

**SYNTHESIS OF POLYAMIDOAMINE DENDRIMER-METHOTREXATE
CONJUGATES AND *IN VITRO* CHARACTERIZATION**



SEZEN GURDAG

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DEDICATION



To my family; for their endless love and support

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CHAPTER 1

DENDRITIC DRUG DELIVERY SYSTEMS

1.1. CANCER AND CANCER THERAPY

Cancer is a leading cause of death around the world threatening the human health. Overall, every year 10.9 million new cases develop out of which 6.7 million ends with death leaving 24.6 million people living with it within three years of the diagnosis (1). The National Cancer Institute estimates about 1.4 million new cases to occur in 2006 (2).

It appears that both external factors such as smoking, chemicals, radiation, and infections, and internal factors like inherited metabolism mutations, hormones, and immune conditions are relevant to the initiation and promotion of carcinogenesis. The National Institute of Health estimates overall costs for cancer in 2005 at \$209.9 billion (2). Given the seriousness of the socioeconomic issues related to cancer, it is clear there is urgent need for thorough curative methods, which unfortunately could not have been established yet.

Current treatment methods are based on prevention of the growth; given that cell growth is a general property of the living organisms, eventually they have unwanted side effects on the normal tissue. Conventional treatment methods include surgery, chemotherapy, hormone therapy, radiotherapy, and immunotherapy. Surgery is preferred and considered as the primary procedure for established tumors large enough to manipulate (3). However, it has

limitations; cancers of blood cells like leukemia and solid tumors in inaccessible areas of the body like bone and brain are not amenable to surgical removal (4). Furthermore, surgery may have undesirable side effects, e.g. changing the growth rate of remaining cancer, triggering a faster metastatic process. In fact, in many cases, patients die of metastatic cancer after the primary tumor has been removed. For all these reasons, chemotherapy, radiotherapy, hormone therapy, and immunotherapy, or their combinations become the main treatment procedures (3).

1.1.1. CHEMOTHERAPY

Chemotherapy is defined as the use of chemotherapeutic agents aimed at killing or controlling the cancer cells. For most of the cancer types, it is used as an adjuvant to surgery and/or radiation therapy. Additionally, it is exploited as the main method of treatment of few cancer types such as leukemias (5). It can be used at different stages of cancer progression. However, one important complication about it is the high risk of drug toxicity, which is mostly true for the effective anticancer drugs since the rationale behind it to disrupt the cell proliferation (3). In the conventional classification of chemotherapeutic drugs, they are divided into four main classes: Alkylating agents, antimetabolites, antitumor antibiotics, and natural products (4, 5).

Alkylating agents: This type of chemotherapeutic agents interferes with DNA synthesis either by breaking the helix or the synthesis of DNA precursors as they have the ability to form covalent bonds with DNA, which results in intra- and

interstrand DNA crosslinks. Most common examples to this class are cyclophosphamide, platinum compounds such as carboplatin and cisplatin, melphalan, and nitrogen mustards.

Antimetabolites: They interfere with the enzymes contributing to DNA synthesis, which explains their S-phase specificity. Their structures are similar to normal metabolites of cells. As structural analogs of normal metabolites, they strongly bind to an enzyme, thereby inhibiting it. This substitution of antimetabolites for normal metabolites results in nonfunctional macromolecules. Commonly used antimetabolites are; methotrexate (MTX), 5-fluorouracil (5-FU), cytosine arabinoside (Ara-C), 6-mercaptopurine, raltitrexed, and gemcitabine. In the figure, molecular structure of MTX is given.

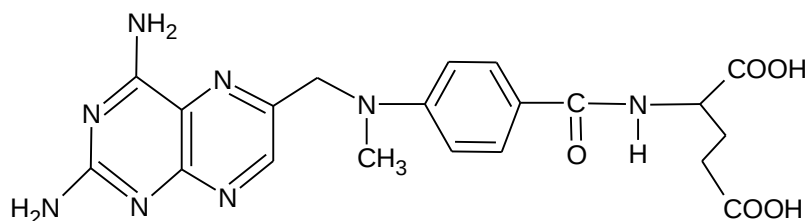


Figure 1. Structure of MTX

Antibiotics: Many antibiotics are natural metabolites made by fungi. They exert their cytotoxic effect by intercalating into DNA and causing DNA strand breaks. Some specific antibiotics obtained from genus *Streptomyces* have been proven to be useful as antitumor agents. Doxorubicin, daunomycin, bleomycin, and mitomycin C are some of the widely used antibiotics.

Natural products: Vinca alkaloids such as vinblastine and vincristine, L-asparaginase, and paclitaxel fall into this category. They are obtained from either

plants (e.g. vinblastine, vincristine, and paclitaxel) or bacteria (enzyme product of bacteria: L-asparaginase). Their action mechanism varies; for example, vinca alkaloids arrest the cells at mitosis by interfering with microtubules formation involved in the separation of chromosomes (6).

1.1.2. RADIOTHERAPY

In radiation therapy, ionizing radiations generate reactive oxygen species, which will produce breaks in the DNA strands leading to cellular reproductive inability. An irradiated cell is considered dead due to irreparable damage to the DNA. Membrane damage is also possible. In clinical practice, dose of radiation is restricted by the damage done to the normal tissues. However, the adverse effects on normal tissues can be prevented by focusing multiple radiation beams from different angles onto the cancerous tissue as well as dividing the total dose into fractions given over a period of time.

1.1.3. HORMONE THERAPY

The normal progenitor cells require estrogens or androgens for their proliferation. This mitogenic effect of the sex steroids is mediated by specific intracellular receptors. Antagonizing this effect generates a cytostatic response. Since relatively a few number of cells contain these receptors, drugs aimed at their disruption have selectivity for them, which is the main advantage over chemotherapy. Clinically, it is also possible to test tumor tissue for the presence

of hormone receptors so that clinicians can determine the responsiveness of the neoplasm to hormones.

1.1.4. IMMUNOTHERAPY

The basic rationale behind immunotherapy is the stimulation of certain parts of the immune system to reject and destroy tumors. People with weakened immune system are more likely to develop cancer. On the other hand, those with normal immune system still develop cancer. It is because immune system may not recognize cancer cells as foreign object or body's response may not be strong enough to kill the cancer cells. To overcome this, researchers designed different types of immunotherapies: cancer vaccines (active specific immunotherapies), monoclonal antibody therapy (passive immunotherapy), and nonspecific immunotherapies and adjuvants. In clinical practice combination of two or more is used depending on tumor's response.

1. 2. DRUG DELIVERY SYSTEMS

Effectiveness of a therapeutic depends on the physicochemical properties of the drug itself. However, along with the existing drugs used in the treatment of many diseases, most of the drug candidates suffer from toxicity, low aqueous solubility, poor transport through the cellular membrane, and short blood circulation times resulting in a failure at the early stages of their development.

It has been acknowledged that cancer is becoming the leading cause of deaths in the industrialized countries. Since it is invasive with aggressive growth profile, unfortunately an efficient therapy could not have been developed yet. Chemotherapeutics offer several challenges, which determine the efficacy of the treatment. The main drawback of chemotherapy is their serious side effects on fast dividing tissues such as bone marrow. Another limitation on the usage is resistant phenotypes, which do not respond to the therapy. Overcoming these hurdles towards creating drugs of maximal therapeutic efficacy yet with minimal side effects on the healthy tissues offers great challenges to the scientists. In this respect, a number of drug delivery devices have been developed, including polymeric micelles, liposomes, linear, hyperbranched polymers, and dendrimers (7). Since their introduction to pharmaceutical research by Helmut Ringsdorf in 1975, design and synthesis of polymeric materials have created tremendous impact on the development of new therapeutics spanning from chemotherapeutics to anti-inflammatory agents, ocular agents to genes and antidepressants (8).

The simplest rationale behind the controlled drug delivery concept is to alter the pharmacokinetics and pharmacodynamics of the biologically active molecules by using novel delivery systems. Hence, both the duration and the localization of the active agent can be manipulated as desired (9-11). Drug delivery system should serve as a tool for the regulation of essential characteristics such as appropriate time scale of release, continuous maintenance of drug levels within the therapeutic range, targeting and reduction

of harmful side effects, and improved compliance (9, 10, 12, 13). While putting the emphasis on these advantages, potential disadvantages associated with the polymeric material should be kept in mind such as possible toxicity or nonbiocompatibility of the polymer, any surgery needed to implant or to remove the delivery system, patient's discomfort due to the device, and high cost of the production of the polymeric material (9, 12). An ideal carrier candidate should be inert, biocompatible and nonimmunogenic, capable of achieving high drug loading, safe from accidental drug release, simple to administer and remove, easy to fabricate and scale up, water-soluble and able to solubilize the drug even if it is highly hydrophobic, have sufficient functional groups for drug attachment (in case on conjugation), if nonbiodegradable should be eliminated from the body through renal excretion (9, 14).

1.3. DRUG RELEASE MECHANISMS FROM THE DELIVERY SYSTEMS

Drug release from the delivery device can be classified into:

1. Diffusion-controlled release
2. Solvent-controlled release
3. Chemically controlled release (15)

Diffusion-controlled release is observed in reservoir and matrix type delivery systems.

