# [16] Tuftsin-Bearing Liposomes as Antibiotic Carriers in Treatment of Macrophage Infections

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## Abstract

Tuftsin is a tetrapeptide (Thr-Lys-Pro-Arg) that specifically binds monocytes, macrophages, and polymorphonuclear leukocytes and potentiates their natural killer activity against tumors and pathogens. The antimicrobial activity of this peptide is significantly increased by attaching at the C-terminus a fatty acyl residue through the ethylenediamine spacer arm. This activity is further augmented by incorporating the modified tuftsin in the liposomes. The tuftsin-bearing liposomes not only enhance the host's resistance against a variety of infections but also serve as useful vehicles for the site-specific delivery of drugs in a variety of macrophage-based infections, such as tuberculosis and leishmaniasis.

## Introduction

Tuftsin is a macrophage natural killer activator tetrapeptide (Thr-Lys-Pro-Arg) that is an integral part of the Fc portion of the heavy chain of the leukophilic immunoglobulin G (residues 289–292) and is released

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physiologically as the free peptide after enzymatic cleavage (Najjar, 1987). It is known to possess specific receptors on monocytes, macrophages, and polymorphonuclear (PMN) leukocytes (Najjar, 1987) and potentiates the natural killer activity of these cells against tumors and, presumably, also against a variety of pathogens (Agrawal and Gupta, 2000). These properties of tuftsin make it an attractive candidate for use as a cell-specific ligand for targeting of drugs to the pathogens that reside and proliferate within macrophages and monocytes. Since these cells can be infected by a number of pathogens, including *Mycobacterium tuberculosis* and *Leishmania donovani*, and provide nonspecific resistance against a variety of infections, it is expected that grafting of tuftsin on the surface of the liposomes that are loaded with antibacterial/antiparasitic drugs should significantly increase their therapeutic efficacy against these infections.

Tuftsin, due to its hydrophilic character, cannot be grafted on the surface of liposomes without being attached to a sufficiently long hydrophobic anchor. Structure–function studies of this tetrapeptide indicate that its binding and subsequent activation of the mononuclear phagocyte system (MPS) is dependent upon rather strict conservation of its molecular structure. Thus, modifications of the peptide at its N-terminus or within the chain lead to a significant reduction or even loss of its biological activity (Fridkin and Gottlieb, 1981). However, the activity is largely retained if modifications are restricted only to the C-terminus (Gottlieb *et al.*, 1982). All the modifications are, thus, limited to the carboxyl group of the Arg residue. Direct attachment of a fatty acyl group to the Arg residue, without any spacer arm, leads to modified tuftsin, which does not allow formation of liposomes, presumably due to perturbation of the phospholipid polar head group packing by the bulky Arg residue (Singhal *et al.*, 1984). This problem is, however, circumvented by introducing an ethylenediamine spacer arm between the Arg residue and the hydrophobic anchor (Fig. 1).

Liposomes containing palmitoyl tuftsin (**I**) specifically recognize macrophages and PMN leukocytes (Singhal *et al.*, 1984). Treatment of macrophages with these liposomes considerably increases their respiratory burst activity (Singh *et al.*, 1992). Pretreatment of animals with tuftsin-bearing liposomes enables the animals to resist malaria (Gupta *et al.*, 1986), leishmania (Guru *et al.*, 1989), and fungal (Owais *et al.*, 1993) infections. In addition, delivery of antileishmanial (Agrawal *et al.*, 2002; Guru *et al.*,

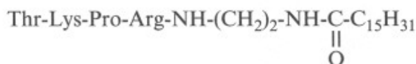


FIG. 1. Structure of palmitoyl tuftsin (**I**).

1989), antitubercular (Agarwal *et al.*, 1994), antifungal (Owais *et al.*, 1993), and antifilarial (Owais *et al.*, 2003) drugs in liposomes containing **I** is shown to increase the therapeutic efficacy of drugs against these infections.

Here, we describe procedures for preparation of **I** as well as the liposomes that contain **I** in their bilayers, entrapment of various drugs in these liposomes, and their delivery to experimental animals with infected *L. donovani*, *M. tuberculosis*, or *Aspergillus*.

