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Advanced Drug Delivery Reviews 41 (2000) 135–146

advanced drug delivery reviews

www.elsevier.com/locate/drugdeliv

Tuftsin-bearing liposomes in treatment of macrophage-based infections

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Received 25 April 1999; accepted 1 October 1999

Abstract

The use of liposomes as drug carriers in treatment of various diseases has been explored extensively for more than 20 years. 'Conventional' liposomes, when administered in vivo by a variety of routes, rapidly accumulate in the mononuclear phagocyte system (MPS). The inherent tendency of the liposomes to concentrate in MPS can be exploited in enhancing the non-specific host defence against infections by entrapping in them the macrophage modulators, and as carriers of antibiotics in treatment of intracellular infections that reside in MPS. This must further be enhanced by grafting on the liposome surface the ligands, e.g. tuftsin, that not only binds specifically to the MPS cells but also enhances their natural killer activity. Keeping this in view, we designed and developed tuftsin-bearing liposomes as drug carriers for the treatment of macrophage-based infections and outline these studies in this overview.

Keywords: Drug delivery system; Liposome; Tuftsin; Immunomodulation; Mononuclear phagocyte system; Malaria; Leishmaniasis; Tuberculosis; Aspergillosis

Contents

1. Introduction

The search for improved ways of administering drugs to patients to achieve maximum efficacy with minimal side effects has been one of the most 'sought-after' dreams of clinicians for years. However, in actual practice, application of drugs in therapeutic and preventive medicine is marred by their indiscriminate action and inability to reach only

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0169-409X/00/$ – see front matter
PII: S0169-409X(99)00061-7

the areas in need of treatment. In spite of the enormous advances made in molecular biology and pharmacology over the last two decades, development of new, more selective drugs is still a very expensive, time consuming and often uncertain process. While the drugs are available to combat a wide range of genetic, malignant, and infectious diseases, their efficacy is often compromised by their inability to reach the intended site at an appropriate concentration. Consequently, much attention has been focussed on an alternative approach, namely the use of drug delivery systems, which are expected to optimise the action of the drugs already in existence by transporting or facilitating their release where they are needed.

Successful homing of drugs to the target depends on the design of the carrier as little can be done to influence the target and its surroundings. In a typical drug targeting sequel, the carrier-drug unit would preserve its integrity, avoid interception by normal cells, penetrate interposing membranes, selectively recognise and associate with the target before the drug is released in the target area. Selective drug delivery to specific foci in the body have two vital benefits. First, it ensures optimal interaction of the drug with the site(s) of its action at the right rate and frequency. Secondly, on reducing the drug dose as well as by increasing the target specificity, the potential for any side effects is greatly diminished.

A large number of macromolecular, cellular, and synthetic carriers have been explored with the aim of perfecting drug delivery systems which would localise drugs to the desired sites in vivo, thus minimising the side effects [1–3]. Amongst these, liposomes bearing cell-specific recognition molecules (e.g., antibody, lectin, glycolipid etc.) on their surface have received wide attention as vehicles for site-specific delivery of drugs and enzymes in vivo [4–7]. Liposomes, soon after their discovery in the mid sixties, have been studied extensively, and employed in virtually every aspect of biotechnology including gene delivery. Liposomes are best suited as carriers for drugs and enzymes as they are formed from naturally occurring phospholipids and therefore possess low inherent toxicity, immunogenicity, and high drug to carrier ratio [1,8].

