CHAPTER II

Tuftsin in the treatment of fibrosarcoma
2.1. Introduction

Solid tumors such as fibrosarcoma have historically provided many challenges to anti-cancer therapy. Therapeutic hurdles to drug penetration in solid tumors include heterogeneous vascular supply and high interstitial pressures within tumor tissue, particularly in necrotic zones, lower pH and presence of leaky vasculature leading to reduced therapeutic response. Intense efforts are underway worldwide to develop newer and more specific strategies to develop effective means for treatment of cancer. This requires a better understanding of genetic alterations that ensued in transformation of cells to tumors. The efforts are contingent upon the identification of appropriate molecular targets, development of modalities that address this target, and delivery of the target recognizing novel agent to the tumor cells. In this regard, tumor microenvironment is considered to be a critical factor in this development since it can both influence the tumor cell phenotype, and thus the appropriateness of a given molecular target, and act as a barrier for the delivery of effective treatment (Brown and Giaccia 1998).

The sheer size and complexity of the human genome present formidable challenges to proliferating cells engaged in the constant manipulation of the DNA double helix. Essential cellular functions such as DNA replication, transcription, and cell division require the frequent packaging and unpackaging of the DNA genome on a regular basis. As key facilitators of such cellular transactions, the DNA topoisomerase family of enzymes have the unique ability to relax and untangle large strands of intertwined DNA, thereby maintaining order within the cell and preserving the structural integrity of the genetic code. The topoisomerases literally prevent the cell from becoming embroiled in a tangled mess. The fundamental importance of the DNA topoisomerases is highlighted by their stringent conservation across prokaryotic and eukaryotic species and by their absolute necessity for
cell proliferation. Because of these functions, the DNA topoisomerases offer formidable target for treatment of various forms of cancer.

Infact, DNA topoisomerases modulate the topology of DNA by modifying the tertiary structure of the double helix without altering the primary nucleotide sequence (Wang et al. 2002, Champoux et al. 2001, Takimoto et al. 2001). They are responsible for relaxing the torsional stress that accumulates when the DNA double helix unwinds to allow DNA or RNA polymerases access to the genetic code. In the absence of topoisomerases, the accumulation of torsionally strained supercoiled DNA would ultimately interfere with vital cellular functions. During cell division, DNA topoisomerases also function to untangle and physically separate the replicated DNA by facilitating the passage of an intact DNA strand through a double-strand nick in the DNA helix. Thus, two linked circular DNA molecules can be physically separated (decatenated) by the action of specific DNA topoisomerases. All DNA topoisomerases act by forming temporary single- or double-strand breaks in the double helix in which the enzyme is covalently bound via a tyrosine residue to one of the nicked ends of the phosphodiester DNA backbone. This normally transient intermediate, called the cleavable complex, allows for the passage of an intact single or double strand of DNA through this break, resulting in the unwinding or untangling of the DNA molecule. Subsequent relegation and release of the enzyme restore the integrity of the DNA double helix. All subsequently characterized DNA topoisomerases can be categorized into two broad families, types I and II, based on structure and function (Wang et al. 2002). Type I DNA topoisomerases generate transient single-strand breaks in DNA and type II DNA topoisomerases generate transient double-strand breaks in DNA. In higher eukaryotes and humans, three groups of topoisomerases have been identified. One group includes topoisomerase I and the mitochondrial DNA topoisomerase, which are both type IB enzymes. The second group includes human DNA topoisomerases IIα and IIβ, which are type II
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enzymes, and the final group consists of human topoisomerases IIα and IIβ, which are both type IA enzymes. The human enzymes with the greatest relevance for cancer chemotherapy are DNA topoisomerase I and DNA topoisomerases IIα and IIβ (Champoux 2001). The majority of topoisomerase interactive agents cause the accumulation of DNA cleavable complexes composed of protein-linked DNA strand breaks. The persistence of these lesions in the presence of ongoing DNA replication or RNA transcription leads to cytotoxic DNA damage, ultimately causing cell-cycle arrest and death by apoptosis or cell necrosis.

2.1.1. Etoposide

The indigenous peoples of North America have a long history of using extracts of the mandrake (mayapple) plant, *Podophyllum peltatum*, for medicinal purposes as a cathartic, emetic, and anthelmintic (Pommier et al. 2001). However, formal pharmaceutical use of the podophyllotoxins in the United States did not begin until 1820, and clinical studies in oncology were not initiated until 1946 (Hande et al. 1998). Initially, severe systemic toxicities limited the use of podophyllotoxins to the topical treatment of condylomata acuminata. Subsequent efforts in the 1960s to develop less toxic podophyllotoxins led to the synthesis of its glycoside derivatives etoposide (VP-16-213) and teniposide (VP-26; Kellner et al. 2003). Clinical testing of these newer epipodophyllotoxins in the 1970s demonstrated predictable toxicity profiles and meaningful antitumor activity in diseases such as acute myelocytic leukemia, non-Hodgkin's lymphomas, and breast, ovarian, gastric, and lung cancers. In 1983, etoposide (VePesid) was approved for the treatment of testicular and small cell lung cancer, and in 1993, teniposide was approved for treating pediatric leukemias and lymphomas. Currently, etoposide is commonly used in cancer chemotherapy and is active against multiple cell lines such as L929 fibroblasts, HL-60, K562, A549 human lung
carcinoma cells (Spurgers et al. 2006, Natalie et al. 2002, Huang et al. 1997). It is a part of the first-line therapy for small-cell lung carcinoma (Smit et al. 1989), malignant lymphoma (Dollery 1999), drug-resistant testicular cancer (Williams et al. 1987) etc.

Etoposide (ETP), like other podophyllins, can alter microtubule assembly, it does so only at concentrations several fold greater than those achieved in vivo. Upon incubation with cells, etoposide caused breaks in single-strand and double strand DNA in dose dependent manner (Loike et al. 1976). Upon its removal DNA breakage gets quickly repaired. Incubation of etoposide with purified DNA does not produce DNA strand breaks. However, when etoposide acts well isolated nuclei, leading to breaks in DNA (Wozniak et al. 1983). This clearly suggests that factors present in the nuclei, in addition to DNA, are required to obtain DNA strand breakage. The relationship between topoisomerase II inhibition and etoposide's antitumour activity was, in part, delayed as information about this important enzyme was being elucidated for quite some time. It was not until 1979 that the
presence of enzyme ‘DNA topoisomerases’ was reported (Liu et al. 1994). Extensive biochemical analysis of this group of enzymes was being undertaken, at the same time ETP was also being brought to the clinic. In 1984, several laboratories demonstrated that mammalian topoisomerase II was the target for etoposide action. Topoisomerase II enzymes are multisubunit proteins, require ATP for overall catalytic activity and modulate DNA topology by passing an intact helix through a transient double-stranded break created in the DNA backbone (Liu et al. 1994, Froelich-Ammon et al. 1995). As a result of their double-stranded DNA passage reaction, type II topoisomerasers are able to regulate over- and under-winding of the double helix and resolve nucleic acid knots and tangles. ETP and other topoisomerase II inhibitors do not kill cells by blocking topoisomerase catalytic function. Rather they poison these enzymes by increasing the steady-state concentration of their covalent DNA cleavage complexes. This action converts topoisomerasers into physiological toxins that introduce high levels of transient protein-associated breaks in the genome of treated cells. The potential lethality of these drug-induced cleavage complexes rises dramatically when replication machinery or helicases attempt to traverse the covalently bound topoisomerase roadblock in the DNA. This disrupts the cleavage complex and converts transient single- or double-strand breaks into permanent double-stranded fractures, which are no longer held together by proteinaceous bridges. These breaks become targets for recombination, sister chromatid exchange, the generation of large insertions and deletions and the production of chromosomal aberrations and translocation. When these permanent DNA breaks are present at sufficient concentration, they trigger a series of events that ultimately culminates in cell death by apoptosis.

Ironically, ETP causes dose limiting haematological (Sinkule et al. 1984) as well as gastro-intestinal toxicity when administered orally (Postmus et al. 1984). Further, the lipophilic nature of ETP poses
difficulty in its use as anti-cancer agent; as a consequence different solubilizers are generally used as co-additives to develop suitable ETP formulations (Hande et al. 1992). These additives are often associated with adverse effects such as hypotension, anaphylaxis, bronchospasm, etc. (O'Dwyer et al. 1984 and Sinkule et al. 1984).

Keeping into consideration toxicity constraints associated with most of the available chemotherapeutic agents including ETP, many workers have successfully tried to use liposome based delivery systems to circumvent these problems (Marina et al. 2002, Batist et al. 2001, Chidiac et al. 2000, Gabizon et al. 1997, Gabizon et al. 1992, Vaage et al. 1992). The higher lipophilicity of etoposide in fact may help in its better intercalation in lipid-based liposomal formulations (Tyagi et al. 1999, Sengupta 1998). Liposomes accumulate in the tumors in a gradual passive fashion due to increased 'leakiness' of the tumour vasculature (Yuan et al. 1994). In this regard, antineoplastic agents encapsulated in long circulating pegylated liposomes are of special significance as they attain higher concentrations in tumor interstitium ultimately eliminating tumors with great efficiency (Papahadjopoulos et al. 1991). Similarly the encapsulation of certain anticancer agents into cationic liposomes was also found to enhance their antitumour potential. However, cost factor, cumbersome methods of their preparation and more importantly intrinsic toxic manifestations of cationic lipids do not argue in favor of their usage as suitable drug vehicle.

It is indisputable that some degree of immune response against cancer exists in animals and humans. Components of the immune system that are capable of recognizing cancer cells have been identified in patients with certain cancers. In the laboratory, cells of the immune system can kill tumor cells. Even more convincing are clinical results showing that stimulation of the immune system with bacterial products or components of the immune system itself can
lead to tumor regression in some patients. The link between cancer and the immune system is also suggested by the fact that people with an impaired immune system, such as AIDS patients, are more likely to develop certain cancers, including Kaposi's sarcoma, rectal cancer and some specific types of lymphomas (Kiessling et al. 1999).