## Synthesis of Palmitoyl Tuftsin (I)

Synthesis of **I** is carried out by the standard solution phase methods of peptide synthesis. The first intermediate, $H_2N\text{-}(CH_2)_2\text{-}NH\text{-}CO\text{-}C_{15}H_{31}$, is prepared by reacting palmitoyl chloride with an excess of ethylenediamine in a dilute ether solution. The amine thus obtained is coupled without purification with *t*-butyloxycarbonyl (Boc)-Arg($NC_2$)-OH, using the *N*, *N*-dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole (DCC/HOBt) procedure. The poduct is purified by silica gel chromatography to obtain Boc-Arg($NC_2$)-NH-$CH_2$-$CH_2$-NH-CO-$C_{15}C_{31}$. The Boc group is removed by treatment with 4 *N* HCI in dioxane. The resulting amine hydrochloride is then coupled with Boc-Pro-OH using DCC/HOBt to obtain the dipeptide Boc-Pro-Arg($NO_2$)-NH-$CH_2$-$CH_2$-NH-CO-$C_{15}H_{31}$ as a white powder. The Boc group is removed by treatment with 4 *N* HCI/dioxane, and the resulting amine is coupled with Boc-Lys[benzyloxycarbonyl (Z)]-OH using benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) reagent to obtain the tripeptide Boc-Lys(Z)-Pro-Arg($NO_2$)-NH-$CH_2$-$CH_2$-NH-CO-$C_{15}H_{31}$. Removal of the Boc group from the tripeptide and subsequent coupling of the resulting amine with Boc-Thr[benzyl (Bz)]-OH using BOP reagent affords the fully blocked tetrapeptide Boc-Thr (Bz)-Lys(Z)-Pro-Arg($NO_2$)-NH-$CH_2$-$CH_2$-NH-CO-$C_{15}H_{31}$. The crude tetrapeptide is purified by silica gel chromatography and crystallized from ethylacetate/hexane. The tetrapeptide is treated with 4 *N* HCl/dioxane to remove the Boc group, and the resulting product is subjected to catalytic hydrogenation over Pd black to obtain the desired compound as a white powder. The detailed synthetic procedure is given below.

### *Preparation of* $H_2N\text{-}CH_2\text{-}CH_2\text{-}NH\text{-}CO\text{-}C_{15}H_{31}$ (**II**)

Palmitoyl chloride (2 ml) is added dropwise to a solution of ethylenediamine (5 ml; Sigma-Aldrich) in anhydrous ether (50 ml) at 0° under vigorous stirring. A white precipitate separates out in the reaction mixture. It is further stirred for 1.5 h, and then the precipitate is filtered and washed with cold ether. The solid is taken in chloroform and washed with saline.

The organic layer is dried over anhydrous sodium sulfate and concentrated under reduced pressure to obtain $H_2N$-$CH_2$-$CH_2$-NH-CO-$C_{15}H_{31}$ as a gummy material (2.1 g). This product is dried over $P_2O_5$ and KOH pellets under vacuum and used as such without further purification. Fast atom bombardment mass spectrometry (FAB-MS): nuclear magnetic resonanance $MH^+$, 299; (NMR) ($CDCl_3$) $\delta$ ppm: 0.88 [triplet (t), 3H, $CH_3$], 1.24 [broad singlet (bs), 26H, $CH_2$], 2.17 (t, 2H, CO-$CH_2$), 3.36 (bs, 4H, NH-$CH_2$-$CH_2$-$NH_2$), 5.7–5.84 [broad hump (bh), N$H$].

*Preparation of Boc-Arg ($NO_2$)-HN-$CH_2$-$CH_2$-NH-CO-$C_{15}H_{31}$ (**III**)*

To a solution of Boc-Arg($NO_2$)-OH (2.1 g, 6.5 m$M$) in anhydrous dimethylformamide (DMF) (15 ml), HOBt (0.9 g, 6.5 m$M$; Janssen Chimica) is added, and the reaction mixture is cooled to 0°. To this solution DCC (1.4 g, 7 m$M$; Janssen Chimica) is added under vigorous stirring. After 10 min, a solution of **II** (2 g, 7 m$M$) in dichloromethane (10 ml) is added, and the reaction mixture is stirred for 1.5 h at 0° and then kept in a refrigerator overnight. Dicyclohexylurea (DCU) is filtered, and the filtrate is evaporated under reduced pressure. The residue is partitioned between ethylacetate (100 ml) and saline (25 ml). The organic layer is washed with 5% citric acid (3 × 20 ml), saline (3 × 20 ml), 5% $NaHCO_3$ (3 × 20 ml), and finally with saline. The organic layer is dried over anhydrous sodium sulfate and concentrated under reduced pressure to obtain the crude compound (3.2 g). The crude product is subjected to silica gel (Merck) chromatography using 2% methanol (MeOH) in chloroform as the eluent to obtain 2.5 g chromatographically homogeneous compound. It is characterized by mass and NMR spectroscopy. Melting point (M.P.) 120–121°; $[\alpha]_D^{25}$ − 3.5 ($c$ = 1, DMF); FAB-MS: $MH^+$, 599; NMR ($CDCl_3$) $\delta$ ppm: 0.88 (t, 3H, $CH_3$), 1.24 (bs, 26H, $CH_2$), 1.4 [singlet (s), 9H], 1.68–1.79 (bs, 4H, $\beta$ and $\gamma$ $CH_2$ Arg), 2.17 (t, 2H, CO-$CH_2$), 3.36 (bs, 6H, NH-$CH_2$-$CH_2$-NH and $\delta$ $CH_2$ Arg), 4.23 [multiplet (m), 1H, $\alpha$ $CH$], 5.7–8.24 (bh, NH).