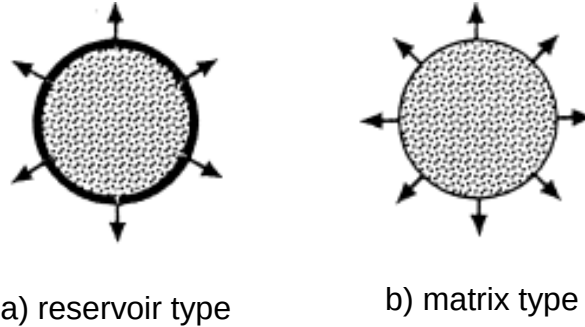
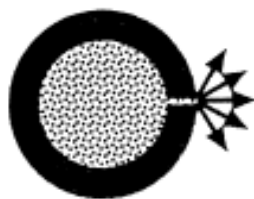


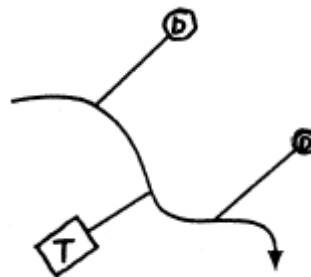
Figure 2. Diffusion controlled delivery system [Adapted from (16)]

In reservoir type release systems as shown in Figure 2.a, the active agent is contained in a core that is surrounded by a rate-controlling membrane. The drug delivery from the system is relatively constant for long times. Since the polymer coating is uniform, the diffusion rate can be kept fairly stable throughout the lifetime of the delivery system (9, 16). However, ionic species or species with molecular weights above 400 can't diffuse through the polymer. For this reason, matrix-type release systems were developed.

Matrix-type systems (Figure 2.b) have the active agent thoroughly mixed with the polymer to form a homogeneous system. The active agent could be either dissolved in the polymer or just mixed with it to achieve high enough concentrations to create interconnecting pores through which the drug could slowly diffuse out (9, 16).



a) osmotic system



b) controlled delivery system

Figure 3. Solvent-controlled (a) and chemically controlled (b) delivery systems
[Adapted from (16)]

Solvent-controlled release systems (Figure 3.a) are also called osmotic systems where active agent is dispersed in a glassy polymer. Since glassy polymer is impermeable, no diffusion could take place through the solid polymer. However, when such device is placed in an aqueous environment, water starts to penetrate the matrix and as a result swelling occurs. Due to chain relaxation upon swelling, active agent begins diffusing out of the polymer (15).

Finally in chemically controlled devices (Figure 3.b), the rate of active agent release from the polymer is manipulated by a chemical reaction which occurs via either hydrolytic or enzymatic cleavage of a labile bond as will be discussed later in Chapter 4 (15). This type of delivery system offers advantage of incorporation of targeting agents and imaging agents as well towards attaining the improved pharmacodynamics of the active agent and traceability of the device *in vivo*.

1.4. PREFERENTIAL ACCUMULATION OF POLYMERIC DELIVERY SYSTEMS IN SOLID TUMORS

Large percentage of the pharmaceuticals does not have selectivity for the target organ, tissue or cells. Most of the time, pharmaceuticals are rather evenly distributed within the body. Furthermore, drug has to overcome biological barriers such as other organs, cells, and intracellular compartments where the inactivation of the drug in addition to undesirable serious side effects might occur before reaching the target site. Therefore, to achieve a therapeutically effective drug dose at the target site, it has to be administered at very high concentrations, which could be detrimental to the healthy tissue also. To tackle these problems, drug targeting is used to spare the healthy tissues while still attaining high enough concentrations in the diseased areas to produce a therapeutic response to the bioactive agent. In a broader term, drug targeting is the ability of the drug to accumulate in the target organ or tissue selectively and quantitatively, independent of the site and methods of the administration (17).

Attachment of small molecular weight drugs to macromolecules serves as a mean of targeting. Blood vessels in most of the solid tumors possess different characteristics from normal blood vessels such as: a) active angiogenesis and high vascular density, b) high degree of extravasation due to extensive production of vascular mediators such as vascular permeability factor (VPF) and vascular endothelial growth factor (VEGF), c) defective vascular architecture, and d) poor lymphatic clearance of macromolecules and lipids from the interstitial space of the tumor tissue (18-20).

Angiogenic tumor vessels are poorly formed with thin walls, mostly having only membrane and endothelial cells. Gaps between endothelial cells lead to the leakiness of vascular structure of the cells due to fast growth allowing extravasation of circulating macromolecules into tumor tissue (7, 14). Additionally, since the lymphatic clearance system is not functioning as well as it is in normal tissues, macromolecules are retained in the interstitial tumor space for longer periods of time (18). In contrast, in normal tissues, the intact endothelial layer constructing the blood vessels allows the diffusion of small molecules but excludes macromolecules from the tissue (7). Therefore, small molecules such as drugs used in the chemotherapy do not discriminate normal and malignant tissue diffusing freely throughout the body. These unique anatomical properties of tumor tissues set the rationale behind the exploitation of the polymeric materials as carriers, thereby revolutionizing the design of novel anticancer drug delivery vehicles. This was first coined into the phrase “Enhanced permeability and retention (EPR) effect” by Maeda (21).

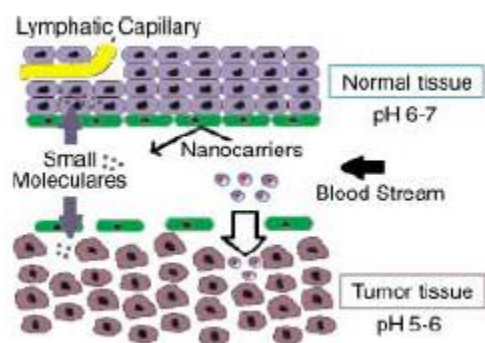


Figure 4. Schematic representation of EPR model [adapted from (7)]

According to the EPR concept, macromolecules are able to accumulate in tumor tissue at concentrations much higher than in normal tissues, even higher

than in plasma (22). However, the EPR effect applies to macromolecules with a molecular weight of at least 45 kDa with long plasma half-lives (23-25). For example, lipidic formulation of SMANCS (poly[styrene-co-maleic acid n-butylester]-conjugated neocarzinostatin) with Lipiodol showed a remarkable increase in the survival rates of the patients with unresectable hepatocellular carcinoma, ranging from 30 to 90%, due to increased accumulation in the tumor (24). Even though SMANCS has a molecular weight of 15000 only, it can bind to albumin, hence reaching on apparent molecular weight of 83000, which leads selective accumulation in the tumor (21). Additionally, Duncan et al. demonstrated strong dependence of HPMA accumulation in s.c. sarcoma 180 and B16F10 melanoma models on the molecular weight of the carrier. Only after 10 min, all HPMA polymeric fractions, from 22kDa to 778 kDa, were present in the tumor. However, only those with a MW higher than renal threshold, ~ 40kDa, showed progressive accumulation, up to 20% of the administered dose per gram, over 24 h in the sarcoma 180 model with higher tumor to normal tissue values (25). Particularly, highly vascularized tumors such as Kaposi's sarcoma and head and neck cancer show good uptake via EPR effect (24, 26). Furthermore, Frechet's bow-tie hybrid polyester dendrimers of 20-160 kDa demonstrated similar profiles where carriers with molecular weight (MW) > 40 kDa had half-lives longer than 24 h resulting in increased accumulation in s.c B16F10 tumors due to EPR effect (23). It was hypothesized that longer circulation half-lives of these highly branched dendritic structures could be because of their slower glomerular filtration probably as a consequence of the

decreased flexibility of the branched molecule and the inability to reptate through the pores (23).

However, there is still much room for the better understanding of contribution of EPR effect in the tumor accumulation of macromolecular carriers both in investigational and clinical medicine.

1.5. SYNTHESIS AND STRUCTURAL PROPERTIES OF DENDRIMERS

The word dendrimer comes from the Greek word “dendron” which means “tree” or “branch” (27). Followers of the work by Vögtle et al. to synthesize dendrimer-like molecules in an innovative “cascade” methodology developed two major strategies (27). These techniques gave rise to larger structures, which are later called arborols, cascade dendrimers or dendrimers in short.

In the first synthesis method developed, divergent method, the growth of dendrimer starts from the core site as the root and goes outward building up generation by generation.

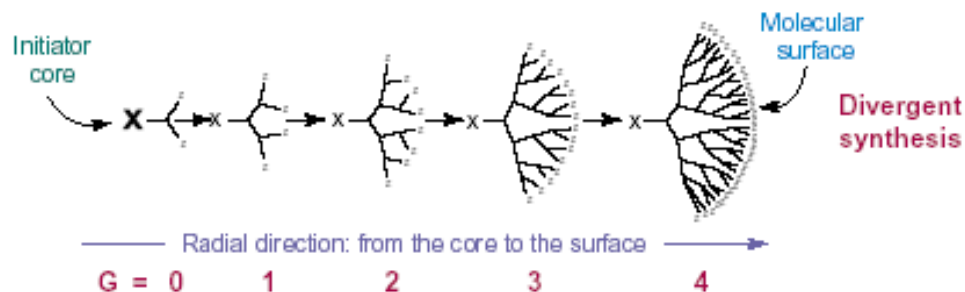


Figure 5. Divergent synthesis method [Adapted from (28)]

Conversely in the convergent method, the synthesis process proceeds from the dendron surface inward to a reactive focal point at the root (29).