2.1.2. Macrophage infiltration and molecular mechanisms for recognition of tumor cells

As first line of defense against cancer, the macrophages must first infiltrate the lesions. The presence of inflammatory macrophages in growing tumors is maintained through recruitment of circulating monocytes and in certain tumors, the proliferation of mononuclear phagocytes, and ultimately their transformation into macrophages play important role in tumor regression (Mantovani 1990, Bugelski et al. 1987a,b). In regressing murine sarcomas, tumor-associated macrophages (TAMs) are found throughout the tumors, whereas in progressing sarcomas, TAMs are confined to the periphery of the tumor (Russell and Gillespie 1977). The presence of noncytotoxic (nonactivated) macrophages in neoplasms could actually enhance tumor growth (Mantovani et al. 1992). Macrophages (and lymphocytes) produce many diffusible growth, angiogenic, and cytotoxic factors, some of which would be favored tumor growth while others will regress tumors (Nathan, 1987). According to the type and level of such mediators, TAMs, therefore, may enhance or inhibit the growth of neoplasms (Prehn, 1972).

Activated macrophages can destroy syngeneic, allogeneic, and xenogeneic tumor cells, but leave normal nontumorigenic cells unharmed, suggesting that histocompatibility and tumor-specific antigens are not involved in this recognition (Fidler and Schroit 1988). In addition, differences in metastatic potential, chromosome number, resistance to chemotherapeutic agents, or the antigenic properties of
tumor cells did not appear to be important factors for macrophage recognition of tumors cells (Killion et al. 1993, Pak and Fidler 1989).

The ubiquitous role of macrophages in homeostasis suggests that macrophages recognize numerous molecules via a wide variety of plasma membrane receptors that interact with extracellular proteins, as well as with adhesion molecules such as the integrins (Gordon et al. 1988). Although carbohydrates and proteins have long been known to play an important role in cell-cell interactions and antigenic structures on cell surfaces, an increasing body of evidence suggests that phospholipids are also involved in macrophage recognition of target cells (Fidler and Schroit, 1988). All cell membranes are composed of a phospholipid bilayer containing four major phospholipids, the choline phospholipids phosphatidylcholine (PC) and sphingomyelin and the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS). In addition, the distribution of phospholipids in the bilayer membrane is extremely asymmetric (Gordesky and Marinetti 1973, Zwaal and Hemker 1982). In this regard, PC and sphingomyelin are preferentially positioned in the outer leaflet of the lipid bilayer, whereas PE is preferentially distributed in the inner leaflet. PS displays absolute asymmetry in its distribution; normal cells contain PS only in the inner leaflet of the cell membrane. Experiments based on several lines of evidence now suggest that when PS is expressed on the outer leaflet, it serves as a recognition molecule for macrophages and is also correlated with their increased binding to tumor cells (Schroit et al. 1985, Ratner et al. 1986). The mechanism for this recognition is nonimmunologic and requires cell-to-cell contact. Because activated macrophages can destroy phenotypically diverse tumor cells, including cells resistant to killing by other host-defense mechanisms and by anticancer drugs, macrophage-directed therapy represents a potential strategy for elimination of biologically heterogeneous metastatic cells.
2.1.3. Activation of macrophages to the tumoricidal state

The activation of macrophages can transform them from noncytotoxic stage to acquire tumoricidal properties (Hibbs 1974 a & b, Allison 1974). There are two major pathways to produce macrophage activation in vivo. Macrophages can become activated subsequent to interaction with microorganisms or their products such as endotoxins and bacterial cell-wall components. Although this interaction is very common, intentional attempts to activate macrophages systemically by administering microorganisms or their products were limited by side effects such as allergic reactions and granuloma formations (Allison 1974). For this reason, the field did not progress until the discovery of muramyl dipeptide (MDP), a defined component of the bacterial cell wall (Lederer 1980). MDP is a water soluble, low molecular weight synthetic moiety of N-acetyl-muramyl-L-alanyl-D-isoglutamine; it has potent effects on a variety of host-defense cells, including macrophages (Fogler and Fidler 1986). A synthetic, lipophilic MDP derivative, N-acetethylmuramyl-L-alanyl-D-isoglutamyl-L-alanyl-L 2-(19,29-dipalmitoyl)-sn-glycero-39-phosphoryl-ethylamide (MTP-PE), was also synthesized (Gisler et al. 1979). Although muramyl peptides influence several macrophage functions in vitro, comparable effects have not been observed in vivo because they are rapidly cleared after parenteral administration (Fogler and Fidler 1985). Even when injected at very high doses, muramyl peptides fail to induce significant macrophage-mediated antitumor activity (Fidler 1988).

Macrophages can also be activated by their interaction with lymphokines. Mitogen- and antigen-stimulated T-cells release diffusible mediators that interact with specific receptors on target cells. A family of lymphokines, referred to as macrophage-activation factors (MAF) (Fidler and Schroit 1984), can render macrophages tumoricidal activity. For example, IFN-γ (member of IFN family) prime
macrophages for their tumoricidal activity. Besides, IFN-α and IFN-β, can also prime macrophages in a synergistic fashion to that of IFN-γ (Saiki et al. 1986a, Pace 1988). It has been reported that IFN-γ-mediated priming of macrophages renders them sensitive to tumoricidal activation by tumor necrosis factor (TNF) and interleukin (IL)-1, suggesting that interactions of lymphocyte and macrophage products (IFNs and ILs) with macrophage receptors can lead to both transitory and sustained tumoricidal activity (Hori et al. 1989). Studies indicated that the priming stimulus delivered to macrophages by IFN-γ (or complete activation by MAF) involves the binding and subsequent internalization of cell-surface receptors (Poste et al. 1979a), a process that requires a sustained presence of ligand. The tumouricidal properties of lymphokine-activated macrophages are shortlived, and the cells become refractory to reactivation by free lymphokines (Poste et al. 1979b). Recently it has been demonstrated that mitogen-activated protein kinases are necessary for activation of macrophages (Dong et al. 1993), but diverse gene expression and release of cytokines by activated macrophages depend upon the immunomodulator, suggesting that intracellular pathways may be distinct for different activation signals. For example, human monocytes activated with either lipopolysaccharide or a synthetic lipopeptide [Ciba-Geigy Protein 31362 (CGP 31362)] secreted IL-1, TNF, and prostaglandin E2, whereas monocytes activated with MTP-PE secreted TNF without the additional presence of IFN-γ (Fidler et al. 1990). These studies indicate that not all macrophages share identical phenotypes.

2.1.4. Activation of macrophages by liposomised immunomodulators

Considerable attention has been focused on the use of synthetic phospholipid vesicles, liposomes, to target various drugs to different organ sites in vivo (Fidler 1988, Schroit et. al. 1983). Most attempts to
target liposomes to solid neoplasms have failed because these phospholipid vesicles are unable to extravasate from the circulation and are rapidly taken up by circulating and fixed phagocytic cells. This natural fate of liposomes can be utilized to target encapsulated immunomodulators to cells of the RES, which activate phagocytic cells for their tumoricidal properties (Schroit and Fidler 1982). Preferential delivery of the liposome-encapsulated substances can be achieved in vivo because mainly phagocytic cells are exposed to the liposome-entrapped agents. This natural localization pattern also allows efficient targeting of liposomes and their contents to various macrophage compartments in the body. Through the systematic evaluation of multilamellar vesicles (MLV) with different phospholipid compositions, certain classes of phospholipids have been preferentially recognized by macrophages. The inclusion of negatively charged phospholipids, such as phosphatidyl serine (PS) or phosphatidylglycerol (PG), in phosphatidyl choline (PC) based MLVs enhances their uptake by macrophages (Schroit and Fidler 1982). Indeed, liposomes composed of PC:PS result in significant accumulation by various cells of the RES (Kleinerman et al. 1983, Xu and Fidler 1984). Since the lung is a major site of disseminated metastatic form of disease, it is possible to identify liposomes with increased efficacy for localization to the lung parenchyma using intravenous injection of radiolabeled liposomes. Large MLV (>0.1 mm) were retained in the lungs more efficiently than small unilamellar liposomes of identical lipid composition. In addition, liposomes of the same structural class were more efficiently retained in the lungs when they contained negatively charged lipids (PS) as compared to neutral charge PC containing liposomes (Schroit et al. 1983).

The intravenous injection of PC:PS MLV containing MDP or MTP-PE immunomodulators results in the in situ activation of alveolar macrophages (AM). In order to assess whether AM activated by liposomised MDP maintained their tumoricidal properties over an
extended period of time, the AM were harvested and assayed for tumoricidal activity on days 1, 2, 3, or 4 post infusion of a single dose of MLV containing MDP, MTP-PE, or a combination of both of these agents.

On day 1, AM harvested from all of the mice that received MLV with hydrophilic MDP exhibited less than 20% cytotoxicity against the murine tumor target cells. At every other time point, AM harvested from mice that received MLV containing MTP-PE consistently exhibited significantly higher levels of tumoricidal activity (up to 55% cytolysis of target cells) (Fogler and Fidler 1984).

2.1.5. Tuftsin

In 1970, Najjar and Nishioka demonstrated that Leukokinin, a leukophilic fraction of Immunoglobulin IgG, splits under the action of specific enzyme (Leukokininase) located in the outer membrane of neutrophils. The biological activity of leukokinin rests in a peptide tuftsin- so called because it was discovered at Tufts University (Najjar et al. 1970). Tuftsin is a 289-292 (Thr-Lys-Pro-Arg) sequence in the CH2 domain of the Fc fraction of the IgG molecule.

The tetra-peptide is released physiologically as a free fragment after enzymatic cleavage (Najjar et al. 1987). Two enzymes are responsible for the production of tuftsin from leukokinin, tuftsin endocarboxypeptidase, a specific enzyme that cleaves the heavy chain at the Arg-Glu bond between residues 292-293, 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. Tuftsin is known to bind specifically to macrophages, monocytes and PMN leukocytes and possess a broad spectrum of activities primarily related to the function of immune system (Najjar et al. 1987, Fridkin et al. 1989). These include potentiation of various cell functions, such as phagocytosis, pinocytosis, motility, immunogenic response,
fungicidal, bactericidal and tumoricidal activity (Fridkin et al. 1989). The features of tuftsin coupled with its low toxicity make the tetrapeptide a promising candidate for immunotherapy (Nishioka et al. 1986; Khare et al. 1997). Tuftsin capacity to augment cellular activation is mediated by specific receptors that have been identified, characterized and isolated from rabbit granulocytes (Bump et al. 1986). Tuftsin and many of its analogs have been chemically synthesized and studied extensively for structure-function relationship (Nishioka et al. 1995, Gershonov et al. 1996).