*Preparation of Boc-Pro-Arg($NO_2$)-HN-$CH_2$-$CH_2$-NH-CO-$C_{15}H_{31}$ (**IV**)*

Compound **III** (3.0 g, 5 m$M$) is dissolved in 4 $N$ HCl/dioxane (25 ml), and the solution is kept at room temperature for 1.5 h. Solvent is removed under reduced pressure, and the residue is precipitated by addition of an excess of dry ether. The hydrochloride salt is collected by filtration and washed with anhydrous ether three times. It is dried over $P_2C_5$ and KOH pellets in a vacuum desiccator.

To a solution of Boc-Pro-OH (1.1 g, 5.2 m$M$) and HOBt (0.75 g, 5.5 m$M$) in dry DMF (25 ml) is added the hydrochloride salt of **III**,

obtained as above, in dry DMF (15 ml) and *N*-methyl morpholine (1.2 ml) at 4° under vigorous stirring. To this vigorously stirred mixture, DCC (1.2 g, 6 m*M*) in dry dichloromethane (10 ml) is added dropwise. The reaction mixture is stirred further for 1.5 h at 0° and 8 h at room temperature. DCU is removed by filtration, and the filtrate is evaporated under reduced pressure. The residue is extracted in ethylacetate (150 ml) and the organic layer washed with 5% citric acid (3 × 20 ml), saline (3 × 20 ml), 5% $NaHCO_3$ (3 × 20 ml), and finally with saline. The organic layer is dried over anhydrous sodium sulfate and concentrated under reduced pressure to obtain an oily residue. The residue is crystallized with ethylacetate/hexane to obtain chromatographically homogeneous **IV** as a white solid. Yield: 2.8 g (83%). It is characterized by mass and NMR spectroscopy. M. P. 140–142°; $[\alpha]_D^{25}$ – 14.2 ($c = 1.1$, DMF); FAB-MS: $MH^+$, 697; NMR ($CDCl_3$) $\delta$ ppm: 0.88 (t, 3H, $CH_3$), 1.24 (bs, 26H, $CH_2$), 1.4 (s, 9H), 1.68–1.79 (bs, 4H, $\beta$ and $\gamma$ $CH_2$ Arg), 1.95–2.1 (bs, 4H, $\beta$ and $\gamma$ $CH_2$ Pro), 2.17 (t, 2H, CO-$CH_2$), 3.4 (bs, 2H, $\delta$ $CH_2$ Pro), 3.36 (bs, 6H, NH-$CH_2CH_2$NH and $\delta$ $CH_2$ Arg), 4.31 (m, 2H, $\alpha$ *CH*s), 5.7–8.24 (bh, NH).

*Preparation of Boc-Lys(Z)-Pro-Arg($NO_2$)-HN-$CH_2$-$CH_2$-NH-CO-$C_{15}H_{31}$ (**V**)*

Boc-Lys(Z)-OH (1.6 g, 4 m*M*) and HOBt (0.68 g, 4.5 m*M*) are dissolved in dry DMF (25 ml), and the solution is cooled to 0°. A solution of dipeptide amine H-Pro-Arg($NO_2$)-HN-$CH_2$-$CH_2$-NH-CO-$C_{15}H_{31}$, obtained by treating **IV** (4 m*M*) with 4 *N* HCl/dioxane and subsequent neutralization with *N*-methyl morpholine, is added to the above mixture. After 5 min, diisopropylethyl amine (0.5 ml) and BOP reagent (2.2 g, 4.5 m*M*; Novabiochem) are added to the reaction mixture, and the stirring is continued for another 4 h. Solvent is removed under reduced pressure, and the residue is extracted in ethylacetate (50 ml × 3). The organic layer is washed with 5% citric acid (3 × 20 ml), saline (3 × 20 ml), 5% $NaHCO_3$ (3 × 20 ml), and finally with saline. It is dried over anhydrous sodium sulfate and concentrated under reduced pressure to obtain an oily residue. The residue is crystallized with ethylacetate/hexane to obtain **V** as a white solid. Yield: 2.7 g (72%). It is characterized by mass and NMR spectroscopy. M.P. 103–105°; $[\alpha]_D^{25}$–12.4 ($c = 1$, DMF); FAB-MS: $MH^+$, 960; NMR ($CDCl_3$) $\delta$ ppm: 0.88 (t, 3H, $CH_3$), 1.24 (bs, 26H, $CH_2$), 1.4 (s, 9H), 1.45–1.82 (bs, 6H, $\beta$, $\gamma$, and $\delta$ $CH_2$ Lys), 1.68–1.79 (bs, 4H, $\beta$ and $\gamma$ $CH_2$ Arg), 1.95–2.1 (bs, 4H, $\beta$ and $\gamma$ $CH_2$ Pro), 2.17 (t, 2H, CO-$CH_2$), 3.21 (bs, 2H, $\varepsilon$ $CH_2$ Lys), 3.4 (bs, 2H, $\delta$ $CH_2$ Pro), 3.36 (bs, 6H, NH-$CH_2$-$CH_2$-NH and $\delta$ $CH_2$ Arg), 4.3–4.81 (m, 3H, $\alpha$ *CH*), 5 (s, 2H, $CH_2$), 7.3 (s, 5H, *CH* aromatic), 5.7–8.24 (bh, N*H*).