Despite their resemblance to the cell surface, ‘conventional’ liposomes, when administered in vivo, rapidly accumulate in the MPS [9–12]. The major sites of their accumulation are Kupffer cells of the liver and the resident macrophages of the spleen. About 70 to 80% of intravenously injected liposomes are concentrated into the Kupffer cells of the liver, 5 to 8% into macrophages in the spleen, and even fewer into phagocytic cells in bone marrow [8,13,14]. The rate and the site of the uptake of the liposomes by the MPS are influenced by the injected dose, blood flow, local tissue damage, and interaction of liposomes with the serum proteins [13,15]. The interaction of serum proteins with the particles depends on the physicochemical properties of the particles, i.e. charge, size, hydrophobicity, and fluidity of the particle surface [13,15]. This tendency of the liposomes to localise in MPS can be turned into an advantage in the treatment of a variety of infections in two ways: (1) As the MPS cells play an important role in non-specific host defence against infections in general, liposomes can be used to activate them by encapsulating in them, thus enhancing non-specific resistance to infections caused by various micro-organisms [11,12]. (2) Liposomal encapsulation of antiparasitic and antimicrobial agents can result in enhancement of their therapeutic efficacy against intracellular infections involving the MPS [9–12]. This must potentially be achieved by grafting on the liposome surface the ligands, e.g. tuftsin, that not only bind specifically to the MPS cells but also stimulate them non-specifically against infections.

In the early 1980s, our research group embarked on a project of drug delivery in macrophages. Over the years we have successfully used tuftsin-bearing liposomes [16–23] in our laboratory as carriers for immunomodulator/drug homing to macrophages. In this review we present the overview of the tuftsin-bearing liposomes and their usefulness in the treatment of macrophase-based infections.

2. Tuftsin-bearing liposomes

Tuftsin is a natural macrophage activator tetrapeptide (Thr–Lys–Pro–Arg) which is a part of the Fc-portion of the heavy chain of the leukophilic

immunoglobulin G (residues 289–292). The tetrapeptide is released physiologically as the free peptide fragment after enzymatic cleavage [24]. Two enzymes are responsible for the production of tuftsin from leucokinin; tuftsin-endocarboxypeptidase, a specific enzyme, cleaves the heavy chain at the Arg–Glu bond between residues 292 and 293, and the membrane enzyme leucikinase acts on the bound leucokinin-S to cleave it at the amino end of threonine between residues 288 and 289. The peptide is known to bind specifically to macrophages, monocytes and PMN leukocytes, and possesses a broad spectrum of activities related primarily to the immune system function [24,25]. These include potentiation of various cell functions, such as phagocytosis, pinocytosis, motility, immunogenic response, bactericidal and tumoricidal activities [25]. The features of tuftsin, coupled with its low toxicity, make the tetrapeptide a promising candidate for immunotherapy [26–28]. Tuftsin's capacity to augment cellular activation is mediated by specific receptors that have been identified, characterised, and isolated from rabbit peritoneal granulocytes [29]. Tuftsin and many of its analogs have been chemically synthesised and studied extensively for structure–function relationships [30,31].

Grafting of tuftsin on the liposome surface would therefore enable us not only in homing the liposomized drug to macrophages but also to stimulate these cells non-specifically against infections. Structure–function studies of tuftsin indicate that its binding and consequent MPS activation 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 biological activity and also its ability to bind to PMN leukocytes [32]. However, it has been shown that extending the peptide at its C-terminus does not affect the biological activity [33]. As tuftsin is a hydrophilic molecule, it would preferentially reside in the aqueous compartment of liposomes and will thus lose its ability to enhance the interactions of liposomes with phagocytes via its receptor. We, therefore, facilitated its grafting on the liposome surface by attaching a sufficiently long hydrocarbon fatty acyl residue to the C-terminus through an ethylenediamine spacer arm (Thr–Lys–Pro–Arg–NH–$(CH_2)_2$–NH–CO–$C_{15}H_{31}$)(Fig. 1)and used

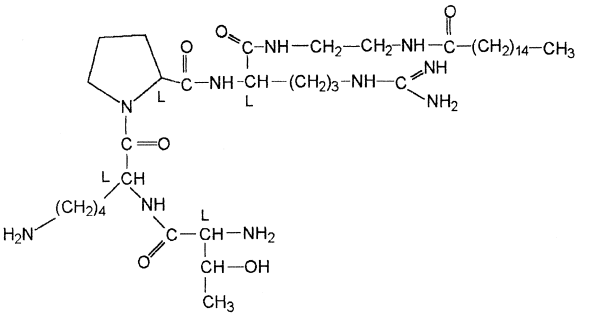

Fig. 1. Hydrophobic derivative of the tetrapeptide Thr–Lys–Pro–Arg (tuftsin) (reproduced with permission [16]).

thus formed liposomes in all the studies described below.