The grafting of tuftsin on the liposomal surface would, therefore, enable it not only in homing the liposomised-drugs to the cell population possessing receptors to recognize it but also stimulate key cells of the immune system non-specifically against various infections (Singhal et al. 1984). Structure-function studies of tuftsin indicate that its binding and consequent MPS activation is dependent upon rather strict conservation of its molecular structure. Thus the modification of the peptide at its N-terminus or within the chain leads to a significant reduction or even loss of biological activity and also reduction in its ability to bind to PMN leukocytes (Fridkin et al. 1981). As tuftsin is a hydrophilic molecule, it would preferentially reside in the aqueous compartment of liposomes and would not have accessibility to its putative receptors present on the surface of various immune cells. Therefore, the tuftsin was specifically modified to facilitate its grafting on liposomal surface by attaching a long hydrocarbon fatty acyl residue to the C-terminus through an ethylene-diamine spacer arm (Thr-Lys-Pro-Arg-NH-(CH$_2$)$_2$-NH-CO-C$_{15}$H$_{31}$) (Singhal et al. 1984).

The incorporation of tuftsin at a percentage of >10 mol% tuftsin in the egg PC/cholesterol (7:3; mol/mol) liposomes was not possible as the resulting mixture could not be dispersed even by the long sonication (Singhal et al. 1984). On the other hand, the liposome
containing lower mol% of tuftsin was founded to poorly bound to PMN leukocytes. It was observed that 7-8 mol% tuftsin in the liposomes elicits optimal immunomodulatory effect. The leakage rate of 6-carboxy fluoroscen (CF) a fluorescent probe from egg PC/Cholesterol/tuftsin liposomes in buffer, pH 7.4 at 37 °C was about 2-4%/hour. This was probably due to binding of the dye with positively charged Arg residue in the 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 head group packing in liposomes bilayer (Hauser et al. 1981). The binding/uptake of the egg PC/Chol/tuftsin liposomes to PMN leukocytes was saturable, time dependent and the cell bound liposomes are apparently taken up by the cells by receptor-mediated endocytosis without losing their structural integrity. This was further supported by the fact that endocytosis was inhibited by lowering the incubation temperature to 0° C (Singhal et al. 1984). The specificity of these liposomes was also examined with other blood cells i.e. erythrocytes, lymphocytes and found that no binding with erythrocytes was observed but there appeared some binding with lymphocytes which was thought to be presumably due to the presence of PMN leucocytes/monocytes as contamination in the lymphocyte preparation (Singhal et al. 1984).

It has been demonstrated that tuftsin could enhance non-specific defense against infections by activating the macrophages (Singh et al. 1992). 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, O₂, H₂O₂ and myeloperoxidase (MPO). Both O₂ and H₂O₂ damage proteins, nucleic acids and membranes sufficiently to kill the cell or even the whole organism. Nevertheless, for macrophages hypohalous acid produced by action of MPO on H₂O₂, has been identified as the major killer agent (Klebanoff et al. 1980).
2.1.5.1. Immunopotentiating effect

The primary effect of tuftsin, after binding to receptors, consists of stimulation of macrophages and polymorphonuclear (PMN) cells. Specific binding sites of tuftsin are also localized on human monocytes. Tuftsin administered to the cell cultures stimulates the production of some cytokines. Intraperitoneal (i.p.) injection of tuftsin increases the production of TNF-α in serum and supernatants of cultured splenic and peritoneal cells (Wleklik et al. 1987). Robey et al. showed that tuftsin as well as its analogs, [Gly¹]-tuftsin, [Leu⁴]-tuftsin, and [Gln⁴]-tuftsin (all being fragments of human C-reactive protein), induce monocytes to produce IL-1 (Robey et al. 1987). Recently, it was also found that treatment of mouse peritoneal macrophages with tuftsin or tuftsin-THF-γ2 chimeras in the presence of antigen augments the IL-6 production (Granoth et al. 1997). In this way, tuftsin may perform its immunoregulatory functions and may influence inflammatory processes by enhancing the IL-2 formation induced by IL-1. It was initially found that tuftsin stimulates phagocytosis after binding to PMNs. Subsequently phagocytosis stimulating activity of tuftsin in monocyte-macrophages was also demonstrated by some workers (Coleman, 1986).

2.1.5.2. Anti-tumor activity

Many workers have reported the anti-tumor activity of tuftsin against experimental tumor models (Nishioka et al. 1981; Nishioka et al. 1983). Florentin et al. stated that tuftsin is able to potentiate various types of immune response when injected into mice, and can be used as a potent activator of macrophages in cancer therapy (Florentin et al. 1978). This was also confirmed by the finding that tuftsin treated mouse peritoneal macrophages exert cytostatic activity for tumor cell proliferation (Bruley-Rosset et al. 1981). Tuftsin was also reported to enhance the cytotoxic response of human monocytes against K562 tumor cell line at the doses of $5 \times 10^{-2}$ to $5 \times 10^{-1}\mu g/ml$. 

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The natural killing activity of lymphocytes against that particular cell line was not affected by tuftsin (Caroll et al. 1982). Tuftsin was also used for the treatment of cancer in humans with corresponding experiments in animals (Catane et al. 1983). Tuftsin at the doses ranging between 50 and 500µg/kg of the body weight enhances the cytotoxic activity of mononuclear cells in mice and human. The effect of tuftsin was accompanied by leucocytosis induction (Catane et al. 1983). It was also stated that tuftsin significantly increases survival rates among Rauscher virus leukemia infected mice and demonstrates antitumor activity against murine melanoma in vivo (Knyszynski et al. 1983; Noyes et al. 1981).

Earlier attempts have demonstrated usage of liposomised ETP in delaying tumour progression, but such treatment failed to regress tumor in model animals (Sengupta et al. 1998, Sengupta et al. 2000). In the present study, we have evaluated tuftsin-mediated augmentation of antitumorogenic potential of ETP against fibrosarcoma induced in Swiss albino mice by the carcinogen benzo (a) pyrene. Development of tumor was ascertained by measurement of tumor volume and confirmed by histopathological examination of the tissues. The tumor-bearing animals were treated with free form as well as liposomal formulation of ETP with or without tuftsin. The efficacy of various formulations was ascertained on the basis of regression of tumor volume and survival study. The present study also tries to unravel the molecular mechanism involved in the regression of tumor by the tuftsin-associated liposomal formulation of etoposide against fibrosarcoma. The tumor suppressor p53 is a predominantly nuclear transcription factor activated by various stresses including exposure to chemotherapeutic (Jimenez et al. 1999) as well as chemopreventive agents (Huang et al. 1999; Hsieh et al. 1999). Activation of p53 entails cell cycle arrest or apoptosis, both detrimental to the uncontrolled growth of tumors; as a result tumor cells are frequently selected for defects in the p53 pathway. When cells do respond adequately to p53-
activating signals, the actual biological outcome may vary greatly. A question that has received great attention, in part owing to its paramount relevance to the successful application of cancer chemotherapy, is how p53 opts between induction of apoptosis versus induction of a viable growth arrest. As it appears now, much of the choice is not in the hands of p53; rather, it is the cellular context, as defined by the balance of intracellular and extracellular signaling events, which dictates whether p53 activation will spare the cell or lead to its apoptotic demise. This is of particular relevance to cancer cells, which often acquire genetic alterations affecting directly or indirectly the functional status of p53. The simplest and most common event is direct mutational activation of the p53 gene itself, which will eliminate all wild-type p53 (p53wt) from the affected cells. Any genetic alterations that impact on the competence of these and many other proteins associated with apoptosis, cell cycle control and DNA damage repair are expected to be capable of modulating the likelihood that p53 will be activated in response to particular types of stress, as well as the biological outcome of such activation. The molecular basis for the differential activation of particular sets of target genes by p53 is not fully understood. Most certainly multiple molecular mechanisms contribute to p53 target gene selectivity. There are ample evidences advocating that covalent modifications on p53 may play a critical role in its target gene preference. The consequences of p53 activation are mostly mediated through the enhanced expression of cell cycle regulating proteins such as the p21 Waf1/Cip1 (CDKN1A) inhibitor of cyclin-dependent kinases (CDKs) and through a number of pro-apoptotic proteins, among them Bax (Miyashita and Reed, 1995), Noxa (APR) (Oda et al. 2000a) and p53AIP1 (Oda et al. 2000b) acting at the mitochondrial membrane, the cell surface death receptors Fas/APO-1 (Owen-Schaub et al. 1995) and KILLER/DR5 (Wu et al. 1997), the IGF-BP3 inhibitor of the IGF-R1 survival receptor (Buckbinder et al. 1995), the death domain-containing PIDD protein (Lin et al. 2000), endothelial cell nitric oxide synthase (Mortensen et
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al. 1999), the p85 regulator of the phosphatidyl-3-OH kinase involved in oxygen damage-response (Yin et al. 1998), enzymes that can produce reactive oxygen species (Polyak et al. 1997), and the plasma membrane protein PERP (Attardi et al. 2000). Furthermore, p53 itself can be associated with mitochondria in apoptotic cells (Marchenko et al. 2000).

To evaluate the efficacy of various liposomal formulations the in vivo study was divided into two phases, short term and long term. In short-term study, animals were exposed to with benzo (a) pyrene and subsequently treated with single dose various formulations. To study the mechanism operative for observed involved in the efficacy of various formulations expression of p53wt, p53mut, p21/waf1 was ascertained at mRNA level by RT-PCR. In long-term study the tumor-bearing animals were treated with free drug and with liposomal formulations of etoposide with or without immunomodulator tuftsinn. Drug formulations were administered at 10mg/kg b.w/day by intravenous route for five consecutive days. The efficacy of various formulations was assessed on the basis of tumor size, expression of p53wt, p53mut and p21/Waf1, Caspase-9, Bcl and Bax at protein and mRNA level by performing western blot analysis and RT-PCR respectively.