*Preparation of Boc-Thr(Bz)-Lys(Z)-Pro-Arg(NO$_2$)-HN-CH$_2$-CH$_2$-NH-CO-C$_{15}$H$_{31}$ (**VI**)*

Boc group from **V** (2.7 g, 2.8 m*M*) is removed using 4 *N* HCl/dioxane in the usual manner, and the resulting amine hydrochloride is neutralized with *N*-methyl morpholine (Sigma-Aldrich) in dry DMF (25 ml). To this solution, Boc-Thr(Bz)-OH (0.86 g, 2.8 m*M*) and HOBt (0.45 g, 3 m*M*) are added, and the reaction mixture is stirred at 0°. After 5 min, diisopropylethyl amine (0.5 ml; Sigma-Aldrich) and BOP reagent (1.7 g, 3.5 m*M*) are added, and stirring is continued for 4–5 h. Solvent is removed under reduced pressure, and the residue is extracted in ethylacetate (3 × 50 ml). The organic layer is washed with 5% citric acid (3 × 20 ml), saline (3 × 20 ml), 5% $NaHCO_3$ (3 × 20 ml), and finally with saline. It is dried over anhydrous sodium sulfate and concentrated under reduced pressure to obtain an oily residue. The crude product is subjected to silica gel column chromatography using 3% MeOH in chloroform as the eluent to obtain 2.1 g (62%) chromatographically homogeneous **VI**. It is characterized by mass and NMR spectroscopy. M.P. 82–83°; $[\alpha]_D^{25}$ −19.5 ($c$ = 1.2, DMF); FAB-MS: $MH^+$ 1150; NMR ($CDCl_3$) $\delta$ ppm: 0.88 (t, 3H, C*H*$_3$), 1.24 (bs, 26H, C*H*$_2$), 1–4 (s 9H), 1.44 (s, 3H, C*H*$_3$ Thr), 1.45–1.82 (bs, 6H, $\beta$, $\gamma$, and $\delta$ C*H*$_2$ Lys), 1.68–1.79 (bs, 4H, $\beta$ and $\gamma$ C*H*$_2$ Arg), 1.95–2.1 (bs, 4H, $\beta$ and $\gamma$ C*H*$_2$ Pro), 2.17 (t, 2H, CO-C*H*$_2$), 3.21 (bs, 2H, $\varepsilon$ C*H*$_2$ Lys), 3.4 (bs, 2H, $\delta$ C*H*$_2$ Pro), 3.36 (bs, 6H, NH-C*H*$_2$-C*H*$_2$-NH and $\delta$ C*H*$_2$ Arg), 4.3–4.81 (m, 4H, $\alpha$ C*H*), 5–5.1 (2s, 4H, C*H*$_2$), 7.3–7.4 (2s, 10H, aromatic C*H*), 5.7–8.24 (bh, N*H*).