We subsequently demonstrated that the tuftsin (Fig. 1) could enhance non-specific defence against infections by activating the macrophages [21]. The biological activity of the peptide was due to the induction of the macrophage respiratory burst and activated macrophages exhibited enhanced levels of NADPH oxidase, O_2^-, H_2O_2 and myeloperoxidase (MPO). Both O_2^- and H_2O_2 are known to damage proteins, nucleic acids and membranes, sufficiently to kill the cell or even the whole organism. Nevertheless, for macrophages hypohalous acids, which are produced by the action of MPO on H_2O_2, have been identified as the major killer agents [34].

The incorporation of >10 mol% tuftsin in the egg PC/cholesterol (2:1; mol/mol) liposomes was not possible as the resulting mixtures could not be dispersed even by prolonged sonication [16]. On the other hand, the liposomes containing <5 mol% tuftsin were only poorly bound to PMN leukocytes. Therefore, it is absolutely important to have 7–8 mol% tuftsin in the liposomes for their optimal effect. The leakage rates of 6-CF from egg PC/cholesterol/tuftsin liposomes in buffer, pH 7.4 at 37°C were about 2–4%/h. Incidentally, this leakage rate was dramatically enhanced upon incorporating another tuftsin derivative, Thr–Lys–Pro–Arg–NH–$C_{18}H_{37}$ in the liposomes bilayer [16]. This was probably due to the binding of the dye with the positively charged Arg residue in this analogue. Since this amino acid residue should be aligned just at the bilayer interface, the effect of its binding with the 6-CF on the liposomes permeability must have

been mediated through perturbation of the egg PC headgroup packing in the liposomes bilayer [35]. The binding/uptake of egg PC/cholesterol/tuftsin liposomes to PMN leukocytes was saturable, time dependent and the cell-bound liposomes were apparently taken up by the cells presumably by receptor-mediated endocytosis without losing their structural integrity. That these liposomes were actively endocytosed by the cells was further suggested by the observation that lowering of the incubation temperature to 0°C inhibited the uptake [16]. We also examined the specificity of the interaction of these liposomes with other blood cells, i.e. erythrocytes, lymphocytes and found that virtually no binding with erythrocytes was observed but there appeared to be some binding with lymphocytes which slightly increased with time, which we concluded was primarily due to the presence of PMN leucocytes/monocytes as contamination in the lymphocyte preparation.

3. Malaria

Malaria is still considered the most prevalent and devastating disease world wide which affects about 300–500 million people and claims 1.5–2.7 million human lives. Furthermore, one-third of the world human population lives in areas which are infested with the disease [36]. Numerous efforts have been made towards the development of effective vaccines against malaria as an effective vaccine may elicit a protective immune response in individuals of diverse genetic makeup and could complement other strategies for prevention and control of this serious public health problem in future. Although these studies provided important knowledge of the nature of the protective host immunological mechanisms and their respective target antigens but there is no effective malaria vaccine as yet on the horizon, and only the chemotherapy remains the major practical method for managing all forms, i.e. 'exoerythocytic' and 'erythrocytic' stages, of infection. Moreover, the situation is aggravating as the malarial parasites are rapidly developing resistance to the existing antimalarial drugs when given in classical pharmaceutical form [37,38]. One of the mechanisms that the malarial parasite employs to resist the drug action is by enhancing the drug efflux from the infected cells, thus preventing the drug accumulation to the toxic levels within the cytosol [39]. A protein likely to be involved in chloroquine resistance has recently been identified which poses some important questions/answers [40].