2.2. Materials and Methods

2.2.1. Chemicals

All the reagents used in the study were of the highest purity available. Egg PC was isolated and purified from hen egg yolk following the published procedure (Owais et al. 1993). Cholesterol was bought from Centron Research Laboratory, Mumbai, India and used after crystallizing three times with methanol. Tuftsinn was modified at C-Terminus by attaching a sufficiently long hydrocarbon fatty acyl residue to the C-terminus through an ethylenediamine spacer arm.
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(Thr-Lys-Pro-Arg-NH-(CH$_2$)$_2$-NH-CO-C$_{15}$H$_{31}$), which permits almost quantitative incorporation into liposomes, following the procedure standardized in our lab [Gupta et al. 1986]. Anti-p53 antibody specific for wild-type (wt) protein (clone PAb 1620, Ab-5), Anti-p53mut (clone PAb 240), Anti p21/Waf1, Anti Bcl-2, Anti caspase-3, Anti Bax and Anti β-actin were purchased from Merck India Ltd. The horseradish peroxidase-conjugated isotypes were obtained from Bangalore Genei (Bangalore, India).

2.2.2. Isolation and purification of egg phosphatidylcholine

Egg PC was isolated and purified following the published procedure (Singleton et al. 1965). Briefly, egg yolks of one dozen eggs were separated carefully, and washed with acetone (100 ml) in a mixer 5-6 times to get rid off the colored impurities. The resulting white solid was powdered and dried in vacuum for 2 hr to remove traces of the solvent. The extraction was performed by stirring the solid mass with one liter of absolute alcohol for 2-3 hours. After filtration, the residue was re-extracted with another 500 ml of ethanol. The filtrates were mixed and evaporated off at 40-45 °C. The sticky mass thus obtained was dried under high vacuum to remove traces of the solvent, dissolved in minimum amount of petroleum ether (b.p.60-80 °C) and poured onto chilled acetone to obtain a white sticky precipitate. The solvent from the precipitated material was removed by decantation. The sticky solid precipitate was redissolved in petroleum ether and again poured onto the chilled acetone. The process was repeated 2-3 times to obtain crude PC. For further purification, it was chromatographed on a neutral alumina (grade III) column. The elution was performed using increasing amount of methanol in chloroform. Fractions obtained with 5-7 % methanol in chloroform corresponded to pure PC. It was further purified by chromatography over Sephadex LH-20 column (2.5x 100 cm), using chloroform/methanol (1:1 v/v) as the eluant. The elution rate was maintained at 60-70 ml per hour.
Fractions were collected and checked for the presence of pure PC. The purity was ascertained by thin layer chromatography using silica gel G-60 TLC plates. The plates were developed using chloroform/methanol/water (65:25:4) as the solvent system, and stained with iodine vapour followed by molybdenum-blue spray to visualize various phospholipid spots.

2.2.3. Liposome preparation

Etoposide bearing unilamellar liposomes were prepared from egg PC (49 μmol) and cholesterol (21 μmol) with or without tuftsin (6.66 mol % of PC) following the published procedure (Owais et al. 1993). Briefly, the solution of egg PC and ETP (in ratio of 40:1 w/w) along with cholesterol and tuftsin in chloroform was reduced to a thin dry film with slow jet of N₂ gas. The dried lipid film was hydrated with 150-mM sterile normal saline, followed by sonication for 1 hour at 4 °C under N₂ atmosphere in bath type sonicator. The sonicated preparation was centrifuged at 10,000 g for 1 h at 4 °C to remove un-dispersed lipid. Finally, liposomal preparation was extensively dialyzed against saline for 24 hour at 4 °C in the dark to remove free form of the drug from the liposomal preparations. Both tuftsin free as well as tuftsin bearing liposomal preparation was found out to be of unilamellar type with size range of 80 ± 10 nm as revealed by electron microscopy (data not shown).

2.2.4. Estimation of liposome intercalated ETP

The intercalation efficiency of ETP in the liposomes was estimated by HPLC method (Khan et al. 2003). Briefly, specific volumes of standard drug solution were injected onto a Hypersil octyldecyl-silane (5 μm particle size) analytical column (150 X 4.6 mm). Detection of ETP was accomplished with a UV-visible-light detector set at 254 nm. A standard curve of ETP was plotted by calibrating peak area versus amount of the drug injected into column. The elution buffer was consisting of an isocratic mixture of 0.005 M
EDTA and methanol (2: 8 v/v). The flow rate was kept 1.2 ml/min and retention time was found out to be 4.5 min. Finally a known volume of liposomal formulation was injected and amount present in the sample was determined by area under curve. The intercalation efficiency of ETP both in plain egg PC and tuftsin bearing liposomes was found out to be of the same order (90 ± 4 percent).

2.2.4 Estimation of liposome intercalated tuftsin

The tuftsin entrapped in the drug containing liposomes was estimated by BCA method as modified in our lab (Owais et al. 2000). Briefly, the liposomes (given volume) were lysed with Triton X-100 solution and incubated with the mixture of solutions A and B of BCA reagent. The absorbance of the colored complex formed was measured at 600 nm and tuftsin content was determined using a standard curve of tuftsin plotted in the presence of Triton X-100. The incorporation of tuftsin was found out to be ~ 98% for both sham as well as ETP containing tuftsin bearing liposomes.

2.2.5. In vitro drug release assay

The release kinetics of etoposide from tuftsin bearing ETP liposomes into surrounding phosphate buffer saline (PBS pH 7.4) was determined by HPLC method following standardized protocol as described above (Khan et al. 2003). The vials containing Lip-ETP and Tuft-Lip-ETP were incubated with PBS at 37 °C under continuous shaking. An aliquot was withdrawn at two different time points (24h and 48h) and mixture was ultracentrifuged at 100,000g for 15 min. Resulting supernatant was analyzed by HPLC method as described earlier. The percent in vitro release was calculated with respect to the total drug content in liposomes.

2.2.6. ETP mediated lysis of human erythrocytes

The human red blood cells (RBCs) were diluted with isotonic buffer to get 50 % hematocrit. Etoposide (free as well as liposomised
form) was added at the concentration of 10μg/ml to erythrocyte suspension. The free form of the drug was dissolved in 50 μl of DMSO and final volume was made up to 1 ml with PBS (5 % DMSO). In order to study drug induced hemolysis, a suspension of RBCs was incubated with 1 ml of free as well as liposomised drug (10 μg/ml) at 37 °C for 1 hour. The mixture was centrifuged at 1500 g and supernatant analyzed for the released hemoglobin by measuring the absorbance at 576 nm.

The percent hemolysis was determined by following equation:

\[
\text{Percent Hemolysis} = 100 \left( \frac{\text{Abs} - \text{Abs}_0}{\text{Abs}_{100} - \text{Abs}_0} \right),
\]

Where \(\text{Abs}\) = Absorbance of the sample

\(\text{Abs}_0\) = Absorbance of -ve control (erythrocytes in 5 % DMSO) with no ETP

\(\text{Abs}_{100}\) = Absorbance of +ve control (erythrocytes in 1% Triton X-100) in presence of 10 μg/ml ETP.

2.2.6. Animals

Female Swiss albino mice weighing 18 ± 2 g were used in the whole study. The animals were given a standard pellet diet (Hindustan Lever Ltd.) and water ad libitum. Animals were checked daily for their mortality and morbidity prior to commencement of the study and only healthy animals were included in the experiment. The techniques used for bleeding, injection as well as sacrifice of animals were strictly performed following mandates approved by the institute’s Animal Ethics Committee (Committee for the purpose of control and supervision of Experiments on Animals, Govt. of India).

2.2.7. Maximum tolerated dose

The maximum tolerated dose (MTD) of the tuftsin bearing liposomal formulation of ETP (administered intravenously) was determined in healthy Swiss albino mice. Doses were escalated in increments starting with 0, 10, 20 40, 100, 200, 400 and 500 mg/kg.
body weight daily for 5 days. After completing the preliminary experiments, the approximate MTD was defined using eight groups (consisting of 9 mice each). The drug induce toxic manifestations were determined by careful surveillance of weight changes and survival of the experimental animals. The highest non-lethal dose of ETP causing >10% weight loss within 1 week of cessation from start of the dosing schedule was defined as the MTD. Out of total nine animals in each group, three mice received free drug, three were given Lip-ETP and the remaining three were administered with Tuft-Lip-ETP.

2.2.8. Hematological toxicity tests

Swiss albino mice were used to analyze the changes in haematological parameters following administration of Tuft-Lip-ETP, Lip-ETP and free form of ETP, with respect to time and dose. The mice were randomized into four groups. Group I served as controls and received vehicle treatment while Gp. II, Gp. III and Gp. IV mice were treated with free ETP, Lip-ETP and Tuft-Lip-ETP formulations respectively, at a dose of 10 mg/kg b.w/day for 5 days. Drugs were administered through the tail vein. On the day 7th post last treatment dose of analysis, the animals were sacrificed under anesthesia by cervical dislocation and blood withdrawn by cardiac puncture. Total and differential leukocyte and platelet counts were determined using a Sysmex cell counter.

2.2.9. Short term studies

To study the effect of various formulations of ETP on the expression of p53wt, p53mut and p21/waf1 at mRNA level, animals were challenged with benzo (a) pyrene subcutaneously (10mg/kg body weight) into the flanks of the left hind limb. Chemotherapy treatment (ETP formulations) was given intravenously on day seven post benzo (a) pyrene administration with various formulations. The drug and vehicle were slowly injected into the tail vein with a 26-gauge needle.
and the total volume of administration never exceeded 100 µl per dose. The animals were divided into seven groups consisting of 12 animals each as given below.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Normal untreated</td>
</tr>
<tr>
<td>Group II</td>
<td>BaP + PBS (Vehicle Control)</td>
</tr>
<tr>
<td>Group III</td>
<td>BaP + Sham liposomes</td>
</tr>
<tr>
<td>Group IV</td>
<td>BaP + Sham tuftsin liposomes</td>
</tr>
<tr>
<td>Group V</td>
<td>BaP + Free etoposide (10mg/kg b.w)</td>
</tr>
<tr>
<td>Group VI</td>
<td>BaP + Liposomal etoposide (10mg/kg b.w)</td>
</tr>
<tr>
<td>Group VII</td>
<td>BaP + Tuftsin bearing liposomal etoposide (10mg/kg)</td>
</tr>
</tbody>
</table>

Four animals from each group were euthanized by cervical dislocation after 24, 48 and 72 h of treatment with various formulations, respectively. The tissues were excised out, cleaned, snap frozen in liquid nitrogen and stored at -80 °C until used for further experimentation.