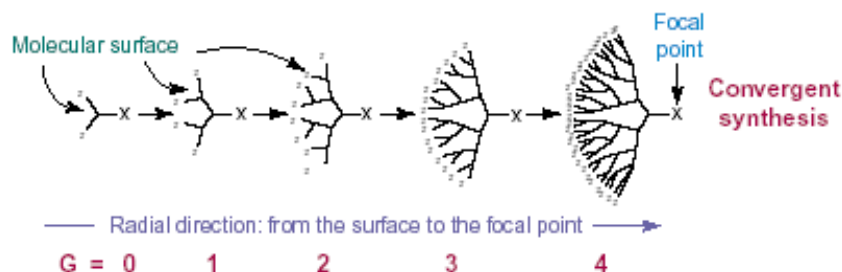


Figure 6. Convergent synthesis method [Adapted from (28)]

This gives only a single reactive dendron. To obtain a full dendritic structure, several dendrons have to be reacted with a multi-functional core. Classical examples of the convergent synthesis can be found in the work by Frechet (29).

In divergent synthesis scheme, large number of reactions has to be performed on a single molecule, which requires for very effective transformations (99+% yield) to avoid any possible structural defects. However, in reality, for example, the yield of a G5-PPI dendrimer is only 25% even with efficient transformations. The convergent approach pioneered by Frechet to dendrimer synthesis affords better control on the synthetic scheme giving “closer to perfect” dendritic architecture since only a small number of reactive sites are activated in each step leading to a very small number of unwanted side reactions. On the other hand, both of these methods involve tedious and time-consuming stepwise processes. Therefore, the need for easier and faster synthesis led scientists to explore new strategies. For example, Frechet’s group from UCLA came up with a “double-stage” convergent growth approach. In this approach, researchers

synthesized first what is called a 'dendritic hypercore' having numerous reactive surface groups followed by attachment of convergent dendritic 'wedges' (29).

The dendritic structure (Figure 7) is comprised of several compartments of different characteristics:

1. The multivalent surface with densely packed tailorable reactive sites
2. The branching units and internal cavities just beneath the surface protected from outside by the dendrimer surface
3. The core

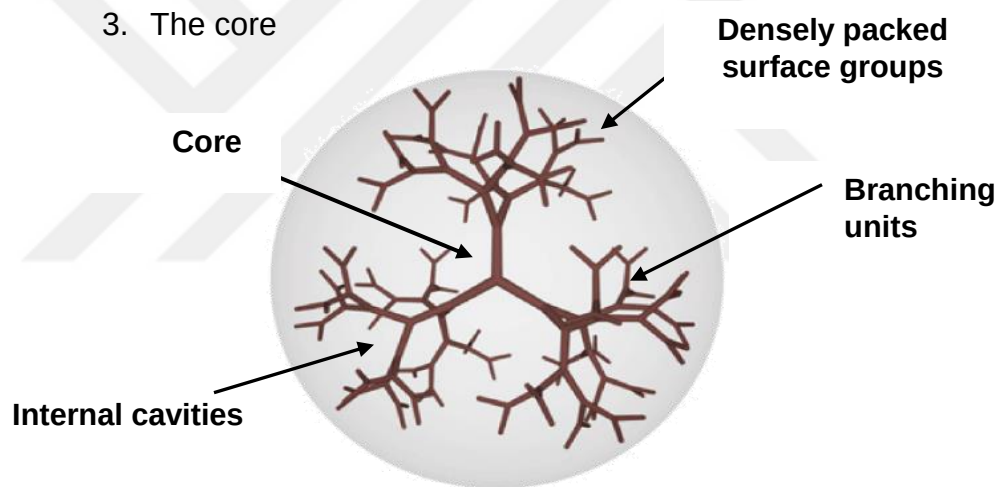


Figure 7. Schematic representation of a dendritic tree

Despite their large molecular size, dendrimers are well-defined structures with a low polydispersity in comparison with linear/hyperbranched polymers. On the molecular level, dendritic branching results in semiglobular to globular structures with a high density of functionalities on the surface in a relatively smaller volume (27). It has been observed that when a dendrimer reaches a certain generation, generally higher than 4, a conformational change occurs imparting a densely packed globular shape. As a result, this conformational

change imparts different solution and bulk properties to dendrimers compared to their linear counterparts (30). Great efforts of researchers afforded the synthesis of over 100 compositionally different dendrimer families. (28) In the figure, two commercialized dendrimers, poly(amidoamine) ([a] PAMAM) and poly(propyleneimine) ([b] PPI) by Aldrich and DSM Fine Chemicals are shown (31).

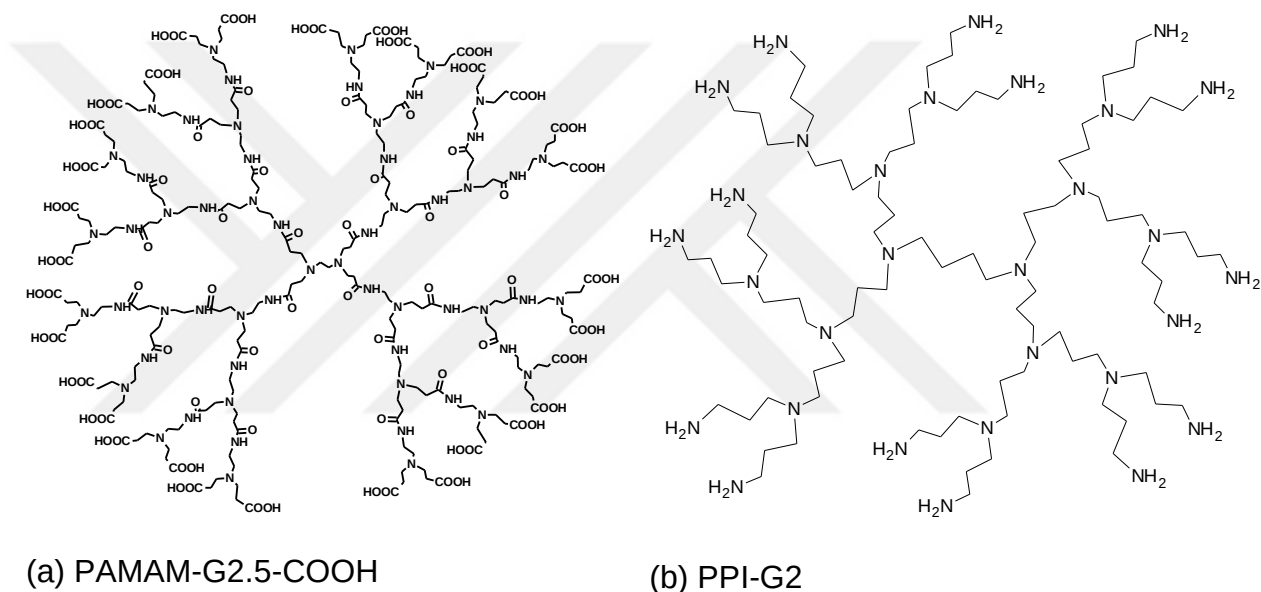


Figure 8. Structure of generation 2.5 PAMAM (a) and generation 2 PPI (b) dendrimers

These unique features make dendrimers attractive for various biological applications ranging from gene delivery to magnetic resonance, the development of boron-neutron capture therapeutics, and drug delivery (27, 28, 30-33). For example, the interior is well suited for encapsulation of guest molecules. Also, these three different sections of the dendrimer can be tailored specifically for the desired purposes (27).

1.6. DENDRIMERS IN DRUG DELIVERY

Dendrimers as chemical entities with biological properties provide such an outstanding example of the close relationship between chemistry and biology (34). Based on their systematic dimensional length and biomimetic properties, they are referred to as “artificial proteins” to which PAMAM dendrimers are very good examples (28). Unlike proteins, they provide researchers with a great flexibility to engineer the surfaces to achieve a significant array of tunable characteristics for the development of novel drug delivery systems as shown in Figure 9.