A number of studies have been performed using liposomes as carriers for drugs, and vaccines against malarial infection [41–46]. Experimental liposome-based vaccines against malaria and other infections have been shown to be safe and highly immunogenic in human trials [47]. Analysis of the intracellular trafficking patterns of the liposomal antigens reveals that after being phagocytosed by macrophages, liposomal antigen readily escapes from the endosomes into the cytoplasm of the macrophages [47]. Mice vaccinated with liposomised recombinant proteins have recently been reported to resist a lethal malaria infection. The protection induced with liposomes adjuvants was as good as or better than that achieved with Freund's adjuvant [48].

We have previously shown that binding of the liposomes to red blood cells was considerably enhanced (20–25-fold) by covalently attaching $F(ab')_2$ fragments to their surface [49,50]. It has further been shown that chloroquine encapsulated in these liposomes was very effective in suppression of the chloroquine susceptible and resistant *Plasmodium berghei* infections, both in terms of reduction in parasitaemia and prolongation in the survival times [51,52]. We further demonstrated that by attaching $F(ab')_2$ fragments of the monoclonal MAb F_{10}, specific to *Plasmodium berghei*-infected erythrocytes, to the liposomes loaded with chloroquine, even the chloroquine resistant malarial infections can be cured [53]. This biologic response may further be enhanced by enhancing the non-specific host resistance against malarial infection [17] as the activated macrophages have been shown to kill the intraerythrocytic malarial parasites in vitro [54].

It has been reported that pre-treatment of mice with tuftsin or one of its derivatives, rendered them at least partially resistant to lethal *Plasmodium berghei* infections [17]. A pre-treatment dose of 50 μg/animal per day of tuftsin for more than 1 day seemed to be necessary to achieve this partial protection, since the mortality rate in animals pre-

treated with a single dose (200 μg) on day 3 or with the lower doses (10 or 25) μg on day 0 to 3 was very similar to that observed in the control animals.

The above effect of tuftsin may further be increased by incorporating it in the liposomes bilayer. For this case, both the mortality and parasitaemias in the animals that received pre-treatment with the liposomal tuftsin were significantly decreased, as compared to those pre-treated with saline or control liposomes [17].

The mean survival times of mice pre-treated with 50 and 100 μg doses of liposomal tuftsin were about 16 and 19 days, respectively, which were greater than that observed with free tuftsin [17]. Since suppression of the host immune response seems to be a regular feature in almost all parasitic diseases [55], agents such as tuftsin may help in bringing the immune status back to normal, which in turn could further enhance the efficacy of chemotherapy.

4. Leishmaniasis

Leishmaniasis is caused by the hemoflagellate protozoan and represents four major clinical syndromes; visceral, cutaneous, mucocutaneous, and diffuse cutaneous leishmaniasis. It is estimated that world wide more than 12 million people are infected and approximately 350 million are at risk. The most devastating clinical form, visceral leishmaniasis (kala azar), is caused by *Leishmania donovani* which causes disseminated disease, characterised by fever, hepatosplennomegaly, anemia, leukopenia and hyperglobinaemia. If untreated, the disease is usually fatal.

The major front line drugs available for leishmaniasis are toxic. Several doses need to be given over a prolonged period of time and development of drug resistance is becoming a problem. Since leishmaniasis affects the MPS cells, a number of studies have been conducted to exploit liposomes as drug vehicles for the treatment of leishmaniasis [9,56–58]. The efficacy of the several liposomal stibanate formulations against *L. donovani* has been shown to be better than the free drug [59]. Different sugar-bearing liposomes, containing drugs viz. pentamidine isethionate and its methoxy derivative, hamycin, and urea stibamine have been found to be more potent in comparison to normal liposome-encapsulated drug or the free drug [60,61].