*Preparation of Thr-Lys-Pro-Arg-NH-CH$_2$-CH$_2$-NH-CO-C$_{15}$H$_{31}$ (**I**)*

Compound **VI** (0.92 g, 0.8 m*M*) is subjected to acidolysis using 4-*N* HCl/dioxane to remove the Boc group of the Thr residue. The hydrochloride salt is then subjected to catalytic hydrogenation over palladium black for 20 h in glacial acetic acid. Solvent is removed under reduced pressure, and the residue is crystallized using anhydrous methanol/ether two times to obtain a chromatographically homogeneous white powder. It is characterized by NMR and FAB-MS. Yield: 0.51 g, (80%). M.P. 149–151° (d); $[\alpha]_D^{25}$ −38.6 ($c$ = 0.7, DMF); FAB-MS: $MH^+$, 781; NMR ($d_6$-DMSO) $\delta$ ppm: 0.85 (t, 3H, C*H*$_3$), 1.24 (bs, 26H, C*H*$_2$), 1.44 (s, 3H, C*H*$_3$ Thr), 1.45–1.82 (bs, 6H, $\beta$, $\gamma$, and $\delta$ C*H*$_2$ Lys), 1.68–1.79 (bs, 6H, $\beta$ and Y C*H*$_2$ Arg and $\beta$ C*H*$_2$ alkyl), 1.95–2.1 (bs, 4H, $\beta$ and $\gamma$ C*H*$_2$ Pro), 2.04 (t, 2H, CO-C*H*$_2$), 3.08 (bs, 2H, $\varepsilon$ C*H*$_2$ Lys), 3.4 (bs, 2H, $\delta$ C*H*$_2$ Pro), 3.36 (bs, 6H, NH-C*H*$_2$-C*H*$_2$-NH and $\delta$ C*H*$_2$ Arg), 4.18–4.51 (m, 4H, $\alpha$ C*H*), 7.24–8.80 (bh, N*H*).

## Preparation of Liposomes

Liposomes are prepared from egg phosphatidylcholine (PC) and cholesterol (CH) by sonication except in the case of rifampin (RFP)-incorporated liposomes (Agarwal *et al.*, 1994). Tuftsin is grafted in the liposome bilayer by mixing 7–8% of **I** by weight of PC in the above mixture. Quantities greater than 7–8% are not well tolerated by the liposome bilayer, whereas lower quantities are inadequate to give optimal results. In case of RFP-incorporated liposomes, only PC is used as the lipid component, as the presence of CH in the lipid mixture reduces the amount of RFP intercalation within the bilayer (Agarwal *et al.*, 1994). Detailed procedures for preparation of tuftsin-bearing liposomes for studies of their interactions with PMN leukocytes, for modulating the nonspecific host resistance against infections, for delivering antileishmanial, antitubercular, and antifungal drugs to macrophages and monocytes are given below.

### *Preparation of Liposomes for Studies of Interactions with PMN Leukocytes*

To monitor the interactions between the tuftsin-grafted liposomes and macrophages/monocytes/PMN leukocytes, the liposomal membrane is labeled with [$^{14}$C]PC to determine the extent of exchange/transfer of lipids between the liposomes and the cells, and the fluorescent dye 6-carboxy-fluorescein (6-CF) (Ralston *et al.*, 1981) is entrapped as the model solute to monitor the extent of solute leakage caused by liposome–cell interactions. Thus, small unilamellar liposomes are prepared from PC (15 $\mu$mol), CH (7.5 $\mu$mol), traces of [$^{14}$C]PC (about 10 $\mu$Ci), and 6-CF (0.2 *M*) with or without **I** (7–8% by PC weight) in 0.8 ml Tris-buffered saline (10 m*M* Tris containing 150 m*M* NaCl, pH 7.4) or sucrose-supplemented Tris-buffered saline [10 m*M* Tris containing 150 m*M* NaCl, 44 m*M* sucrose, and 5 m*M* ethylenediaminetetracetic acid (EDTA), pH 7.4] by probe sonication (Singhal *et al.*, 1984). Typically, a solution of lipids in chloroform is dried in a glass tube under a slow jet of oxygen-free nitrogen gas, resulting in the formation of a thin lipid film on the wall of the tube. Final traces of solvents are removed after leaving the tube *in vacuo* for 1–2 h. The lipid film is dispersed in 0.8 ml Tris-buffered saline. The tube is flushed with nitrogen and stoppered. It is vortexed at 35–40° for 20 min. The lipid dispersion so obtained is transferred to a cuvette and sonicated (1–1.5 h) under nitrogen using a probe-type sonicator to obtain an optically clear suspension. The sonicated preparations are centrifuged at 10,500*g* for 1 h at 5°. Only the liposomes found in the top two thirds of the supernatant are used. Free and liposomal 6-CF are separated by gel filtration over a Sephadex G-50

column (1.4 × 20 cm), using Tris-buffered saline as the eluting buffer. Liposome-rich fractions are pooled together and used in further experiments. The outer diameter of liposomes is determined by negative-staining electron microscopy.

The extent of incorporation of **I** in the liposome bilayer is estimated as follows. To an aliquot (20 $\mu$l) of PC/CH liposomes containing **I** are added 580 $\mu$l phosphate buffer (10 m*M*, pH 8.5), 20 $\mu$l trinitrobenzenesulfonic acid (1.5% in 0.8 *M* $NaHCO_3$, pH 8.5), and 200 $\mu$l Triton X-100 (1% in 0.8 *M* $NaHCO_3$, pH 8.5) or buffer. It is mixed and then incubated in the dark at 18–20° for 1 h. After this period, the reaction is stopped by adding 400 $\mu$l HCl (1.5 *M*). The absorbance for yellow color thus obtained is read at 410 nm. Quantities of **I** incorporated in the bilayers are calculated from the standard curve, which is drawn by reacting varying amounts of **I** with trinitrobenzenesulfonic acid under identical conditions. The absorbance at 410 nm is linear with concentration to at least 0.8 absorbance units. The amounts of **I** incorporated into the liposome bilayer are over 95%, and about 60% of this amount is localized in the outer monolayer.