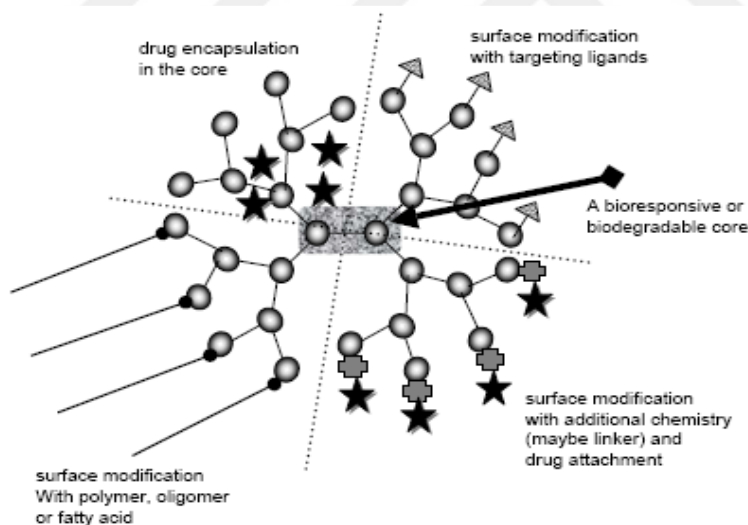


Figure 9. Design strategies of therapeutics and drug delivery systems with dendrimers [Adapted from (35)]

Dendrimers are the most widely used synthetic polymer family employed in pharmaceutical research. Applications of dendrimers in drug delivery can be divided into two different classes:

- 1) encapsulation of drug (noncovalent interaction)
- 2) conjugate systems (covalent interaction)

The first strategy exploits dendrimers as depot systems where the drug can be embedded and released upon physical or chemical stimuli. The advantage of this method is the preservation of the pharmacological properties and the chemical integrity of the drug molecule embedded (32, 36). In the second approach, drug molecules are covalently attached to polymeric surface to form dendrimer-drug conjugates. Release of the drug is intended to occur via chemical/enzymatic cleavage of the bond leading to a better control on the drug release from the delivery device compared to the first approach affording specific tissue targeting as well (30).

1.6.1. DENDRITIC BOXES AS CARGO SPACES

Early examples of dendrimers as candidates for efficient drug delivery systems stem from their use as dendritic boxes for the encapsulation of small molecules (30). The robust covalent structural features of the dendrimers enables guest molecule to enter and exit upon extensive modifications done to dendrimer surface to achieve a variety of defined sizes and shapes (28).

Meijer et al. described the encapsulation of dye molecules into poly(propylene[imine]) (PPI) dendrimers modified with phenylalanine (37-39). Surface modifications done to generation 5 PPI dendrimer with L-Phe allowed it to have highly dense hydrogen-bonded shell, which induced the incarceration of the dye molecules into the dendritic structure. It has been reported that large dye

molecules such as Bengal Rose, Rhodamine B, and New Coccine and small dye molecules such as p-nitrobenzoic acid can be encapsulated simultaneously in the dendritic box at a loading ratio in accordance with the number of internal cavities. Even though the first attempt to release the dye molecule failed due to the bulky amino acid used for capping, further attempts by opening up the outer shell after hydrolysis with hydrochloric acid demonstrated 100% release of the large guest molecules (38). Similarly, encapsulation efficiency of the generation 4 PPI-dendrimer showed strong positive correlation with the extent of the modification done to the amine surface groups to afford amide analogues with alkyl acid chlorides (39).

More recently, Tywman and coworkers synthesized arbonol-type dendrimers starting with PAMAM G1.5 and G2.5 with 8 and 16 ester terminal groups, respectively, which formed infinitively water-soluble complexes with small antifungal and antibacterial drugs at neutral pH (40). However, they were unstable in acidic conditions and formed precipitates, which suggests the enrollment of basic tertiary nitrogens in the dendrimer in binding to the acidic protons of the guest since they are protonated at acidic pH ~2.

Recently, Frechet and coworkers have developed 3rd generation dendritic unimolecular micelles with a hydrophobic core shielded by a hydrophilic shell, PEG chains which increased the water solubility of encapsulated pyrene tremendously by 356-fold from 8×10^{-7} M to 2.85×10^{-4} M (41).

In addition to small dye molecules, large drugs such as anticancer drugs, MTX, adriamycin (ADR), and 5-FU have been embedded in PEGylated PAMAM

dendrimer systems (42, 43). The encapsulation efficiency of the carrier system increased with the increase in PEG chain, length from MW = 550 to MW = 2000, postulated to be due to availability of larger holes created between the dendrimer branches for the guest molecules (42). PAMAM dendrimer with basic interior easily encapsulated the acidic drug MTX 4 times more than the basic drug ADR due to the favored ionic interaction between the host and the guest. Similar to Kojima, Bhadra and coworkers observed up to 12 times enhancement in 5-FU loading after PEGylation (43). On the contrary, Pan et al. reported that grafting generation 3 PAMAM surface with PEG chains of MW=2000 had no significant effect on the encapsulation of MTX (44).

Other than encapsulation of molecules, complexation of anti-inflammatory drug such as Ibuprofen (Ibu) has been performed. Our group prepared the complexes of Ibu with various PAMAM dendrimers (45, 46). It was found that Ibu can be efficiently complexed with 3rd and 4th generation PAMAM dendrimers via ionic interaction between –NH₂ group of the dendrimer and –COOH group of the drug. As generation increases from 3 to 4, there is a significant increase, from 32 to 78, in the number of incorporated drug, similarly observed by Kojima et al. (42). Frechet et al. have developed pH-responsive linear-dendritic block copolymers, composed of polyethylene oxide (PEO) and either a polylysine or polyester dendron with a highly pH-labile acetal linkage (47). At acidic pH upon hydrolysis of acetal units, the hydrophobic dendrimer becomes hydrophilic, eliminating the driving force for self-assembly, thereby destabilizing the micelle. Destabilization of the micellar structure will eventually release the entrapped

molecules from the internal compartment. Drug release upon acid hydrolysis was demonstrated with the use of a hydrophobic dye, Nile Red, embedded in the dendritic architecture. Reduction in the buffer pH yielded time-dependent decrease in the fluorescence intensity as the dye molecule started to be released to the aqueous environment where its fluorescence intensity is lower. This observation strongly suggested that liberation of the Nile Red was triggered by hydrolysis of the acetal linkages proving the validity of the hypothesis (47).

1.6.2. DENDRIMER-DRUG CONJUGATE SYSTEMS

In the polymeric conjugate systems, dendrimers have filled the gap created by the need for macromolecular carriers with a well-defined structure and low polydispersity. Due to their low polydispersity, they can assure reproducible pharmacokinetics of the delivery systems in addition to multivalency, affording the attachment of several different biological moieties such as drugs, targeting and imaging molecules all at once. A wide range of pharmaceuticals has been conjugated with dendritic systems. Some noteworthy examples will be mentioned here in more detail.

Anticancer drug conjugates: Kono and coworkers have synthesized conjugates of folic acid (FA) and the antimetabolite MTX with poly(aryl)ether dendrimers (48). FA showed higher reactivity towards the end functionalities of the carrier, 12.6 FA / dendrimer, compared to MTX, 4.7 MTX / dendrimer. Even though no *in vitro* drug activity tests have been performed, the study was

encouraging, setting an example of how dendrimers can be useful in terms of attaching multiple copies of biologically active molecules.

Baker's group from University of Michigan has vastly utilized this unique class of dendrimers for MTX conjugation which can also target and image with the use of folic acid (FA) and fluorescein isothiocyanate (FITC) / 6-TAMRA moieties, respectively (49-53). They observed the FA-targeted conjugate of MTX with generation 5 PAMAM dendrimer with an ester bond afforded 4 times better *in vivo* activity in FR(+) KB human epidermoid carcinoma cells compared to free MTX (51). On the other hand, amide bonded conjugate performed worse than both free drug and ester bonded conjugate. This was attributed to hydrolytically labile character of the ester bond at low pH found in the lysosomes (pH = 4.0-6.0) releasing more drug than the amide bonded counterpart (51). Latallo et al. investigated *in vivo* activity of the ester conjugate in KB xenografts in SCID mice (50). The conjugate produced a comparable activity at the concentration of 55 mg/kg, equivalent to 5 mg/kg of MTX, to the second highest dose of free MTX, 21.7 mg/kg, in terms of tumor suppression. Interestingly, 5 mg/kg of free MTX did not show any activity. Also, the conjugate achieved a delay of tumor growth based on end-point volume of 4 cm³ by at least 30 days. Overall, their study was a good example of the versatile use of dendrimers in biomedical applications.

Use of high molecular weight polymers, >20000 Da, is crucial for chemotherapy to take the advantage of enhanced tumor accumulation due to EPR effect (21, 25). At the same time, higher molecular weight of the carrier enables the delivery system to circulate in the body for longer times since the

rate of renal clearance is based on the hydrodynamic volume (25). Biodistribution of the 4th generation dendrimer based on 2,2-bis(hydroxymethyl)propanoic acid core in mice demonstrated that its circulation half-life is less than 10 minutes at a molecular weight of 3800 Da. However, the synthesis of well-characterized dendrimers with high molecular weights is time-consuming. Furthermore, there is only a slight change in hydrodynamic volume with increasing generation. Therefore, Frechet and coworkers started seeking alternative approaches to achieving significant increases in the molecular weight without resorting to synthesizing higher generation dendrimers. They prepared three different dendritic scaffolds based on 2,2-bis(hydroxymethyl)propanoic acid for the evaluation of the effects of the composition and total mass of the carrier on the *in vitro* and *in vivo* activity (54). The third scaffold was a hybrid between polyester dendrimer and PEO star polymer representing the core. PEO was selected to increase the molecular weight and hence hydrodynamic volume, plasma half-life, and accumulation of the system in the tumor without sacrificing the biocompatibility and the solubility. It has been suggested that the third carrier with the highest mass exhibited the best properties as a carrier. Coupling of DOX to this carrier through an acid labile hydrazone linkage yielded a conjugate with a total mass of 27200 Da. Even though 80-98% reduced *in vitro* activity of the drug observed with the conjugate, encouraging results have been observed *in vivo*. Conjugation of DOX to a hybrid dendritic carrier increased its half-life significantly from 8 min to 72 min. This study proved the hybrid dendrimers as promising backbones for the development of macromolecular drug carriers.