We encapsulated sodium stibogluconate, one of the major front line drugs for leishmaniasis, in tuftsin-bearing liposomes and demonstrated that the efficacy of the drug was markedly enhanced (at least 200 times) against *Leishmania donovani* infections in hamsters [19]. Free drug, when given at 10 mg/kg per day dose for five consecutive days produces over 90% inhibition of the infection on day 28 post-treatment. However, a single treatment at 250 μg or 500 μg/kg drug for one day only was very effective ($P < 0.01$) after encapsulating in tuftsin-bearing liposomes. At a 500 μg/kg dose, the drug encapsulated in the liposomes showed much better ($P < 0.01$) effects than that observed with free drug or tuftsin free liposomal drug on day 28 post-treatment. But this difference was reduced ($P < 0.01$ on day 7 but > 0.05 on day 28 post-treatment) by reducing the drug dose to 250 μg/kg, although at this dose the liposomised drug exhibited the maximum antileishmanial activity. On further decreasing the liposomised drug dose, no difference was obtained between the effects of drug encapsulated in tuftsin-free and tuftsin-bearing liposomes. The failure to observe the enhanced antileishmanial effect of the drug loaded in tuftsin-bearing liposomes, as compared to that in the tuftsin-free liposomes, at low drug doses is possibly due to dilution of the tuftsin-bearing liposomes below their optimal concentration required to observe optimal activation of the host's macrophages [16]. However, optimisation of parameters like, route of administration, surface charge, cholesterol content, and single vs. multiple treatment regimens, treatment at lower parasite burden may further improve the efficacy. The improvement in the therapeutic efficacy of the liposomised drug may result from the respiratory burst-inducing activity of tuftsin, in addition to the effect of targeted drug delivery of the drug to the macrophages [19,21].

We also studied the course of the infection in animals pre-treated with both free and liposomal tuftsin which considerably enhances their resistance to leishmania infection. However, the inhibitory effect on this infection was greater ($P < 0.01$) on day 30 post-infection for single treatment when tuftsin was incorporated in the liposomal bilayer. Moreover,

a single treatment with tuftsin or liposomal tuftsin was more effective than the multiple treatment [19].

The susceptibility of the peritoneal macrophages, derived from the pre-treated animals to *L. donovani* infection was examined in vitro. These experiments demonstrated that the infection of macrophages was markedly reduced when these cells were derived from the liposomal tuftsin pre-treated animals. Also, the parasite multiplication inside such macrophages was considerably decreased, as compared to the macrophages of untreated animals [19].

Successful management of leishmaniasis cases has been reported to be difficult due to increased resistance of the parasite to the front line drugs, like antimonials [62]. Although the second front line drug, amphotericin B (Amp B), is quite effective as an antileishmanial agent, it suffers from severe side effects. The toxic effects of Amp B can be significantly minimised, keeping intact the antileishmanial property, by encapsulating this drug in the liposomes [20,63].

To examine whether this property of Amp B was further enhanced by its incorporation in the tuftsin-bearing liposomes, we evaluated the efficacies of Amp B incorporated in tuftsin-free liposomes (Lip-Amp B) and tuftsin-bearing liposomes (Tuft-Lip-Amp B) against *L. donovani* infections in vitro. The antileishmanial activity of Lip-Amp B, at all the tested Amp B concentrations, was higher than that observed with free Amp B in identical conditions. This activity further increased when tuftsin was grafted on the surface of Lip-Amp B liposomes. The difference observed between the antileishmanial activities of Lip-Amp B and Tuft-Lip-Amp B was very significant ($P < 0.01$) at Amp B concentrations lower than 10 μg/ml, but it became smaller at the higher Amp B concentrations. These results clearly indicate that encapsulation of Amp B in liposomes helps not only in reducing the drug toxicity but also in increasing the drug efficacy against *L. donovani* infections in vitro (Agarwal and Gupta, unpublished observations).

The in vitro results were validated by observing an enhancement of drug efficacy at all the tested drug doses in vivo. Besides enhancing the efficacy, liposomisation of the drug also led to a marked decrease in the drug toxicity. While the animals died at a 2 mg/kg dose of free Amp B, drug administration in liposomes did not show any toxicity even at 5 mg/kg (Agarwal and Gupta, unpublished observations). These results were consistent with the earlier studies, showing an increase in the LD_{50} of Amp B after liposomisation [63]. Over 95% efficacy in reducing the spleen parasite burden was observed at a dose of 5 mg/kg even after 30 days post-infection. Besides, reducing the parasite load in the spleen, this preparation was also effective in eliminating the liver and bone marrow parasite load.