2.2.10. Long term studies

2.2.10.1. In vivo tumor model

Solid fibrosarcoma was induced in mice according to published method with slight modification (Faiderbe et. al, 1992b). Briefly, single dose of benzo (a) pyrene 250µg/animal was administered subcutaneously into the flanks of the left hind limbs. The mice were observed daily for any change along the dimensions of the two hind
limbs. Only those mice that developed palpable tumors between 90 and 100 days of post-administration of benzo (a) pyrene were included in the study.

2.2.10.2. Treatment schedule and assessment parameters

The tumor-bearing mice were pooled and randomized into seven groups as follows: Normal untreated mice were included in Gp. I.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Normal Untreated</td>
</tr>
<tr>
<td>Group II</td>
<td>PBS (Vehicle Control)</td>
</tr>
<tr>
<td>Group III</td>
<td>Sham liposomes (Vehicle Control)</td>
</tr>
<tr>
<td>Group IV</td>
<td>Tuftsin bearing liposome (No etoposide)</td>
</tr>
<tr>
<td>Group V</td>
<td>Free drug (Etoposide)</td>
</tr>
<tr>
<td>Group VI</td>
<td>Liposomal etoposide</td>
</tr>
<tr>
<td>Group VII</td>
<td>Tuftsin bearing liposomal etoposide</td>
</tr>
</tbody>
</table>

The tumors were measured regularly with a Vernier Caliper until they reached a volume of 200 mm$^3$. At this juncture the mice were treated with 10-mg/kg b.w/day of etoposide for five consecutive days, the first day of treatment was considered as the day 0. The drug and vehicle were slowly injected into the tail vein with a 26-gauge needle, taking care that the total volume of administration never exceeded 100 μl per dose. The tumors were measured regularly after treatment. Anti-tumor activity was assessed by calculating the tumor volume according to the formula

$$V = D \times d^2 \times \frac{\pi}{6},$$

where $V = $ tumor volume, $D = $ biggest dimension, and $d = $ smallest dimension.
2.2.10.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from the tissues with TRIzol reagent (Invitrogen). Tumor tissue samples were homogenized with TRIzol (1 ml TRIzol per 100 mg of tissue) using a glass-teflon homogenizer and RNA was extracted and purified as per the manufacturer's protocol. For RT-PCR reactions, 3 µg of total RNA was combined with 500 ng of oligo dT primer (Invitrogen) in a final volume of 12 µl, heated at 70°C for 10 min and then placed on ice. Reverse transcription reactions (20 µl) were then assembled with final concentrations of 1X reaction buffer, 10 mM DTT, 0.5 mM dNTP's and 100 U of Superscript II reverse transcriptase (Invitrogen). Two percent of the RT reaction was used as input for each PCR. PCR reactions (50 µl) were assembled with final concentrations of 1X buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of each primer and 2.5 U Taq polymerase (Invitrogen). Primers were obtained from Sigma-Aldrich and PCR was performed with forward (F) and reverse (R) forward primers specific to mouse p53wt, p53mut, p21/waf1, caspase-3, bax, bcl2 genes and primers specific for glyceraldehydes-3-phosphate dehydrogenase (GAPDH); Table 2.1. GAPDH, a housekeeping gene, PCR reaction employing primers for GAPDH were used as control. The GenBank accession no. AF051368, AB021961, U09507, NM_009810 XM_991820, NM_007527, DQ080431, BC145810 for p53wt, p53mut, p21/waf1, caspase-3, bax, bcl-2 and GAPDH respectively. PCR cycle parameters were as follows: initial step of 95°C for 5 min and then 20-35 cycles of 95°C for 30s, annealing temperature of 59-61°C for 30 s, 72°C for 45s and a final step of 72°C for 5 min. Cycle number that allows for endpoint analysis during linear amplification was determined empirically. The size of the PCR amplicon was determined by comparison with 100-bp DNA ladder. PCR products of p53wt (723 bp), p53mut (768 bp), p21/waf1 (595 bp), caspase-3 (668 bp), bax (589), bcl-2 (606 bp) and GAPDH
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(382 bp) were resolved on a 1.5% agarose gels and observed in UV-transilluminator.

Table 2.1: Forward and reverse primers specific to mouse p53wt, p53mut, p21/waf1, caspase-3, bax, bcl2 and GAPDH.

<table>
<thead>
<tr>
<th></th>
<th>F: 5'-TTACCAGGGCAACTATGGCTTCCA-3'</th>
<th>R: 5'-TCCCGGAACATCTCGAAGCGTTTA-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53mut</td>
<td>F: 5’- TTACCAGGGCAACTATGGCTTCCA-3’</td>
<td>R: 5’-TCCTCTGTAGCATGGGCATCCTTT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21/waf1</td>
<td>F: 5’- TGGCTGAACTCAACACCACCTTA-3’</td>
<td>R: 5’- AGACCATCTGCGCTTGAGAGTGAT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>caspase-3</td>
<td>F: 5’- AGAGCCTGGAATGTCATCTGCT-3’</td>
<td>R: 5’-TGAGCATGGACACAATACACGGGA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bax</td>
<td>F: 5’-TGTCTCCCAGGCAATGGAGATGAA-3’</td>
<td>R: 5’-ACTCCAGCCCAAAGATGGTCACT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bcl-2</td>
<td>F: 5’-AAAGCACCCACTCAAGCAGCAAAAG-3’</td>
<td>R: 5’-TTATTGAGCAGAGTCCTTGACGGCT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5’-TAAAGGGCATCCTGGGCTACACT-3’</td>
<td>R: 5’-TTACTCTCTGGGAGGCCATGTAGG-3’</td>
</tr>
</tbody>
</table>

2.2.10.4. Preparation of cell lysate

The skin/tumor tissue (maintained on ice) was removed with sharp scalpel blades. The samples were homogenized in lysis buffer (containing 20% glycerol, 20 mmol/L HEPES, 10 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.1% NP40, 0.2 mmol/L EDTA, 1 mmol/L DTT, 1 µg/mL pepstatin A, 1 µg/mL aprotinin, 100 µg/mL phenylmethylsulfonyl fluoride) and processed according to the
published method (Serpi et al. 1999). The homogenate was kept for 15 minutes in lysis buffer on ice and then centrifuged for 15 minutes 20,000 \( g \) at 4 °C and the supernatant was collected.

2.2.10.5. Western Blotting

Western blotting was carried out in the cell lysate of skin/tumor tissue following published procedure (Towbin et al. 1979). Protein content of each sample was estimated by the routine method using bovine serum albumin as a standard (Lowry et al. 1951). Proteins (30μg) were resolved on 10 % SDS-PAGE gels and electroblotted onto nitrocellulose membranes. The blots were blocked overnight with 3 % nonfat dry milk and probed with appropriate antibodies [i.e., anti-p53wt (clone PAb 1620) and anti-p53mut (clone PAb 240) antibody, anti-Bax (clone 5B7/3A2), anti-Bcl-2 (10C4), Anti Caspase-3 (clone AM1.4.1-1B)] at dilutions recommended by the suppliers. Immunoblots were detected by horseradish peroxidase-conjugated anti-mouse IgG using chromogen 3,3'-diaminobenzidine tetrahydrochloride. To quantify equal loading, membranes were reprobed with β-actin antibody. Data are presented as the relative pixel density of each bands normalized to band of β-actin. The intensity of the bands was quantitated using Alpha Image Analysis software on Alpha Image Gel Documentation System.

2.3. Results

While developing a novel formulation of a given therapeutic agent, it is desirable to have idea about its in vitro stability profile. The in vitro drug release kinetic studies revealed that incubation of liposomised ETP in PBS resulted in slow release of the drug. There was 12 % and 23 % leakage after 24h and 48h respectively of ETP (active component) from tuftsin bearing ETP liposomes. On the other hand, there was 19 % and 38 % drug release after 24h and 48h respectively in case of Lip-ETP formulation (Fig. 2.2A).
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The toxicity studies suggest that incorporation of ETP in liposomes markedly lowers its toxicity to erythrocytes. Among various formulations, free ETP was found to show maximum toxicity resulting in significant hemolysis of erythrocytes. The incorporation of ETP in conventional as well tuftsin bearing liposomes resulted in diminution of its toxic property to a surprisingly low level. Tuft-Lip-ETP formulation imparts less toxicity to erythrocytes (~7 % hemolysis) than that of conventional liposomal preparation (~12 % hemolysis) of same drug (Fig. 2.2B).

Loss in body weight in response to the administration of antineoplastic agent ETP was used as another parameter to assess its toxicity. As shown in Fig. 2.3, the maximum tolerated dose (MTD) for free, liposomal as well as tuftsin bearing liposomal ETP was found out to be 165.53, 279.77 and 413.25 mg/kg, respectively (P value: Lip-ETP vs free ETP p<0.001; and the animals treated with Tuft-Lip-ETP over free as well as liposomal ETP p<0.001).

Haematological tests were performed to evaluate the effect of liposomised ETP on rapidly proliferating hematopoietic cells. The immune cell depletion was significantly greater in the group of animals treated with free ETP with a mean total leukocyte count of 1764/mm$^3$ as compared to 4,457/mm$^3$ counts in the Lip-ETP treated group (p<0.001) on day 7 post last dose of ETP. Myelo-suppression was significantly lower in the group of animals treated with Tuft-Lip-ETP with a mean total leukocyte count 7364/mm$^3$ as compared to groups of animals treated with free (p<0.001) as well liposomal ETP (p<0.001). Control healthy mice had a total leukocyte count of 12,160/mm$^3$ at the same time point (Fig. 2.4A). No substantial change was observed in the differential leukocyte profile following therapy suggesting that the proliferation of all the components was equally suppressed. A reduction in platelet count was also observed on day 7 in drug treated groups as compared with control. The mean platelet
counts in the free ETP, Lip-ETP and Tuft-Lip-ETP-treated groups were $1.4 \times 10^5 / \text{mm}^3$ (p<0.001 vs control), $2.1 \times 10^5 / \text{mm}^3$ (p<0.001 vs free ETP) and $2.5 \times 10^5 / \text{mm}^3$ (p<0.001 vs free as well as Lip-ETP) respectively. Vehicle treated control animals had a mean platelet count of $2.8 \times 10^5 / \text{mm}^3$ (Fig. 2.4B).