Majoros et al. prepared a single-molecule based nanodevice functioning in three distinct ways as a result of incorporation of biologically different units, FA as folate receptor targeting agent, Taxol as anticancer drug, and FITC as imaging unit (55). Since the inherent insolubility of Taxol is one of the major problems associated with its delivery, conjugation of the drug with water-soluble molecules is desired. Three molecules of Taxol were conjugated to the difunctional dendrimer through an ester linkage. KB cells with up-regulated folate receptor (FR) showed green fluorescence documenting the uptake of the trifunctional dendrimer conjugate whereas FR down-regulated KB cells did not exhibit any green fluorescence indicating the receptor-mediated uptake of the conjugate. Additionally, at low concentrations only FR(+)-KB cells were affected by the activity of the conjugate.

Zhuo et al. have synthesized PAMAM dendrimers with a cyclic core of 1,4,7,10-tetraazocyclodecane (56). 5-FU has remarkable antitumor activity but it has high toxic side effects at the same time. They attempted to subdue its toxicity by slowly releasing it from generations 4 and 5 PAMAM dendrimers. Even though slow release of the drug was achieved in buffer at pH 7.4, the *in vitro* evaluation of the conjugate system has not been performed.

1- β -D-Arabinofuranosylcytosine (Ara-C) is one of the most effective antitumor agents used in the treatment of leukemias and lymphomas (57, 58). However, the main hurdle for sufficient Ara-C delivery to tumor tissues is the degradative enzymes such as cytidine deaminase and deoxycytidylate deaminase giving rise to inactive metabolite 1- β -D-Arabinofuranosyluracil (Ara-U)

especially in the liver and other tissues (57). To tackle this problem, two different approaches toward PEG-Ara-C hybrid conjugates have been used. The first study describes the synthesis of tetrameric and octameric PEG-Ara-C amide prodrugs starting from aspartic acid units including various linkers such as amides and carbamates (57). The second approach utilizes the functionalization of hydroxyl units of PEG with a bicarboxylic amino acid, L-2-amidoadipic acid (58). Both of the reports have demonstrated that when the conjugates of different molecular masses and composition, and free Ara-C were tested in cytidine deaminase assay, only free Ara-C was completely deaminated to inactive metabolite Ara-U thus providing an indication of the effective conjugation strategy of the parent drug via an acyl substitution on the N⁴ of Ara-C for the protection from enzyme deamination. Furthermore, approximately 20 times improved circulation time was observed with the conjugate. On the other hand, linear conjugates of Ara-C with the PEG polymer did not accomplish such enhancement in the pharmacokinetics of the free drug.

Malik et al. described the synthesis of cisplatin-G3.5-PAMAM dendrimer conjugates (59). Unfortunately, the conjugate showed several orders of magnitude less cytotoxic activity compared to free drug, which was attributed to significantly hampered release of the drug from the conjugate, less than 1% of the loaded drug after 74 h of incubation. Despite poor release characteristics, conjugate preferentially accumulated in the B16F10 xenografts in C57 mice, which was also reflected in the better pharmacodynamics of dendrimer-cisplatin, such as decreased systemic toxicity and selective tumor accumulation.

For the targeting of cancer with traceable dendrimeric conjugates Onoi et al. demonstrated the proof of concept synthesis of DNA-directed assembly of dendritic tectodevice (Figure 10) where DNA acts as a functional zipper between the two dendrimer molecules derivatized with either FA or FITC (49).

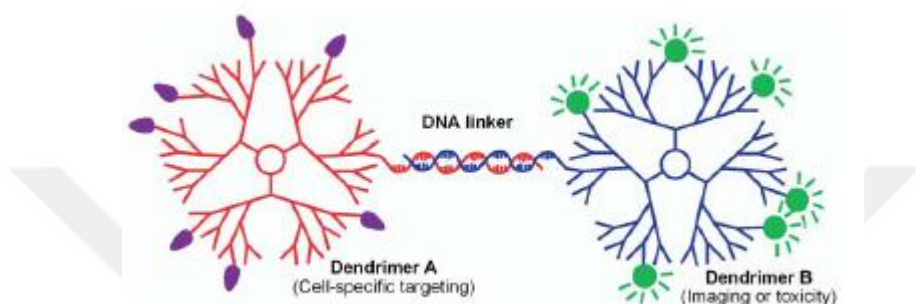


Figure 10. DNA-dendrimer conjugates as potential cancer targeting imaging agents/therapeutics [Adapted from (34)]

In this strategy, one dendrimer molecule will provide targeting exclusively to cancer cells while the other will have the imaging or anticancer agent. Hence, the study made a big contribution to the design and production of novel dendrimeric nanodevices assembled by DNA zippers toward the development of better therapeutics targetable to different cancer cells with imaging and therapeutic agents.

Immunoconjugates: Coupling of dendrimers to antibodies produced immunoconjugates for the use in radioimmunotherapy (60-62). Targeted therapy of cancer sparing the normal cells is crucial which can be also realized with the use of antibodies specific to antigens on the cancer cells. In addition, for more effective radioimmunotherapy, binding of a large number of radiometals to a single antibody molecule is important while still retaining the high specificity of

the monoclonal antibody. Prevailing issues with immunoconjugates such as decreased immunoreactivity and poor solubility can be resolved using dendrimers as carriers. Patri et al. showed that anti-PSMA antibody J591 conjugated to a 5th generation PAMAM dendrimer containing a fluorochrome, 6-TAMRA, can be used for targeting prostate cancer having a potential as a drug delivery system (61). The conjugate has been proven to successfully target antigenic site positive LNCaP cells with minimal loss of immunoreactivity compared with the control PC-3 cells without the receptor. Kobayashi and coworkers incorporated indium and gadolinium into a OST7-PAMAM-G4 conjugate at approximately a 49 times higher amount than into a murine monoclonal OST7 antibody alone with minimal loss of immunoreactivity. The conjugate exhibited specific accumulation in KT005 tumor in mice compared to control with nonspecific antibody (60). In an earlier report by the same group, the positive effects of saturation of dendrimer chelates with yttrium(III) or indium(III) for the application as a MRI contrast agent in imaging or in neutron capture therapy has been demonstrated to enhance the therapeutic effects in cancer cells (63).

The therapeutic activity of an immunoconjugate of MTX with cetuximab monoclonal antibody based on 5th generation PAMAM dendrimer was tested on F98_{EGFR} glioma cells transfected to express EGFR, which is crucial for several metabolic activities such as cell division, adhesion, motility, invasiveness, resistance to apoptosis, and angiogenesis (64). Due to these important cellular functions of EGFR, targeting of this receptor with the antibody may increase the

in vitro activity of the drug. The conjugate had 12.6 MTX molecules per molecule of dendrimer, which was also attached to one molecule of the antibody. Binding assay revealed that the antibody-mediated uptake of 2 nmol/l of the conjugate was completely inhibited by free antibody cetuximab at a concentration of 2 μ mol/l. Interestingly, the addition of same concentration of dendrimer resulted in an increase in the intracellular amount of the conjugate. This was possibly due to the bending of the cellular membrane upon electrostatic interaction with the cationic dendrimer as reported by several groups as the morphological changes induced in the cells (65, 66). Administration of bioconjugate in receptor(+) F98^{EGFR} glioma cell model resulted in a tumor/brain ratio of approximately 10 which was less than 2 in receptor(-) model created with F98^{WF} glioma. On the other hand, no significant difference was observed between the median survival times of the mice treated with conjugate in comparison to the control. However, the study displayed successful realization of bioconjugate formation with high drug payload with efficient targeting ability both *in vitro* and *in vivo*.

Anti-inflammatory and antiviral drug conjugates: Apart from anticancer drugs, the conjugation of anti-inflammatory drugs such as 5-aminosalicylic acid (5-ASA), ibuprofen, and methylprednisone (MP) was also investigated. Diseases of the colon can be effectively treated if the anti-inflammatory agents are delivered directly to the diseased tissue. In this respect, conjugates of 5-ASA and 3rd generation PAMAM dendrimer using two different azo linkers, p-aminobenzoic acid (PABA) and p-aminohippuric acid (PAH), were synthesized. It has been shown that the choice of linker is crucial in terms of both drug loading

and drug release from the conjugate such that utilization of PAH as the spacer resulted in conjugates with approximately 3 times more 5-ASA than resulted using PABA as the spacer. This was indicative of lower steric hindrance at the –COOH group of PAH than that of the PABA molecule toward coupling with the dendrimer surface amine group (67). In our group, conjugation and *in vitro* characterization of Ibu and MP with various PAMAM dendrimers have been carried out (45, 68, 69). The conjugate of Ibu with G4-PAMAM-OH dendrimer had approximately 58 Ibu molecules as the highest payload on a dendrimer achieved so far.