The Tuft-Lip-Amp B were more effective than the Lip-Amp B against leishmaniasis even at a low single dose of 0.5 mg/kg. The tissue distribution experiments demonstrated clearly the higher and faster uptake of Tuft-Lip-Amp B liposomes from the circulation with most of the liposomes being cleared from the circulation within 1 h after administration as compared to Lip-Amp B (Agarwal and Gupta, unpublished observations). The increased uptake of Tuft-Lip-Amp B observed in infected organs suggested that the drug distribution in the biophase was also influenced by infection besides the liposomisation. Although liposomisation of Amp B did not significantly alter its distribution in the macrophage rich organs, except when tuftsin was grafted on the liposomes surface, but it did lead to a decrease in the drug concentration in the kidney of these animals. It may thus be suggested that the higher efficacy of Lip-Amp B observed could partly be due to the reduced drug concentration in the kidney, which should not only lead to the decreased drug toxicity but also an increased drug tolerance. In case of the tuftsin-bearing liposomes, the higher efficacy observed was perhaps because of both the increased drug tolerance and enhanced uptake of liposomised drug by the macrophage rich organs of infected mice. Tuftsin-bearing liposomes also increased the drug accessibility to areas like bone marrow which are otherwise inaccessible to free Amp B, and were the main cause of relapse of the leishmanial infection.

5. Fungal infections

Fungal infections continue to be a major problem in management of immunocompromised patients. The presence of any fungal disease in humans

commonly implies that the host defence systems have been compromised due to some reason. Thus, the fungal infections often represent the opportunistic infections which are the major cause of morbidity in immunocompromised/deficient human subjects. The clinical effects of the fungal disease in the immunocompromised host vary widely according to the nature of the underlying disorder as well as the fungus involved. In general, these diseases are very likely to be progressive in nature, and often disseminated and life threatening. Patients with acute leukaemia, especially following hospitalisation and administration of antibiotics, are prone to various fungal infections and their early diagnosis in cancer patients still remains elusive.

Chemotherapy with antifungal agents is a priori indication for all systemic and superficial fungal infections in humans. Various antifungal chemotherapeutic agents available include; polyenes, azoles, allylamines, morpholines, flucytosine, griseofulvin, iodides, hydroxy–stibamine, and imidazole classes of drugs [64–66]. As elimination of fungi from the tissues of normal healthy subjects is often associated with stimulation of the cell-mediated immune defences which involve activation of the mononuclear phagocytes by sensitised T cells [67], treatment with antifungal drugs along with the agents that provoke macrophages/monocytes should find useful application in general in fungal chemotherapy. Amphotericin B (Amp B), a polyene antibiotic, has been widely used in clinical practice to treat various systemic fungal infections, e.g. candidiasis, histoplasmosis, aspergillosis, etc. [64–66,68]. Side effects associated with Amp B, vary from mild headache, chills and fever to severe hemolytic anemia and acute nephritis, limiting its fuller exploitation as a proper therapeutic measure. Nephrotoxicity is the most serious problem associated with Amp B therapy and its severity may necessitate interruption of treatment.

Chemical derivatisation of amphotericin B has been done to eliminate its toxic side reactions, but without much success [69]. The toxicity of Amp B to cells originates from its binding to sterols present in the cellular membranes; ergosterol in the case of fungal cells, and cholesterol in mammalian cells. The binding of Amp B to membrane sterols results in disorganisation of the cellular membranes, possibly by formation of specific pores composed of small aggregates of Amp B and sterol. These defects cause depolarisation of the membrane, and consequently an increase in the membrane permeability to protons and monovalent cations. The leakage of metabolites from cells leads to cell death.

