft-Lip-Amp B (PT(+),Tuft-Lip-Amp B); Group IV, untreated animals given no drug (PT(-),DT(-)); Group V, untreated animals given Lip-Amp B (PT(-),Lip-Amp B); Group VI, untreated animals given Tuft-Lip-Amp B (PT(-), Tuft-Lip-Amp B).

For CFU determination, 3 animals were taken out from each group of all the three sets. The animals were sacrificed and livers, lungs, kidneys and spleens were dissected out aseptically. The organs were minced in normal saline (5 ml) and 200  $\mu$ l of this suspension was plated on rich media, and the plates were incubated for 48 h at 37°C. The

Table I

Effect of Lip-Amp B on *A. fumigatus* infections in Balb/c mice

| Formulation    | Survival on day 7 post-treatment |         |          |         |
|----------------|----------------------------------|---------|----------|---------|
|                | Exp. I                           | Exp. II | Exp. III | Exp. IV |
| Saline         | 0/4                              | 0/5     | 0/10     | 0/14    |
| Free drug      | —                                | 0/5     | 0/10     | —       |
| Empty Liposome | 0/4                              | —       | —        | —       |
| Empty Tuft-Lip | 0/4                              | —       | —        | —       |
| Lip-Amp B      | 3/8                              | 3/10    | 9/25     | 4/11    |
| Tuft-Lip-Amp B | 6/8                              | 11/15   | 19/25    | 9/13    |

Drug dose, 0.5 mg/kg (single, i.v.); challenge dose,  $\sim 1.8 \times 10^7$  spores/animal.

colonies were counted and the fungal load calculated by multiplying with the dilution factor.

## 3. RESULTS AND DISCUSSION

Balb/c mice infected with *A. fumigatus* ( $\sim 1.8 \times 10^7$  spores/animal) were treated with various doses (single, intravenous) of Amp B encapsulated in egg PC/cholesterol liposomes. No significant antifungal effects were observed when the administered dose of Amp B was  $< 0.5$  mg/kg, but at a dose of 0.5 mg/kg, at least 35% of the infected animals survived after the treatment. This dose was therefore selected for comparing the antifungal effects of Lip-Amp B with Tuft-Lip-Amp B. Table I shows that the % survival of the infected animals was considerably increased (70–75%) by treating the animals with Tuft-Lip-Amp B. In addition, the animals that had survived by day 7 after the Tuft-Lip-Amp B treatment were virtually free of fungal infection as compared to those treated with Lip-Amp B (Table II). These results demonstrate that the efficacy of Lip-Amp B against *A. fumigatus* infection is considerably increased by grafting tuftsin onto the liposomes surface.

Table II

Fungal load in animals that survived infection after treatment with liposomised Amp B

| Organ  | Fungal load (CFU) |                 |                |                 |
|--|-------------------|-----------------|----------------|-----------------|
|  | Exp. I            | Exp. II         | Exp. III       | Exp. IV         |
| <i>Animals that survived from the Lip-Amp B-treated group</i>      |                   |                 |                |                 |
| Liver  | 187 $\pm$ 88      | 307 $\pm$ 75    | 258 $\pm$ 38   | 192 $\pm$ 21    |
| Lung   | 625 $\pm$ 0       | 657 $\pm$ 50    | 508 $\pm$ 52   | 692 $\pm$ 63    |
| Kidney   | 625 $\pm$ 176     | 533 $\pm$ 74    | 475 $\pm$ 25   | 483 $\pm$ 16    |
| Spleen   | 2,312 $\pm$ 618   | 1,282 $\pm$ 122 | 1,525 $\pm$ 44 | 1,785 $\pm$ 194 |
| <i>Animals that survived from the Tuft-Lip-Amp B-treated group</i> |                   |                 |                |                 |
| Liver  | 0                 | 0               | 0              | 0               |
| Lung   | 0                 | 0               | 0              | 0               |
| Kidney   | 0                 | 50*             | 25*            | 0               |
| Spleen   | 0                 | 0               | 25*            | 0               |

The fungal load was determined on day 7 post-treatment. Values are means of 3 animals  $\pm$  S.D. \*Fungal load in one animal.