2.3.1. Short term studies

Short-term study at mRNA level after 24h and 48h treatment with various formulations revealed low expression of p53wt and p21/waf1 while upregulation of p53mut in animals subsequent to treatment with benzo a pyrene (Gp. II) as compared to untreated normal mice (Fig. 2.5, 2.6 and 2.7). Tuftsin-bearing liposomal formulation of etoposide significantly raised expression of p53wt and p21/Waf as compared to free and liposomal formulation of etoposide and this expression profile was maintained even after 72h post treatment. No normalization in expression of p53wt and p21/waf was evident in animals treated with tuftsin-bearing liposomes without etoposide or free etoposide (Fig. 2.5 and 2.7).

The data of the present study demonstrate detectable amount of p53mut mRNA in normal healthy animals. After 24h post-treatment significant decreased in expression of p53mut was observed in the animals treated with free etoposide and tuftsin-liposome (sham liposomes) devoid of etoposide (Fig. 2.6). In the animals treated with liposomal etoposide the expression of p53mut was significantly lower in comparison to animals treated with tuftsin liposomes and free etoposide (Fig. 2.6). The expression of p53mut in the animals treated with tuftsin-bearing liposomal etoposide was just only in detectable amount and was comparable to that of control animals that were not treated with benzo a pyrene. The same level of mRNA expression was observed after 48 and 72h post treatment (Fig. 2.6).
2.3.2. Long-term studies

To evaluate in vivo efficacy of various ETP formulations against experimental fibrosarcoma, tumors were developed in model animals. The treatment with benzo (a) pyrene ensued in development of tumors in 90 percent of the animals within 90-100 days (Fig. 2.8). The establishment of the tumors was confirmed by histopathological examinations of the tissues. The fibrosarcoma was found to be accompanied with increased vascularization, large pleomorphic, hyperchromatic spindle shaped cells with anisonucleosis and irregularly dispersed chromatin (Fig. 2.9).

Among various formulations tuftsin bearing liposomised ETP was found to be most effective as compared to tuftsin free liposomised ETP or free form of the drug in treatment against fibrosarcoma. Treatment of the animals with Lip-ETP was started when the tumors attained the size of approximately 200 mm$^3$. Chemotherapy with Tuft-Lip-ETP at a dose of 10 mg/kg b.w/day for 5 days significantly reduced the growth of the tumour as compared with Lip-ETP (p<0.001), free ETP (p<0.001), Sham-Tuft-Lip (p<0.001) as well as controls treated with sham liposomes (no tuftsin) and PBS (p<0.001). Liposomised ETP delayed tumour growth and was superior to free form of ETP and sham tuftsin liposomes, however remained unable to regress the tumour significantly. On the other hand, tuftsin bearing ETP liposomes reduced the growth upto 165.7 ± 2.63 mm$^3$ from 200 mm$^3$ within 52 days after the initiation of chemotherapy (Fig. 2.10A).

Mortality was seen in all benzo (a) pyrene induced tumor, however animals treated with appropriate chemotherapy had comparatively longer life span. Table 2.1 shows the median day of death in each group. Treatment with free form of ETP increased the life span of the tumour-bearing mice as compared to those treated with PBS and sham liposomes (p<0.001). As depicted in Fig. 2.10B, treatment with Lip-ETP was superior to free form of ETP and Sham-
Tuft-Lip treatment ($p<0.001$) in increasing the life-span of the tumor-bearing mice. While the animals treated with Tuft-Lip-ETP significantly increase the life span of tumour bearing animals as compared to animals treated with Sham-Tuft-Lip, ETP as well as Lip-ETP ($p<0.001$).

The western blot as well as RT-PCR analysis showed significantly low level of expression of p53wt protein in tumors of animals treated with vehicle controls (PBS and Sham liposomes) in comparison to normal untreated healthy mice (Fig. 2.11A, lanes 1, 2 and 3). As depicted in Fig. 2.11(A), comparatively high expression of p53wt protein was recorded in the animals treated with various formulations than the animals treated with vehicle controls. Treatment with liposomal etoposide ensued in upregulation of p53wt, which was superior to the animals treated with sham tuftsin liposomes and free etoposide ($p<0.001$). Significantly higher expression of p53wt was recorded in the animals treated with tuftsin-bearing liposomal etoposide as compared to animals treated with liposomal etoposide ($p<0.001$), indicating that tuftsin-bearing liposomal formulation of etoposide regulate expression of p53wt protein significant manner and help in inhibition of DMBA-induced neoplastic changes.

Animals not treated with benzo a pyrene had detectable level of p53mut. Administration of benzo a pyrene resulted in development of fibrosarcoma in the treated animals and also upregulated p53mut levels. Treatment with PBS or sham liposomes did not abolish this effect (Fig. 2.11B, lanes 1, 2, 3). As depicted in the Fig. 2.11 (B), the level of p53mut was significantly reduced ($p<0.001$) in the animals treated with liposomal etoposide in comparison to free etoposide as well as tuftsin liposomes (sham). In animals treated with tuftsin-bearing liposomal etoposide, the expression of p53mut was significantly low ($p<0.01$) in comparison to liposomal etoposide.
(without etoposide). These results clearly show that etoposide mediates down-regulation of p53mut, which is more apparent upon its intercalation in liposomes along with tuftsin used in the present study (Fig. 2.11B).

The effect of various formulations on the expression of p21/waf1 was also assessed, which is transcriptionally up-regulated in the presence of p53wt. Immunoblot as well as RT-PCR analysis showed increased expression of p21/waf1 in animals treated with various formulations of etoposide. Increased expression (p<0.001) was observed in the animals treated with liposomised etoposide in comparison to free etoposide as well as sham tuftsin liposomes (Fig. 2.11C). The expression of p21/waf1 was significantly higher in the animals treated with tuftsin bearing liposomal etoposide in comparison to liposomal etoposide.

Immunoblot as well as RT-PCR analysis showed an increased expression of proapoptotic gene bax in liposomal etoposide treated groups. The expression of bax was reduced in the animals treated with vehicle controls when compared with untreated control animals. The expression of bax increased significantly (p<0.01) in the animals treated with etoposide in comparison to vehicle controls. In the groups of animals treated with tuftsin bearing liposomal etoposide as well liposomal etoposide without tuftsin, the expression of bax was similar but significantly higher (p<0.001) than those treated with free etoposide (Fig. 2.11D). However no significant difference in Bax level was observed in the animals treated with sham tuftsin liposomes when compared with vehicle controls.

The level of bcl-2 was also found to be significantly low (p>0.001) in the groups of animals treated with free etoposide in comparison to vehicle controls. As depicted in the Fig. 2.11E, the level of bcl-2 was reduced significantly (p<0.001) but equal to the animals treated with liposomal etoposide as well as tuftsin bearing liposomal
etoposide in comparison to free etoposide. While no significant difference was observed in animals treated with sham tuftsin liposome when compared with vehicle controls. These results clearly show that immunomodulator tuftsin play not much significant role in down-regulation of bcl-2, in etoposide mediated chemotherapy against fibrosarcoma (Fig. 2.11E).

We also studied effect of liposomised etoposide on caspase-3. The expression level of caspase-3 was significantly reduced in animals treated with vehicle controls (PBS and Sham liposomes) in comparison to normal untreated mice (Fig. 2.11F, lanes 1, 2 and 3). As depicted in Fig. 2.11F, comparatively high level of expression of caspase-3 (p<0.01) was recorded in the animals treated with free etoposide. In the groups of animals treated with liposomal etoposide with or without tuftsin the expression level of Caspase-3 was identical but significantly higher (p<0.001) when compared to free etoposide. No significant difference was observed in the animals treated with sham tuftsin liposomes in comparison to animals treated with vehicle controls, indicating that liposomal formulation of etoposide with or without tuftsin plays a determining role in the expression of caspase-3 protein.
Figure 2.2(A): Leakage of ETP from liposomised formulations in surrounding PBS buffer. Data is mean ± SD of three independent experimental values. *p<0.001 (Tufts-Lip-ETP) versus Lip-ETP at 24h and 48h.

Figure 2.2(B): Hemolysis of human erythrocytes induced by various formulations of ETP. Data is mean ± SD of three independent experimental values. a p<0.001 versus free ETP and b p<0.001 versus Lip-ETP.
Figure 2.3: Effect of increasing doses of ETP on body weight of the treated animals.

The dose resulting in the loss of more than 10% body weight is identified as the MTD. Data shown are means of three individual animals at each dose point.
Figure 2.4(A): Effects of ETP chemotherapy on leukocyte counts of the treated animals. Data are means ± SD with n = 5 per group. 
\( a \) \( p < 0.001 \) versus free ETP and \( b \) \( p < 0.001 \) versus Lip-ETP.

Figure 2.4(B): Effects of ETP chemotherapy on platelet counts of the treated animals. Data are means ± SD with n = 5 per group. 
\( a \) \( p < 0.001 \) versus free ETP and \( b \) \( p < 0.001 \) versus Lip-ETP.
Figure 2.5: Effect of various formulations on the mRNA expression of p53wt after the administration of benzo (a) pyrene in Swiss albino mice.