The usefulness of the liposomes as vehicles for Amp B has been demonstrated in fungal diseases, such as candidiasis, histoplasmosis and cryptococcosis by reducing its toxicity and increasing its therapeutic index [70–74]. Recently, targeted delivery to lung tissues, the organs first infested by many fungi, via inhaled liposomal Amp B aerosol has been shown to be a more effective approach [75]. The liposomal preparation permitted the use of high amounts of drug which in free form would have been quite toxic. Earlier, it was thought that the decreased toxicity of liposomal Amp B was due to the altered disposition of the drug. However, no association between these alterations and decreased toxicity was found [76]. Moreover, no significant difference in organ distribution was observed between the liposome and free Amp B [77]. Both in vitro and in vivo results show liposomal Amp B to be less cytotoxic. The toxicity to cells is dependent on the attainment of a sufficient level of Amp B in the aqueous phase. Liposomes composed of phospholipids with saturated acyl chains are non-toxic whereas Amp B intercalated in liposomes composed of phospholipids containing unsaturated acyl chains are almost as toxic as Amp B [78]. The reduction in toxicity of liposomal Amp B is also evident in the presence of sterols in liposomes [79]. This may be attributed to the tighter lipid packing in liposomes giving rise to an increased order and stability and thus decreasing the flux of Amp B molecules across the membranes. Following the promising clinical results, three formulations containing Amp B (Abelcet, Amphocil, and AmBisome) are currently available on the market [12].

With a view to further increase the scope of liposomised Amp B (Lip-Amp B), we incorporated Amp B in tuftsin-free liposomes (Lip-Amp B) and tuftsin-bearing liposomes (Tuft-Lip-Amp B) and then studied their efficacy against human *Aspergillus fumigatus* infections in mice. The result of these studies showed that the % survival of the *A. fumigatus*-infected mice considerably increased (70–

75%) by treating them with Tuft-Lip-Amp B, as compared to the animals that received treatment with Lip-Amp B [22]. Also the survived animals from the Tuft-Lip-Amp B treated group were virtually free of any fungal load whereas animals treated with Lip-Amp B still had some residual infection [22]. These results strongly indicate that the efficacy of Tuft-Lip-Amp B against *A. fumigatus* infection considerably increased perhaps due to activation of macrophages/monocytes, the key line of host defence against pathogenic fungi [67]. To further examine the validity of the belief, we first treated the animals with drug-free tuftsin-bearing liposomes prior to the infection and then after challenging them with lethal doses of *A. fumigatus*, the infected animals were treated with Lip-Amp B and Tuft-Lip-Amp B. In these experiments, no difference between the efficacies of Lip-Amp B and Tuft-Lip-Amp B were observed, demonstrating that the higher efficacy of Tuft-Lip-Amp B, as compared to Lip-Amp B, was primarily due to macrophage activation affected by tuftsin [22]. Further, the tuftsin incorporation in the Lip-Amp B did not affect the drug toxicity, as we observed almost identical LD_{50} value (ca. 7 mg/kg) for both Lip-Amp B and Tuft-Lip-Amp B [22]. These studies thus clearly demonstrate that the Tuft-Lip-Amp B formulation was significantly better than the Lip-Amp B preparation in treatment of the systemic fungal infections due to its higher efficacy and comparable toxicity, suggesting that the administration of macrophage activators along with Amp B in liposomes should greatly improve the efficacy of fungal chemotherapy.

6. Tuberculosis

Tuberculosis is a single most infection which results in the largest number of deaths world-wide; nearly 3 million people are killed every year [80]. The association of tuberculosis with HIV infection has significantly exacerbated the situation in developed and developing nations [81]. HIV infection is the highest risk factor identified so far for latent tuberculosis infection to progress to an active disease. This infection also increases the risk to new tuberculosis infection that will progress to disease [81,82].