Table III  
Effect of Tuft-Lip pretreatment on the efficacy of Lip-Amp B against fungal infections

| Treatment            | Survival on day 7<br>post-drug treatment | Fungal load (CFU) 24 h after drug treatment |                |                |                |
|----------------------|--|---|----------------|----------------|----------------|
|                      |  | Liver                                       | Lung           | Kidney         | Spleen         |
| PT(-),DT(-)          | 0/7                                      | 71,042 ± 3,442                              | 53,542 ± 2,602 | 19,166 ± 2,194 | 39,170 ± 1,301 |
| PT(-),Lip-Amp B      | 4/12                                     | 23,125 ± 1,654                              | 13,333 ± 954   | 7,708 ± 360    | 17,916 ± 954   |
| PT(-),Tuft-Lip-Amp B | 9/12                                     | 9,167 ± 954                                 | 2,291 ± 360    | 5,208 ± 360    | 16,875 ± 1,275 |
| PT(+),DT(-)          | 1/7                                      | 29,583 ± 2,525                              | 20,833 ± 2,009 | 14,375 ± 1,875 | 33,958 ± 954   |
| PT(+),Lip-Amp B      | 6/7                                      | 2,916 ± 721                                 | 1,875 ± 625    | 4,792 ± 721    | 13,541 ± 954   |
| PT(+),Tuft-Lip-Amp B | 6/7                                      | 2,708 ± 360                                 | 1,041 ± 360    | 2,500 ± 625    | 12,708 ± 360   |

Drug dose, 0.5 mg/kg (single, i.v.); challenge dose,  $\sim 1.8 \times 10^7$  spores/animal. PT, pretreatment; DT, drug treatment. Values are means of 3 animals ± S.D.

Monocytes/macrophages are the key line of host defence against pathogenic fungi [6]. As tuftsin is known to increase the killer activity of these cells [1], the observed increase in the therapeutic efficacy of Amp B after its encapsulation in Tuft-Lip may partly be attributed to macrophage activation. To further examine the validity of this conclusion, we determined the effects of Lip-Amp B on the initial fungal load, which was measured 24 h after the drug treatment, and the % survival (on day 7 post-drug treatment) in mice that were treated with Tuft-Lip prior to the *A. fumigatus* infection. The data shown in Table III indicate that the Tuft-Lip pretreatment not only reduced the initial fungal load but also increased the % survival in Lip-Amp-treated animals. In the pretreated groups, both Lip-Amp B and Tuft-Lip-Amp B were equally effective, confirming that macrophage activation does increase the therapeutic efficacy of the antifungal drug, Amp B. These results thus strongly indicate that the chemotherapeutic efficacy of some antifungal drugs can be markedly increased by administering them along with macrophage activators in liposomes.

Amp B is a potent antifungal drug but its therapeutic use is limited due to its high toxicity. The toxicity has, however, been shown to considerably decrease without affecting the drug's efficacy by encapsulating this drug in liposomes [7–10], thus rendering Amp B suitable for use in the treatment of fungal infections. The present study further increases the scope of liposomised Amp B formulations in the treatment of fungal infections by demonstrating a significant improvement in the Lip-Amp B efficacy against experimental aspergillosis after grafting tuftsin onto the surface of liposomes. This modification of the Lip-Amp B surface, however, did not affect the drug toxicity, as we observed almost identical LD<sub>50</sub> values (7–8 mg/kg) for both Lip-Amp B and Tuft-Lip-Amp B.

In conclusion, this study shows that the Tuft-Lip-Amp B formulation is considerably better than the Lip-Amp B preparation in the treatment of systemic fungal infections due to its higher efficacy and comparable toxicity. In addition, it suggests that administration of macrophage activators/immunomodulators along with Amp B in liposomes should greatly improve the efficacy of fungal chemotherapy.

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