### *Preparation of Liposomes to Increase Nonspecific Host Resistance against Parasitic and Fungal Infections*

Liposomes are prepared from equimolar amounts of PC and CH with or without **I** (8% by weight of PC) in Tris-buffered saline (10 m*M* Tris containing 150 m*M* NaCl, pH 7.4) by probe sonication, as described above. The sonicated preparation is centrifuged at 12,000*g* for 30 min (10°) to sediment undispersed lipids and titanium particles. The amount of **I** incorporated in the liposome bilayer is estimated as described above.

### *Preparation of Liposomes as Vehicles to Deliver RFP in* Mycobacterium tuberculosis-*Infected Mice*

*Mycobacterium tuberculosis* resides and proliferates primarily within mononuclear phagocytes. Therefore, delivery of antitubercular drugs, like RFP, through tuftsin-bearing liposomes should help to enrich this drug within the diseased cells, which in turn may result in reduction of the drug dose, leading to reduced RFP toxicity (Raleigh, 1972). Tuftsin-bearing liposomes are thus prepared by incorporating RFP in both the bilayers and internal aqueous compartment of the liposomes.

Typically, PC (62.5 $\mu$mol), RFP (0.61 $\mu$mol), and **I** (7–8% by PC weight) are dissolved in chloroform methanol (1:1, v/v). The solution is dried in a glass tube under a slow jet of oxygen-free nitrogen, resulting in the formation of a thin lipid film on the wall of the tube. Final traces of the solvent are removed after leaving the film *in vacuo* overnight at 4°.

The lipid film is dispersed in 1.5 ml of phosphate-buffered saline (PBS; 10 m*M* phosphate containing 150 m*M* NaCl, pH 7.4) and vortexed at 35–40° for 15–20 min. The lipid dispersion thus obtained is transferred to a cuvette and sonicated for 30 min under oxygen-free nitrogen, using a probe-type sonicator. The sonicated preparation is centrifuged at 12,000*g* for 30 min to remove titanium particles as well as the undispersed or poorly dispersed lipids. The supernatant is carefully removed and gel filtered through a Sephadex G-50 column (40 × 1.5 cm) using PBS as the eluent to separate liposome-associated RFP from free RFP. Liposomes are eluted in the void volume. The liposome-rich fractions are pooled together and concentrated in an Amicon Centriflo CF-25 cone. The outer diameter of liposomes is determined by electron microscopy.

RFP is estimated by measuring its absorbance at 334 nm. The amount of liposome-associated drug is determined after lysing the liposomes with Triton X-100 (1%). The RFP absorbance at 334 nm is found to remain unaffected by the presence of liposomes and the detergent and is linear at least up to 100 μg/ml of RFP. About 28–32% of RFP is found to be incorporated in the unilamellar liposomes.

To determine whether RFP is entrapped or intercalated in liposomes, the above liposome preparation is frozen under liquid nitrogen and immediately thawed. The preparation is then gel filtered again over a Sephadex G-50 column (40 × 1.5 cm). RFP elutes under two different peaks. The one in the void volume corresponds to the liposomal form while the other corresponds to the free form, suggesting that the drug resides in both the internal aqueous space and the lipid bilayer.

### *Preparation of Liposomes as Drug Carriers in Treatment of* Leishmania donovani *and* Aspergillus *Infections*

Amphotericin B (Amp B) is a very effective drug for treatment of leishmanial infections that are resistant to treatment with antimonials and fungal infections including aspergillosis. Fungal pathogens, unlike *L. donovani*, do not reside within monocytes and macrophages. It is thought that delivery of Amp-B through tuftsin-bearing liposomes concentrates the drug within macrophages, which migrate to fungally infected lesions and serve as a drug depot, making available high concentrations of the drug in the affected region.

Amp-B-loaded liposomes are prepared from PC and CH (molar ratio 7:3) by probe sonication. Typically, PC (49 μmol), CH (21 μmol), Amp B (1.0 mg), and **I** (7–8% by weight of PC) are dissolved in a round-bottom flask in a minimum volume of chloroform/methanol (1:1, v/v). The solvents are carefully removed under reduced pressure so that a thin film is formed on the wall of the flask. Final traces of solvents are removed by leaving the

flask *in vacuo* at 4° overnight. The dried lipid film is hydrated with 150 m*M* sterile saline (2 ml) under vigorous stirring for 1 h under a nitrogen atmosphere in a bath-type sonicator. The lipid dispersion thus obtained is dialyzed against normal saline for 24 h in the dark at 4°. The dialyzed preparation is centrifuged at 10,000*g* for 1 h at 4° to remove traces of undispersed lipids. The supernatant is analyzed for both Amp-B and tuftsin.