Advances in antimycobacterial chemotherapy have brought tuberculosis from a life threatening situation to a potentially curable one. Still, the most important factor in the treatment of tuberculosis is prolonged chemotherapy, for a minimum period of 6–12 months, which is often associated with serious and undesirable side effects, e.g. hepatotoxicity [83–85]. Also, undesired effects may be caused by the high levels of antitubercular drugs in blood required to achieve an effective intracellular drug concentration. It is, therefore, desirable to develop an approach which would allow the use of lower drug doses by delivering the agent to the infected cells, thereby improving efficacy and potentially reducing the toxicity.

Liposomes could resolve some of the above problems by serving as carriers for site specific and/or sustained delivery of antitubercular drugs. 'Passive targeting' of the liposomes to MPS [9–12] could be utilised as the causative organism, *Mycobacterium tuberculosis*, resides and proliferates primarily within the mononuclear phagocytes, which normally serve as the first line of defence against infections [86].

Rifampicin, isoniazid, streptomycin, pyrazinamide, thioacetazone, *p*-aminosalicyclic acid, ethambutol, and streptomycin are amongst the major front line drugs in the treatment of tuberculosis [83,85,87]. Most of these drugs though possess strong antitubercular activity, but each of these exhibit several undesirable effects, thus further emphasising the necessity of suitable vehicles which may help in diminishing the undesirable effects of these drugs without compromising their efficacy. A number of studies have demonstrated that the efficacy of these antitubular drugs is increased in their liposomised forms [88–91].

Liposomes besides delivering the drug to the infected site could also act as drug reservoirs to provide a slow and sustained release of the drug. This would not only reduce the cost of the treatment but may also shorten the duration of the treatment, the two major drawbacks associated with the chemotherapy of tuberculosis.

We demonstrated the usefulness of the liposomes as vehicles for refampicin (RFP) in treatment of *Mycobacterium tuberculi* infections in mice [23]. The drug acts on DNA-dependent RNA polymerase

in the bacterial cell to block the protein synthesis and kills the micro-organism. There is little or no cross resistance between RFP and other antimycobacterial drugs [92]. The sterilizing activity of RFP is high because of its ability to kill the semidormant bacilli. The drug is thus comparatively safe, but its half-life in circulation is relatively short (ca. 3 h) and most of the drug following oral administration is metabolized and excreted, leaving only a limited amount available for activity against the mycobacterium [93]. Thus, a daily dose of 600 mg of RFP is required for effective treatment, which often leads to toxic side effects [85].

After optimising the various parameters, i.e. route of administration, liposome surface charge, cholesterol content and single vs. multiple treatment regimens on the antitubercular activity, we showed that the antitubercular activity of rifampin was considerably increased, as compared to free drug, when it was encapsulated in conventional PC liposomes probably because of the ability of the liposomes to localise preferentially in macrophages/monocytes, leading to a high intracellular drug concentration. This was consistent with our finding that the intermittent treatments (twice weekly) with these preparations were significantly more effective rather than the continuous treatments. A further increase in the activity was observed when the drug was loaded in tuftsin-bearing liposomes. Rifampin delivered twice weekly for 2 weeks in these liposomes was at least 2000 times more effective than the free drug in lowering the load of lung bacilli in infected animals [23]. However, pre-treatment with the drug-free tuftsin-bearing liposomes did not render the pre-treated animals resistant to the *Mycobacterium tuberculosis* infections, neither did it appreciably increase the chemotherapeutic efficacy of the liposomised rifampin presumably because of inactivation of the primed macrophages by the mycobacterial sulfatides [94].

The dynamics of the distribution of RFP-liposomes in healthy and tuberculous mice showed that a greater liposome concentration in the liver, spleen and lungs of healthy mice was achieved, although the retention time in tuberculous mice was longer [95]. These findings offered support to our observations. Besides tuberculosis, RFP is also effective against leprosy and MAIS complex infections, thus enlarging the scope of RFP-loaded liposomes in the treatment of a variety of mycobacterial infections.

Acknowledgements

We thank Drs. R.K. Jain, A. Puri, A. Singhal, A. Agarwal, M. Owais, P.Y. Guru, S. Chandra and N.B. Singh for their contribution in the work.

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