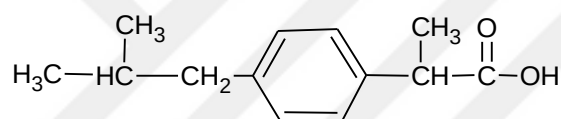


Figure 11. Structure of Ibu

Confocal microscopy along with flow cytometry and UV-Vis spectroscopy revealed efficient transport of the conjugated drug across the cellular membrane as fast as 15 min via nonspecific fluid phase endocytosis. Furthermore, the effect of the use of a spacer molecule on drug payload has been studied as pointed out earlier (67, 68). The conjugation of MP modified first with glutaric acid (GA) produced 12 times higher drug payload on the dendrimer than what was achieved when dendritic arms were extended with GA as the first step. Therefore, it has been suggested that formation of MP-spacer conjugate overcomes the steric hindrance at the dendrimer surface for the large steroid.

Dendrimers are also utilized in the development of novel antiviral agents, artificial mimics of the anionic cell surface, where the dendrimer surface is

functionalized to have anionic surface groups such as sialic acid (SA) residues to compete with the cellular surface for the binding of virus (70). Generation 4 PAMAM dendrimers derivatized with SA moieties were found to be 51-170 times more effective compared to free SA in one strain of influenza A virus. Unfortunately, conjugate was inactive against the other two subtypes, attributed to the differences in the interaction between G4-SA and the individual influenza strains.

In addition, the versatility of highly water soluble PEGylated generation 2 poly(aryl ether) dendrimers for the attachment of several bioactive molecules has been successfully shown by tailoring the surface groups with cholesterol, phenylalanine, and tryptophan to form carbonate, ester, and carbamate linkages, respectively (71).

1.7. BIODISTRIBUTION OF DENDRIMERS

The body distribution of the dendrimeric carriers has been carried out by the attachment of imaging agents such as ^{111}In , ^{88}Yt , Cy5, Gd, ^{125}I , ^3H , FITC, and 6-TAMRA (50, 54, 63, 72-77). However, there is one complication in these indirect methods in terms of the measure of *in vivo* partitions of the dendrimers since body distribution may change after the modification with any kind of ligand mentioned herein (35). For example, the surface modification of PAMAM dendrimers with Gd imparts hydrophobicity to the molecule, increasing its uptake by reticuloendothelial (RES) cells and subsequent liver accumulation (78). As a general trend, positively charged dendrimers are cleared more rapidly than those

with negatively surfaced dendrimers. Similarly to positively charged dendrimers, hydrophobic surfaced macromolecular carriers are taken up mainly by the liver as the clearance route.

Recently, an extensive report has been prepared by Malik and colleagues on the cytotoxicity and biodistribution of positive and negatively charged PAMAM and PPI dendrimers, based on either diaminobutane (DAB) or diaminoethane (DAE) cores, in addition to poly(ethylene oxide) (PEO) grafted carbosilane dendrimers (CSi-PEO) (76). It has been shown that after i.v and i.p administration of ^{125}I -labeled generations 3 and 4 PAMAM dendrimers, they were rapidly cleared from the circulation, less than 1.5 % of the administered dose was recovered in the blood only after 1 h. In contrast, anionic PAMAM dendrimers, generations 2.5, 3.5, and 5.5, displayed longer clearance rates. In the same period of time, their blood retention was 15-40% of the initially administered dose. However, regardless of the significant differences in the blood clearance rates, both anionic and cationic carriers accumulated in the liver to a great extent, 35-65% and 60-90%, respectively. On the other hand, Roberts et al. observed the possibility of toxicity induced by a 7th generation PAMAM dendrimer at a dose of 45 mg/kg as a single dose. One out of five animals died and in a following multiple injection dose study liver vacuolarisation was noticed (79).

In a similar study, positively (PSD) and neutrally charged (NSD) G5 PAMAM dendrimers were evaluated in B16 melanoma and DU145 human prostate cancer models in terms of tumor accumulation with respect to the other

organs (77). Most of the organs showed the highest uptake as fast as 5 min. As expected, positive surfaced dendrimers accumulated at a higher level than the neutral counterpart due to the greater propensity to interact with the negatively charged cell surface. However, tumor tissue did not show any preference for either dendrimer. The following figure summarizes important findings of the study in regard to comparative organ accumulation of the dendrimeric species.

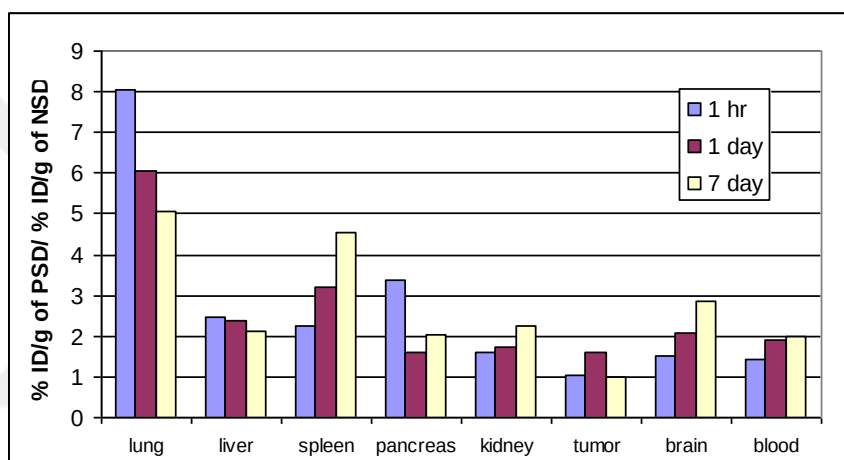


Figure 12. Comparative time-dependent organ accumulation of PSD and NSD in different organs (77)

As can be seen, the lungs had the highest uptake possibly due to larger surface area. Additionally, the endothelial cells in the other organs such as liver and kidney accumulated cationic charged dendrimer at 28-6% ID/g tissue and neutral species at 5-3% ID/g tissue. Levels in B16 melanoma tumor model, heart, spleen, and pancreas were relatively lower, ranging from 1-3% ID/g tissue. Additionally, studies to evaluate the biological safety of the dendrimers revealed no difference between the control group and dendrimer-injected group (77). Similar results were obtained by Kukowska-Latallo et al. with partially acetylated ^3H -G5-PAMAM dendrimers. Due to excretion of the dendrimer mainly via urine,

kidney had the second highest uptake, which was followed by the lungs because of high vascularity of the organ. Liver, heart, spleen, pancreas, and brain had less than 10% of the administered dose. Tumor developed with KB cells in athymic nude mice had approximately 2% ID/g tissue over the period of 7 days (50).

In a more recent study, Frechet's polyester dendritic scaffolds were evaluated in terms of accumulation in various organs (54). The polyester dendrimer with a molecular weight of 3790 Da showed the lowest blood circulation time: at 10 min postinjection only 14% of the injected dose was found in the blood. None of the vital organs had any significant amount of the carrier. Three times increase in the molecular weight did not make any contribution to the blood retention, at 30 min postinjection only 4% of the administered dose remained in the serum after 5 h and subsequently it was completely excreted from the body without any evidence for accumulation in any tissue. On the other hand, building up the molecular weight to 23500 led to pronounced accumulation in the liver. Even after 5 h, the liver level of this compound was at 55% of the initial dose given. Such a difference in the liver accumulation of the carriers was attributed to the location of the radiolabel ^{125}I in the carriers such that in the latter, the marker was attached to the surface groups while the other two carriers had the imaging agent buried in the interior attached to the core unit.

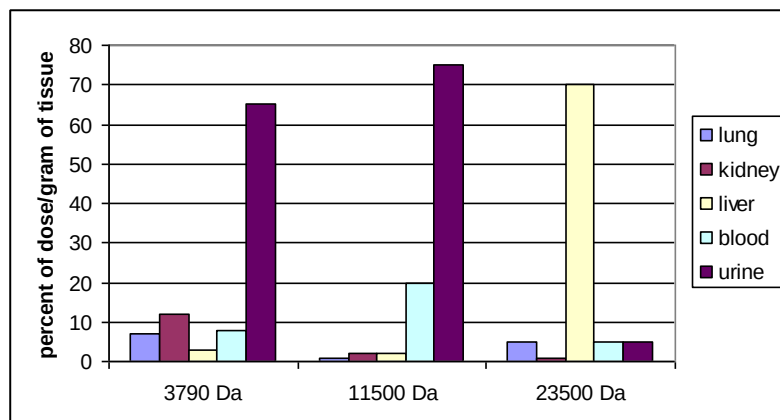


Figure 13. Body distribution of PEO dendrimers at 30 minutes postinjection (54)

Most importantly, conjugation of DOX to this polyester carrier remarkably increased the half-life of the drug from 8 minutes to 72 min, a 9-fold improvement (54).