Treatment with various formulations followed on day 7 after the single subcutaneous dose of benzo a pyrene (10mg/kg). The p53wt mRNA expression were analyzed after (A) 24h, (B) 48h as well as (C) 72h. GAPDH was used as control. Isolation of total RNA, cDNA synthesis and PCR were performed as described in Materials and Methods. Lane 1, Untreated (Normal); lane 2, Vehicle Control (PBS); lane 3, Sham liposomes; lane 4, Tuftsin liposomes (No ETP); lane 5, Free ETP; lane 6, Liposomal ETP and lane 7, Tuftsin bearing liposomal ETP.
(B)
48h

- **p53wt**
- **GAPDH**

![Graph showing pixel density comparison](image)

- **Pixel density**
- **Categories:**
  - Untreated (Normal)
  - Vehicle Control (PBS)
  - Vehicle Control (Sham liposomes)
  - Tuftsin liposomes
  - Free etoposide
  - Liposomal etoposide
  - Tuftsin bearing liposomal etoposide
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(C)

72h

p53wt

GAPDH

1 2 3 4 5 6 7

Pixel density

Untreated (Normal)  Vehicle Control (PBS)  Vehicle Control (Sham liposomes)  Tuftsin liposomes  Free etoposide  Liposomal etoposide  Tuftsin bearing liposomal etoposide

117
Figure 2.6: **Effect of various formulations on the mRNA expression of p53mut after the administration of benzo (a) pyrene in Swiss albino mice.**

Treatment with various formulations followed on day 7 after the single subcutaneous dose of benzo a pyrene (10mg/kg). The p53mut mRNA expression were analyzed after (A) 24h, (B) 48h as well as (C) 72h. GAPDH was used as control. Isolation of total RNA, cDNA synthesis and PCR were performed as described in Materials and Methods. Lane 1, Untreated (Normal); lane 2, Vehicle Control (PBS); lane 3, Sham liposomes; lane 4, Tuftsin liposomes (No ETP); lane 5, Free ETP; lane 6, Liposomal ETP and lane 7, Tuftsin bearing liposomal ETP.
Chapter 2

Tufts in the treatment of fibrosarcoma

(A) 24h

![Image showing gel electrophoresis results for p53mut and GAPDH genes at 24 hours.](image)

![Bar graph showing apoptosis levels for different conditions.](image)
Chapter 2

Tuftsin in the treatment of fibrosarcoma

(B)

48h

p53mut

GAPDH

1 2 3 4 5 6 7

Pixel density

- Untreated Normal
- Vehicle Control (PBS)
- Vehicle Control (Sham liposomes)
- Tuftsin liposomes
- Free etoposide
- Liposomal etoposide
- Tuftsin bearing liposomal etoposide
Chapter 2

Tufts in the treatment of fibrosarcoma

(C)

72h

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![Graph showing pixel density comparison between different groups.](image)
Figure 2.7: Effect of various formulations on the mRNA expression of p21/waf1 after the administration of benzo (a) pyrene in Swiss albino mice.

Treatment with various formulations followed on day 7 after the single subcutaneous dose of benzo a pyrene (10mg/kg). The p21/waf1 mRNA expression were analyzed after (A) 24h, (B) 48h as well as (C) 72h. GAPDH was used as control. Isolation of total RNA, cDNA synthesis and PCR were performed as described in Materials and Methods. Lane 1, Untreated (Normal); lane 2, Vehicle Control (PBS); lane 3, Sham liposomes; lane 4, Tuftsin liposomes (No ETP); lane 5, Free ETP; lane 6, Liposomal ETP and lane 7, Tuftsin bearing liposomal ETP.
Chapter 2  
Tufts in the treatment of fibrosarcoma

(A)

24h

p21/Waf1

GAPDH

Pixel density

1.8
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1 2 3 4 5 6 7

Untreated (Normal)
Vehicle Control (PBS)
Vehicle Control (Sham liposomes)
Tufts in liposomes
Free etoposide
Liposomal etoposide
Tufts in bearing liposomal etoposide
Chapter 2

Tufts in the treatment of fibrosarcoma

(B)

48h

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![Graph showing pixel density comparison](image-url)

- Untreated (Normal)
- Vehicle Control (PBS)
- Vehicle Control (Sham liposomes)
- Tufts (liposomes)
- Free etoposide
- Liposomal etoposide
- Tufts bearing liposomal etoposide
Chapter 2

Tufts in the treatment of fibrosarcoma

(C)
72h

p21/Waf1

GAPDH

1 2 3 4 5 6 7

Pixel density

Untreated (Normal)  Vehicle Control (PBS)  Vehicle Control (Sham liposomes)  Tufts in liposomes  Free etoposide  Liposomal etoposide  Tufts bearing liposomal etoposide
Figure 2.8: Establishment of fibrosarcoma (A) and (B) upon exposure of animals with benzo (a) pyrene. The benzo (a) pyrene was administered into the flanks of the animals subcutaneously at the dose of 250μg/animal.
Figure 2.9: *Establishment of fibrosarcoma upon exposure of animals with benzo (a) pyrene.*

Administration of benzo (a) pyrene (250µg/animal, s.c.) into the flanks of the animals ensued successful tumor induction, in at least 90% of the animals, while 10% of the animals did not develop any tumor. The tumor bearing animals were sacrificed by cervical dislocation and fixed in 10% formaldehyde solution. Subsequently tissues were excised and sections were prepared following standard HE staining of 12-mm-thin sections. (A) Photomicrographs from tumor showing zone of necrosis, increased vascularization and hemorrhage. Large number of hyperchromatic mesenchymal cells with features of fibrosarcoma is present. Some hyperchromatic cells arranged in glandular form also. (B) Photomicrograph from tumor showing soft tissue sarcoma. It shows large pleomorphic, hyperchromatic spindle shaped cells with anisonucleosis and irregularly dispersed chromatin. Some inflammatory cells can also be seen.
Figure 2.10(A): Effects of chemotherapy with various formulations of ETP on tumor development in Swiss mice.

Treatment of tumor bearing animals was started when the tumor size reached a volume of approximately 200 mm³. Free ETP and sham tuftsin liposomes significantly delayed tumor growth (p<0.05) as compared with controls (PBS and Sham liposomes). Liposomal ETP was more superior to free ETP in delaying tumor growth (p<0.001). The regressions of tumors were recorded only in the group of animals treated with tuftsin bearing liposomal ETP. Data are values ± SD (n = 20 at initiation of therapy, the number varies at later time points due to mortality).
The fibrosarcoma was induced by exposure to benzo (a) pyrene. The treatment of tumor bearing animals was started at the time point when tumor size reached a volume of approximately 200 mm$^3$. All the treated mice received a dose of 10mg/kg b.w/day for 5 days with various formulations of the drug. Free ETP significantly enhanced the life span of the mice as compared with the control group (PBS and sham liposomes) ($p<0.01$). Lip-ETP was superior to free ETP in increasing the lifespan ($p<0.001$). The best therapeutic effect was recorded in the group of animals treated with tuftsin bearing liposomal ETP ($p<0.001$). Data shown the number of mice surviving at the given time points.
Figure. 2.11: Effect of various formulations on the expression of p53wt (A) at protein level as analyzed by western blot as well as (B) at mRNA level by RT-PCR in mouse fibrosarcoma.

The treatments were given to the animals for five consecutive days when the tumor reached the size of 200 mm³ with various formulations. All the surviving animals were sacrificed after 25 days from the first administered drug dose. Skin/tumor lysates preparation for Immunoblot analysis and isolation of total RNA, cDNA synthesis and PCR were performed as described in Materials and Methods. Lane 1, Untreated (Normal); lane 2, Vehicle Control (PRSI); lane 3, sham liposomes; lane 4, Tuftsin liposomes (No ETP); lane 5, free ETP; lane 6, liposomal ETP and lane 7, Tuftsin bearing liposomal ETP.
Figure 2.12: Effect of various formulations on the expression of p53mut (A) at protein level as analyzed by western blot as well as (B) at mRNA level by RT-PCR in mouse fibrosarcoma.

The treatments were given to the animals for five consecutive days when the tumor reached the size of 200 mm³ with various formulations. All the surviving animals were sacrificed after 25 days from the first administered drug dose. Skin/tumor lysates preparation for Immunoblot analysis and isolation of total RNA, cDNA synthesis and PCR were performed as described in Materials and Methods. Lane 1, Untreated (Normal); lane 2, Vehicle Control (PBS); lane 3, sham liposomes; lane 4, Tuftsin liposomes (No ETP); lane 5, free ETP; lane 6, liposomal ETP and lane 7, Tuftsin bearing liposomal ETP.
Figure 2.13: Effect of various formulations on the expression of p21/waf1 (A) at protein level as analyzed by western blot as well as (B) at mRNA level by RT-PCR in mouse fibrosarcoma.

The treatments were given to the animals for five consecutive days when the tumor reached the size of 200 mm³ with various formulations. All the surviving animals were sacrificed after 25 days from the first administered drug dose. Skin/tumor lysates preparation for Immunoblot analysis and isolation of total RNA, cDNA synthesis and PCR were performed as described in Materials and Methods. Lane 1, Untreated (Normal); lane 2, Vehicle Control (PBS); lane 3, sham liposomes; lane 4, Tuftsin liposomes (No ETP); lane 5, free ETP; lane 6, liposomal ETP and lane 7, Tuftsin bearing liposomal ETP.
Figure. 2.14: *Effect of various formulations on the expression of bax (A) at protein level as analyzed by western blot as well as (B) at mRNA level by RT-PCR in mouse fibrosarcoma.*

The treatments were given to the animals for five consecutive days when the tumor reached the size of 200 mm³ with various formulations. All the surviving animals were sacrificed after 25 days from the first administered drug dose. Skin/tumor lysates preparation for Immunoblot analysis and isolation of total RNA, cDNA synthesis and PCR were performed as described in Materials and Methods. Lane 1, Untreated (Normal); lane 2, Vehicle Control (PBS); lane 3, sham liposomes; lane 4, Tuftsin liposomes (No ETP); lane 5, free ETP; lane 6, liposomal ETP and lane 7, Tuftsin bearing liposomal ETP.
Figure. 2.15: Effect of various formulations on the expression of bcl-2 (A) at protein level as analyzed by western blot as well as (B) at mRNA level by RT-PCR in mouse fibrosarcoma.

The treatments were given to the animals for five consecutive days when the tumor reached the size of 200 mm$^3$ with various formulations. All the surviving animals were sacrificed after 25 days from the first administered drug dose. Skin/tumor lysates preparation for Immunoblot analysis and isolation of total RNA, cDNA synthesis and PCR were performed as described in Materials and Methods. Lane 1, Untreated (Normal); lane 2, Vehicle Control (PBS); lane 3, sham liposomes; lane 4, Tuftsin liposomes (No ETP); lane 5, free ETP; lane 6, liposomal ETP and lane 7, Tuftsin bearing liposomal ETP.
Chapter 2

Tuftsin in the treatment of fibrosarcoma

Figure 2.16: Effect of various formulations on the expression of caspase-3 (A) at protein level as analyzed by western blot as well as (B) at mRNA level by RT-PCR in mouse fibrosarcoma.