The amount of liposome-intercalated Amp B is determined by measuring its absorbance at 405 nm. The intercalation efficiencies of Amp B in tuftsin-bearing liposomes and tuftsin-free liposomes are about 95% and 85%, respectively. The extent of tuftsin incorporation is estimated as described above.

## Interactions of Tuftsin-Bearing Liposomes with Blood Cells

Specific interactions of tuftsin-bearing liposomes with blood cells are studied by incubating the liposomes with the respective cell population (PMN leukocytes, lymphocytes, erythrocytes, etc.) at 37° and then measuring the transfer/exchange of lipids between the liposomes and cells as well as the solute leakage caused by the interaction of liposomes with cells. These studies show that the tuftsin-bearing liposomes interact specifically with PMN leukocytes, monocytes, and macrophages, but not with lymphocytes or erythrocytes under identical conditions (Singhal *et al*., 1984).

PMN leukocytes and lymphocytes are isolated from freshly drawn rat blood using a Ficoll-Paque gradient. Erythrocytes are isolated from rat blood by simple centrifugation. The detailed procedures are given below (Singhal *et al*., 1984).

### *Isolation of Lymphocytes and PMN Leukocytes*

Heparinized rat blood (10 IU/ml blood) is centrifuged at 1000*g* for 10 min, and the plasma is removed. The cells are suspended in Hanks' balanced salt solution (137 m*M* NaCl, 37 m*M* KCl, 1.0 m*M* $CaCl_2{\cdot}2H_2O$, 0.41 m*M* $MgSO_4{\cdot}7H_2O$, 0.50 m*M* $MgCl_2{\cdot}6H_2O$, 0.44 m*M* $KH_2PO_4$, 0.34 m*M* $Na_2HPO_4{\cdot}2H_2O$, 5.55 m*M* glucose, 4.16 m*M* $NaHCO_3$, pH 7.4) to 50% hematocrit. One milliliter of this suspension is layered on top of 1 ml Ficoll-Paque (Pharmacia product). It is centrifuged at 300*g* for 15 min at 20 ± 2°. The top layer containing lymphocytes is removed and washed several times with Hanks' balanced salt solution. Any contaminating erythrocytes are removed by treating one volume of lymphocytes with seven volumes of ammonium chloride (150 m*M*, pH 7.4) for 5 min at 20°. The mixture is centrifuged, and the cell pellet is washed three times with Hanks'

balanced salt solution. The cell viability is determined by Trypan Blue exclusion; usually over 95% of the cells are viable. The purity of the cells is determined in a thin smear stained with Leishmann stain. These cells are contaminated with 5–7% of PMN leukocytes and monocytes. Without any further purification, these cells are used in further experiments after suspending in the above buffer ($3.6–4.0 \times 10^7$ cells/ml).

The second layer formed in the Ficoll-Paque gradient mainly consists of PMN leukocytes. It is carefully aspirated and washed three times with Hanks' balanced salt solution. Contaminating erythrocytes are removed by the ammonium chloride treatment, but it is not necessary to remove the contaminating lymphocytes (25–30%) from the PMN leukocytes for the study. The viability of these cells is >95%. The cells are suspended in the above buffer ($3.6–4.0 \times 10^7$ cells/ml) and used within 1 h.

*Isolation of Erythrocytes*

Heparinized rat blood is centrifuged at 1000*g* for 5 min. Plasma and buffy coat are removed. The pellet is washed several times with sucrose-supplemented Tris-buffered saline. The washed cells are suspended in buffer ($3.6–4.0 \times 10^7$ cells/ml) and used immediately in further experiments.

*Interactions of Liposomes with PMN Leukocytes*

Liposomes (0.13–2.5 $\mu$mol lipid P/ml) are mixed with PMN leukocytes ($1.8–2.0 \times 10^7$ cells/ml) in Hanks' balanced salt solution. The mixture is incubated at 37° for 1 h in a shaking waterbath. After incubation is complete, the cells are harvested by centrifugation. The cell pellet is repeatedly washed with Hanks' balanced salt solution until the supernatant is free from radioactivity. The amounts of radioactivity and 6-CF in the cell pellet are assayed after disrupting the cells with Triton X-100 (1% final concentration). The 6-CF fluorescence is monitored by using excitation and emission wavelengths of 490 and 520 nm, respectively. Recovery of cells is about 90% and >95% are viable at the end. To determine the effect of incubation temperature on uptake of liposomes by the leukocytes, liposomes (2 $\mu$mol lipid P/ml) are also incubated with PMN leukocytes ($1.8–2.0 \times 10^7$ cells/ml) at 0° for different periods of time. After incubation is complete, the cells are processed, and the amounts of radioactivity and 6-CF associated with the cell pellet are determined.