Extensive studies have been performed on dendrimers exploiting them as MRI agents so that they increase the blood circulation time of the Gd-chetales. Kobayashi et al. compared various PAMAM and DAB dendrimers as MRI agents (80, 81). In these studies, DAB-Gd chelate was found to be excreted from the body more rapidly than the PAMAM-Gd. At the same time, in a following study, DAB dendrimers demonstrated a higher liver accumulation than the same generation of PAMAM dendrimers (81). Even though the dendrimers cleared from the body rapidly, their whole body retention times still were higher than that of Gadomer-17 (80). Additionally, saturation of the dendrimers with radiolabels such as ^{111}In and ^{88}Y had prominent effects on the accumulations in the liver, kidney, spleen and bone (63). Overall unsaturated PAMAM G2 dendrimers had 2-3 times higher uptake in all the organs tested with a significantly slower whole-body clearance time in turn.

Researchers made attempts to decrease the uptake by RES system by modifying the surface groups of cationic dendrimers by attachment of neutralizing groups such as PEG (82). PEGylation of PAMAM G3 dendrimers decreased its liver accumulation by 3 times.

1.8. TOXICITY OF DENDRIMERS

For dendrimers to be used as carriers in biomedical applications, they should exhibit low toxicity. Several studies have been conducted in this respect (27, 54, 76, 83-86).

In all the studies a general trend was observed: cationic dendrimers are more toxic due to destabilization of the cellular membrane and cell lysis (27, 65, 83, 87). Furthermore, Hong et al. reported the disruption of membrane integrity in lipid membranes in KB and Rat2 cells after the treatment with G5 and G7 PAMAM dendrimers (65). It has been noted that G5 PAMAM dendrimer did not show any cytotoxicity up to 500 nM. G7 PAMAM dendrimers at a relatively lower concentration, 10-100 nM, induced hole formation in lipid bilayers in size of 15-40 nm in diameter. On the other hand, even at a higher concentration of 500 nM, G5 dendrimer had only a little effect on the membrane integrity, which was totally reversed upon acetylation of -NH₂ groups of the dendrimer (65).

Kissel and coworkers investigated the toxicity of several polycations including G3 PAMAM in L929 mouse fibroblast cells (83). In all the assays, PAMAM was reported to be the least toxic polycation. This is envisaged as a result of relatively lower molecular weight of the dendrimer in spite of the high

cationic charge on the surface. Additionally, under the physiological conditions, PAMAM dendrimer is only partially protonated. The polymer toxicity was in the order of PEO=PLL>dextran>PAMAM.

Similar findings were reported for PAMAM, PPI, and CSi-PEO dendrimers in B16F10 melanoma, CCRF-CEM human leukemic lymphblastoid, and HepG2 human hepatoma cell lines (76). Cationic PAMAM dendrimers showed generation-dependent cytotoxicity, IC_{50} values of G3 and G4 PAMAM dendrimers were reported as 0.05 mg/ml, close to that of control poly-L-lysine ($IC_{50} = 0.01$ mg/ml). Cationic DAB dendrimers were found to be slightly more cytotoxic than PAMAM of the same number of end groups. On the other hand, anionic PPI, PAMAM and CSi-PEO dendrimers were not cytotoxic against B16F10 cells, with IC_{50} values above 2 mg/ml. Consistent with this cytotoxicity profile, RBC haemolysis experiments confirmed the nontoxic behavior of anionic dendrimers up to 2 mg/ml whereas cationic dendrimers were lytic at half of this concentration. Even though cationic PPI dendrimers showed no generation-dependent activity, haemolysis induced by cationic PAMAMs was generation-dependent. This is attributed to the primary and tertiary amino groups of PAMAM (76).

Frechet's 3-arm PEG star polymer dendrimers based on PEO had a significantly higher IC_{50} value, ~40mg/ml, in B16F10 cell in a 48 h incubation assay mainly due to neutral PEG chains at the periphery. The high molecular weight carrier, 23500 g/mol, has a potential in further studies thanks to its low cytotoxicity (54).

For oral applications of dendrimers, minimal effects on the GI tract are desired. The effect of surface group and molecular weight of PAMAM were assessed on human intestinal adenocarcinoma Caco-2 cell line (84, 86). Partial derivatization of the surface amines of the dendrimer with neutral groups like PEG or fatty acids such as lauroyl chains significantly reduced the cytotoxicity displayed by the unmodified dendrimer. For example, derivatization with the 6 lauroyl chains increased the IC_{50} value by 7-fold compared to the native dendrimers. Reduced toxicity of the modified dendrimer can be explained by the shielding of the positive charge from nitrogen mainly at the periphery by the attached PEG or lauroyl chains decreasing the adherence of the dendrimers to the cell surface as well as encapsulation of the dendrimer's cationic interior tertiary amines (27). However, El-Sayed et al. observed rather higher IC_{50} values for these dendrimers on the same cell line model (88). In their study, except G4-PAMAM with an IC_{50} value of less than 0.1 mM, G2 and G3 PAMAM dendrimer induced toxicity at concentration above 10 and 0.1 mM respectively. The discrepancy between the two studies was attributed to the assay method where the latter used an indirect method, lactate dehydrogenase (LDH) enzyme assay based on cell damage to detect the cytotoxicity.

In a more recent investigation, anionic melamine based dendrimers displayed significantly lower cytotoxicities compared to cationic dendrimers even with smaller molecular weights (85). However, higher concentrations, ~1 mg/ml and above, reduced the cell viability in each case. As observed by the other groups, charge neutralization with PEG reduced the cytotoxicity even further. At

the highest concentration of 10 mg/ml, cell viability of Clone 9 was ~ 60% of the control group.

1.9. RESEARCH STRATEGY

Despite the 30 year history behind the development of polymeric drug delivery system, it is still regarded as a child. Even though there has been a great effort and attention put into the design of targeted drug delivery system, impact of the choice of the carrier on the performance of the prodrug has been much less appreciated. Furthermore, the involvement of certain functional groups of the drug molecule in the conjugation scheme has been much less of a focus in this regard. In the following chapters, there will be discussion on these two unique aspects of the design of prodrug systems.

The versatile use of dendrimers as drug carriers has been demonstrated. The unique structural properties of dendrimers encouraged us to design nanodevices that can not only treat but also target and image with the attachment of appropriate active agents.

In this thesis, antimetabolite drug methotrexate (MTX) is utilized as a model drug due to its well-established pharmacokinetics. Anionic generation 2.5 and cationic generation 3 PAMAM dendrimers were chosen as carriers. The use of two distinct end functional groups on the dendrimer surface will render the final conjugate molecule either anionic or cationic depending on the charge of the native dendrimer. Hence, the impact of the surface charge on the *in vitro* performance of the prodrug can be investigated in terms of both intracellular

transport and intracellular drug release from the conjugate. Moreover, since MTX is a bifunctional drug, the exploitation of different functional groups of the drug in the linking chemistry will provide valuable information about the *in vitro* activity of the conjugate as a function of regioselective conjugation of the drug with the dendrimer. Furthermore, what is so unique about this thesis is that, it also uses isogenic cell lines, explained in Chapter 3, with drug sensitive and drug resistant counterparts. Therefore, we will be able to assess the enhancement of the *in vitro* performance of the conjugate in each cell lines in comparison to each other. After establishing the *in vitro* profile of the conjugate we want to move one step forward and determine the *in vivo* performance of the conjugates in tumor xenografts which will be developed later. The following (Chapter 2) will start first with the synthesis of the dendrimer-drug conjugates and then in the next chapter (Chapter 3) will continue with the *in vitro* characterization of these conjugates. Chapter 4 deals with the *in vitro* cell-free drug release from the conjugates later on.

CHAPTER 2

SYNTHESIS OF POLYAMIDOAMINE DENDRIMER-METHOTREXATE CONJUGATES

2.1. EXPERIMENTAL WORK

Significance of the work: As has been discussed earlier, dendrimers are vastly utilized in drug delivery applications as they offer several advantages over other polymer families. PAMAM dendrimers are especially one unique dendrimer family shown to be useful in many drug delivery systems in addition to gene delivery, imaging, and boron neutron capture therapy. Even though a quite deal of effort has been put into the evaluation of dendrimer-drug activity *in vitro*, so far there is no comparison in terms of the effect of the dendrimer's end functionality on the performance of the conjugated drug. Therefore, we were encouraged to rationalize and investigate the possibility of enhancement in the activity of an antimetabolite drug, MTX, as a result of conjugation with the two PAMAM dendrimers: carboxyl terminated generation 2.5 PAMAM (anionic) and amine terminated generation 3 PAMAM (cationic) dendrimers.

In this first method developed, MTX is directly attached to the dendrimer through an amide bond without any protection of any group of the drug, which will be discussed first. In contrast, in the second approach, conjugates have a spacer in between the drug and the dendrimer to extend the distance for the easier access of the lysosomal enzymes to the linkage. By a proper choice of the

linker we were able to produce two almost complete analogous conjugates of MTX with the dendrimers, where the drug is attached to the spacer through an ester bond whereas the identity of the linkage between the dendrimer and the spacer was different, an amide bond. Similarly to the directly linked conjugates, we expect them to have distinct characteristic features due to the nature of the dendrimer.