The treatments were given to the animals for five consecutive days when the tumor reached the size of 200 mm$^3$ with various formulations. All the surviving animals were sacrificed after 25 days from the first administered drug dose. Skin/tumor lysates preparation for Immunoblot analysis and isolation of total RNA, cDNA synthesis and PCR were performed as described in Materials and Methods. Lane 1, Untreated (Normal); lane 2, Vehicle Control (PBS); lane 3, sham liposomes; lane 4, Tuftsin liposomes (No ETP); lane 5, free ETP; lane 6, liposomal ETP and lane 7, Tuftsin bearing liposomal ETP.
2.4. Discussions

Drugs used in treatment of cancer generally show severe toxic manifestations and undesirable side effects. The usage of most of the anticancer drugs including ETP is also limited by an ever-increasing trend of multi drug resistance displayed by the cancerous cells. This resistance is mainly due to the drug efflux mediated by an array of transporters p-glycoproteins such as p-gp4 and other multi drug resistance proteins chiefly MRP1, MRP2 and MRP3 etc (Borst et al. 2000, Zelcer et al. 2001 and Guo et al. 2002). It is demand of the ordeal to develop newer formulations of potential anticancer agents viz. ETP showing least toxicity and also being able to circumvent the problem of drug resistance. In this regard usage of the drug delivery systems that can specifically deliver anticancer drugs solely to tumor tissues could prove to be an acceptable solution. Etoposide entrapped in liposomes has been found to be much assuring to this end (Sengupta et al. 1998 & 2000). Liposomes are lipid-based vesicles that enable efficient and targeted delivery of drugs to the desired site (Gabizon et al. 1997, Vaage et al. 1992). Moreover, liposomised anticancer formulations can circumvent drug efflux and achieve adequate drug concentration in the target cell to enable tumor cell killing as they deliver overwhelming disposition of the drug that can match well with p-glycoprotein transporters mediated drug efflux (Candoni et al. 2006, Sadva et al. 2002, Lo et al. 2001, Lawrence et al. 2001, Poujol et al. 1999).

We have attempted to further enhance therapeutic efficacy of liposomal ETP by the incorporation of the immunomodulator tuftsin. Immunomodulators are biological response modifiers and can also exert antitumor effects by improving host defense mechanisms against the tumor. Tuftsin has been shown to direct anti-proliferative effect on tumor cells and also enhance the ability of the host to tolerate damage by toxic chemicals that may be used to destroy the cancer cells (Knyszynski et al. 1983, Noyes et al. 1981). Immunomodulatory therapy thus provide an alternative to conventional chemotherapy for a variety of diseased conditions, especially when the host's defense mechanisms is impaired.
and need appropriate activation. To suppress tumors in vivo, macrophages must first infiltrate to the desired site. In fact, the presence of inflammatory macrophages in growing tumors is retained through recruitment of circulating monocytes (Bugelski et al. 1987a, Bugelski et al. 1987b, Evans and Cullen 1984, Normann 1985) and in certain tumors, the proliferation of mononuclear phagocytes (Mantovani, 1990). Activated macrophages can destroy syngeneic, allogeneic, and xenogeneic tumor cells, but leave normal nontumorigenic cells unharmed, suggesting that histocompatibility and tumor-specific antigens are not involved in this recognition (Fidler and Schroit, 1988). In addition, differences in metastatic potential, chromosome number, resistance to chemotherapeutic agents, or the antigenic properties of tumor cells did not appear to be important factors for macrophage recognition of tumors cells (Killion et al. 1993, Pak 1989). Successful treatment of metastases by the intravenous injection of liposomes containing immunomodulators has been reported for several tumor-host models, including mouse fibrosarcoma (Fidler 1986b, Deodhar et al. 1982, Eppstein et al. 1986, Lopez-Berestein et al. 1984), melanomas (Phillips et al. 1985, Phillips and Tsao 1989), lung carcinoma (Brodt et al. 1989) and colon carcinoma (Thrombe and Deodhar 1984) etc. Macrophages are believed to migrate to local regions of inflammation and the sites of tumor growth where they induce further immune reactivity against tumor cells. The treatment of tumors could be achieved by first delivering the drug to the RES and concomitant activation of their components (cf. macrophages) for their tumoricidal activity by a desirable immunomodulator. Being particulate in nature, liposomes are avidly taken up by macrophages that act as secondary depot of the drug. These macrophages after reaching to the tumor site release the drug thereby bring about tumor cell death. At the outset, we speculated our novel formulation comprising the immunomodulator tuftsin grafted on the surface of liposomes along with ETP would be able to eliminate fibrosarcoma in Swiss albino mice.

In previous studies conducted in our lab, it was demonstrated that pretreatment with liposomised tuftsin led to the successful elimination of a range of pathogens in leukopenic mice (Khan et al. 2005, Khan et al. 2004, Khan et al. 2002). Antitumor activity of tuftsin has been known
since long but no sincere effort have been made to evaluate therapeutic potential of its liposomised form or per se tuftsin bearing liposomal preparation of any anticancer agent. In the present study, we evaluated anticancer potential of tuftsin bearing liposomal ETP (Tuft-Lip-ETP). Since ETP has cytotoxic properties and the immunomodulator tuftsin possesses both anti tumor and immunomodulatory properties to co-activate immune system, we considered it of interest to evaluate therapeutic potential of their combination against experimental fibrosarcoma. Our results are well in agreement with proposed hypothesis as novel formulation of liposomised ETP with tuftsin grafted on the surface was found to be more effective than its free form. Median day of death (MDD) studies have also demonstrated that animals treated with Tuft-Lip-ETP have increased life span. More importantly, significant tumor regression was also induced in the animals treated with Tuft-Lip-ETP. Enhanced efficacy of Tuftsin-bearing ETP liposomes might be due to the fact that tuftsin helps in specific targeting to macrophages that will subsequently infiltrate to tumor site. Treatment with free ETP and Sham-Tuft-Lip were not much effective in killing tumor cells.

The tumor suppressor gene p53 is regarded as a key factor in maintaining the balance between cell growth and cell death (Agarwal et al.1998, Mowat et al. 1998). The importance of p53 gene can be drawn from the fact that this gene is reportedly mutated in ~80% of all human malignancies (Hollstein et al. 1991). Because of its role in regulation of cell cycle, alterations in p53 are critical events in carcinogenesis. In tumors loss of p53wt prevents the activation of this growth control pathway (Burns et al. 1991). The failure to induce transcriptionally active p53wt plays a role in the unregulated growth of the tumors and also in the failure to respond to chemotherapeutic agents, which normally trigger p53wt thus regulating cell cycle arrest or cell death (el-Deiry 1994). Because the balance between p53wt and p53mut determines the fate of the cell, many chemotherapeutic agents are known to exert their anticancer effects by modulating their expression levels (Skvortsova et al. 2005, Takimoto et al.2002, Bunz et al. 1999). The results of the present study showed increased up-regulation of p53wt along with its downstream effector p21/Waf1 and down-regulation of p53mut by
Tuftsin-bearing liposomal formulation of etoposide. In fact the intrinsic property of etoposide is to act by modulating the balance between wtp53 and p53mut protein expression by repressing p53mut and enhancing tumor suppressor p53 (Takimoto et al. 2002, Natalie et al. 2002, Kevin et al. 2006, Huang et al. 1997). The effect of etoposide on p53wt and p53mut gets more apparent upon its liposomization along with tuftsin. The up-regulation of p53wt by etoposide is also responsible for the transcriptional induction of p21/waf1 by directly interacting with its regulatory elements (el-Deiry et al. 1994). As observed in the present study, liposomization of etoposide with or without tuftsin results in up-regulation of p21/waf1. The effect is more prominent in tuftsin-bearing etoposide liposomes. The p21 protein is a cyclin-dependent kinase (CDK) and has multiple functions. It mediates p53-induced growth arrest at the G1 or G2 cell cycle checkpoint and can inhibit CDK-cyclin activity and directly inhibit DNA replication (Boulaire et al. 2000, Li et al. 1994, Shivji et al. 1994, Chen et al. 1995). The halt offers time for DNA repair before replication or mitosis and thus links p21 directly to the tumour suppressor function of p53 (Taylor & Stark 2001, Winters 2002, Gartel et al. 1996, Colman et al. 2000). Besides activating p21/Waf1 the p53 protein can also selectively repress one or more antiapoptotic genes, with an outcome essentially similar to transactivation of proapoptotic genes through the process of apoptosis. Genetic changes resulting in loss of apoptosis or derangement of apoptosis-signaling pathways in the transformed cells are likely to be critical components of carcinogenesis (Kastan et al. 1995, Schulte-Hermann et al. 1997). The tumor suppressor p53 thus very sophisticatedly causes tumor cell death; simultaneously up-regulating death-promoting genes and turning off protective genes by the process of apoptosis. Apoptosis is mediated by caspase-3, one member of a family of cysteine proteases (Thornberry et al. 1998) and is thought to be regulated by bax and bcl-2. Bcl-2 is the antiapoptotic protein whose expression is reportedly transcriptionally blocked by p53 (Haldar et al. 1994, Miyashita et al. 1994). The expression of caspase-3 and bax was increased in the animals treated with various formulations of etoposide. Significantly higher expression of caspase-3 was observed in animals treated with Liposomised ETP in comparison to animals treated with free
etoposide. No significant difference was observed in the animals treated with tufts-in-bearing liposomal etoposide in comparison to liposomal etoposide devoid of tufts-in. The expression level of bcl-2 also decreased in the animals treated with various formulations of etoposide in comparison to animals treated with vehicle controls. The bcl-2 level was observed to be significantly lower in the animals treated with liposomal formulations of etoposide over free etoposide. No significant difference was observed in the animals treated with tufts-in-bearing liposomal etoposide in comparison to animals treated with tufts-in-free liposomal etoposide. These results clearly show that the regression of tumor in the animals treated with tufts-in-bearing liposomal etoposide formulations is through the activation of apoptosis as well as cell growth arrest mediated by p53. These results also show that therapeutic treatment of tufts-in alone or in combination with etoposide has no modulatory effect on the mechanism of apoptosis. Free as well as liposomal formulations of etoposide effected the modulation of the gene involved in the mechanism of apoptosis but had no effect on the expression of the inhibitor of cyclin dependent kinase that is p21/waf1. Despite these interesting and promising results it remains to be found out more precisely whether the higher expression of p21/waf1 by tufts-in is p53 dependent or independent. Finally we conclude that Tufts-in-bearing liposomised etoposide is an effective formulation for the treatment of fibrosarcoma and can pave the way for treatment of other forms of cancer as well. Formulation of tufts-in-bearing liposomes in combination of other anticancer drugs may be equally effective against various form of cancer.