Free 6-CF is also incubated with the cells under identical conditions. About 0.01% of the total dye remains bound to the cell pellet.

### *Interactions of Liposomes with Lymphocytes*

Liposomes (2 $\mu$mol lipid P/ml) are mixed with lymphocytes ($1.8–2.0 \times 10^7$/ml) in Hanks' balanced salt solution, and the mixture is incubated for different periods of time. After incubation is complete, the cells are washed and assayed for radioactivity and 6-CF.

### *Interactions of Liposomes with Erythrocytes*

Liposomes (2 $\mu$mol lipid P/ml) are mixed with erythrocytes ($1.8–2.0 \times 10^7$/ml) in sucrose-supplemented Tris-buffered saline, and the mixture is incubated for different periods of time. After completing the incubation, the cells are washed and assayed for radioactivity. In this case, 6-CF cannot be measured due to quenching of its fluorescence by hemoglobin.

## Treatment of Experimental Animals with Tuftsin-Bearing Liposomes to Enhance Their Nonspecific Resistance to Infections

To increase the nonspecific resistance of experimental animals to parasitic infections, tuftsin-bearing liposomes are given intravenously for 3 consecutive days prior to infection (day −3 to day −1). Normally, the dose given is 50 ng tuftsin per day per animal, but this dose may vary depending on the type of infection. Animals are infected on day zero. Under these conditions, animals resist *Plasmodium berghei* (Gupta *et al.*, 1986), *L. donovani* (Guru *et al.*, 1989), and to some extent *Aspergillus fumigatus* (Owais *et al.*, 1993) infection, but no effects are seen on the course of *M. tuberculosis* infection under identical conditions.

## Treatment of Leishmanial, Tuberculosis, and Aspergillosis Infections Using Tuftsin-Bearing Liposomes as Drug Carriers

RFP-loaded tuftsin-bearing liposomes are used to treat *M. tuberculosis* $H_{37}Rv$ infections in Swiss albino mice. Various doses of drug-loaded tuftsin-bearing liposomes and drug-loaded but tuftsin-free liposomes and the free drug are delivered intravenously postinfection. The course of infection is determined by measuring the bacilli load in lungs, livers, and spleens, as well as the mean survival times of animals. The numbers of bacilli in lungs, livers, and spleens of mice are quantitated by measuring colony-forming units (cfu). The efficacy of RFP is considerably improved upon its administration in tuftsin-bearing liposomes compared with tuftsin-free liposomes. Consistently, administration of repeated doses of RFP in tuftsin-bearing liposomes shows significantly better antitubercular effects than the administration of a single dose (Agarwal *et al.*, 1994).

AmpB-loaded tuftsin-bearing liposomes are used to treat *L. donovani* infections in golden hamsters and also *A. fumigatus* infections in BALB/c mice. The liposome-associated drug is administered intravenously in both cases (Agrawal *et al.*, 2002; Owais *et al.*, 1993). The drug loaded in tuftsin-bearing liposomes as well as the free drug are given to the control group of animals. While the drug efficacy in leishmanial infections is assessed by measuring the parasite load in spleens of the infected animals, the efficacy of Amp B in *A. fumigatus* infections is determined by measuring both the fungal load (cfu) in various organs and survival times of the treated animals. Antileishmanial and antifungal effects of Amp B are seen to be considerably better if it is administered in tuftsin-bearing liposomes as compared to tuftsin-free liposomes or in the free form (Agrawal *et al.*, 2002; Owais *et al.*, 1993).

## Concluding Remarks

Tuftsin is a well-known macrophage activator tetrapeptide that specifically binds monocytes, macrophages, and PMN leukocytes. This activity of tuftsin is further increased upon its incorporation in liposomes, after attaching a sufficiently long hydrophobic anchor at its C-terminus. The tuftsin-bearing liposomes not only enhanced the nonspecific host's resistance against a variety of pathogens but also helped in significantly boosting the chemotherapeutic potential of a number of drugs if delivered after their encapsulation in these liposomes. These studies thus clearly demonstrate the usefulness of this tetrapeptide in augmenting the chemotherapeutic potential of antiparasitic, antitubercular, and antifungal drugs by virtue of its ability to home the drug-loaded nanoparticle-like systems (e.g., liposomes) to monocytes and macrophages and also to simultaneously stimulate the nonspecific killer activity of these cells against pathogens.

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