In the second section, attempts to use regioselective protection strategies will be discussed to achieve high reaction yields with possibly higher drug payload and more elaborately structured conjugates to have better targeting over the currently synthesized conjugates, involving folic acid by other research groups.

2.2. MATERIALS

Methotrexate (4-amino-10-methylpteroylglutamic acid) (MTX) was obtained from National Cancer Institute (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis). Polyamidoamine (PAMAM) dendrimers (generation 2.5 with -COOH end groups and generation 3 with -NH₂ end groups) were purchased from Sigma Aldrich Chemical Company and Dendritech, MI. Regenerated cellulose dialysis bag of MW cutoff = 1000 and 3500 were purchased from Spectrum Laboratories Inc. Other reagents were obtained from assorted vendors in the highest quality available, and include *N,N*-dicyclohexyl carbodiimide (DCC) (Fluka), ethyl-6-hydroxy-hexanoate (EHH) (Sigma), triethylamine (TEA) (Fischer), fluorescein

isothiocyanate (FITC) (Sigma), 5-(*boc*-amino)-1-pentanol (Fluka), 4-dimethylamino pyridine (DMAP) (Sigma), di-*tert*-butyl dicarbonate (*t-boc*) (Sigma), 9-fluorenylmethyl-*N*-succinimidyl carbonate (Fmoc) (Sigma), ethyltrifluoroacetate (ETFA) (Sigma), 8-chlorothiophylline (Sigma), tetramethylammonium chloride (TMAC) (Sigma). All the solvents used were purchased from Fisher Scientific except HPLC grade dimethylsulfoxide (OmniSolv methylsulfoxide, VWR).

2.3. SYNTHESIS OF PAMAM-METHOTREXATE CONJUGATES

The following section will describe and discuss first directly coupled conjugates and spacer linked MTX-dendrimer conjugates. Then, the protection strategies will be demonstrated.

2.3.1. DIRECTLY LINKED CONJUGATES

Since the dendrimers differ in the identity of the surface groups in the first conjugate formed, designated conjugate A, the -NH₂ group of the drug has been coupled with the -COOH group of the generation 2.5 PAMAM dendrimer in a DCC-mediated reaction. On the other hand, in the cationic counterpart, the available group on the dendrimer for the conjugation is an -NH₂. Therefore, the formation of the coupling reaction occurred through -COOH group of the drug.

2.3.1.i. Conjugation of MTX to PAMAM-G2.5-COOH (Conjugate A):

MTX (0.0696 g, 0.153 mmol) and molar end-group equivalent of PAMAM-G2.5-COOH (30 mg, 0.0048 mmol, based on 32 end functional groups) were dissolved in 10 ml anhydrous DMSO, followed by the addition of molar equivalent amount of DCC (0.0316 g, 0.153 mmol). The reaction mixture was stirred continuously for 72 hrs at room temperature in the dark. After 72 hrs, it was filtered to remove by-product dicyclohexylurea (DCU) and the filtrate was dialyzed against an excess amount of reagent grade DMSO using membrane (MW cutoff = 3500 Da) for 24 hours to remove unreacted MTX and DCC. The filtrate was dried at room temperature to obtain dendrimer-MTX conjugate. This conjugate was characterized using a Varian 400 MHz spectrometer to estimate the conjugation ratio between MTX and the dendrimer. ^1H NMR spectrum for the conjugate (400 MHz, DMSO): δ (ppm) 2.31-3.57 (m, 356H, CH protons of PAMAM overlapping with the aliphatic singlets of MTX, 9H), 6.53 (s, 2H, aromatic NH_2 of MTX), 6.82 (d, 2H, aromatic CH of MTX), 7.67 (s, 2H, aromatic NH_2 of MTX), 7.91 (d, 2H, aromatic CH of MTX), 4.76 (s, 2H, CH_2 of MTX), 8.5 (s, 1H, aromatic CH of MTX).

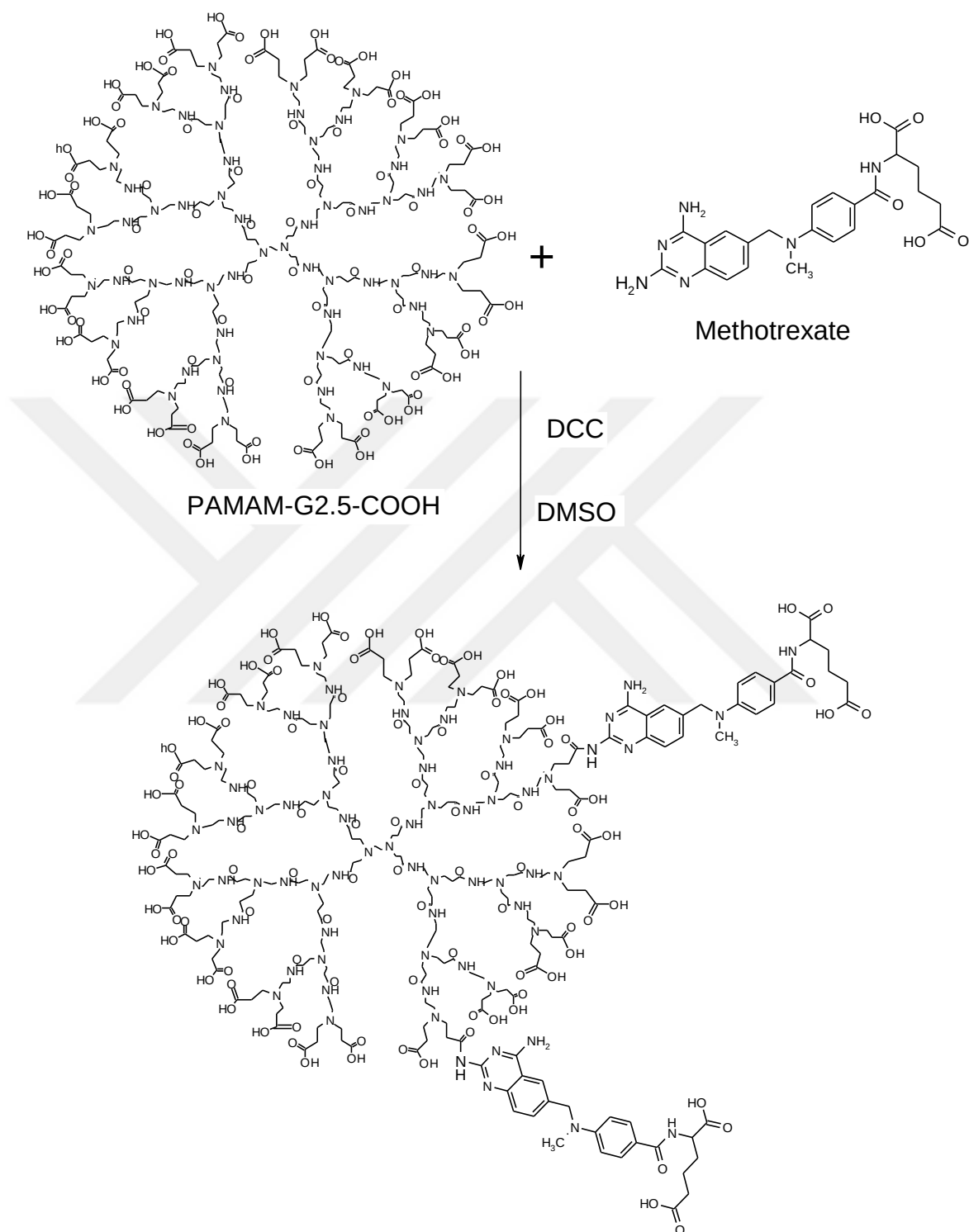


Figure 14. Synthesis scheme of PAMAM-G2.5-COOH-MTX (Conjugate A)

2.3.1.ii. Conjugation of MTX to PAMAM-G3-NH₂ (Conjugate B): MTX

(0.1263 g, 0.278 mmoles) and molar end-group equivalent of PAMAM-G3-NH₂ (60 mg, 0.00868 mmoles, based on 32 end functional groups) were dissolved in 10 ml DMSO, followed by the addition of a molar equivalent of DCC (0.0574 g, 0.278 mmoles). The reaction conditions and the purification steps were similar to those mentioned above. Similarly, a Varian 400 MHz spectrometer was used to determine the drug payload. ¹H NMR spectrum for the conjugate (400 MHz, DMSO): δ (ppm) 2.18-3.46 (m, 484H, CH protons of PAMAM overlapping with the aliphatic singlets of MTX, 9H), 6.77 (d, 2H, aromatic CH of MTX), 7.54 (s, 2H, aromatic NH₂ of MTX), 7.70 (d, 2H, aromatic CH of MTX), 7.95 (s, 2H, aromatic NH₂ of MTX), 4.76 (s, 2H, CH₂ of MTX), 8.51 (s, 1H, aromatic CH of MTX), 4.30 (s, 1H, aliphatic CH of MTX).

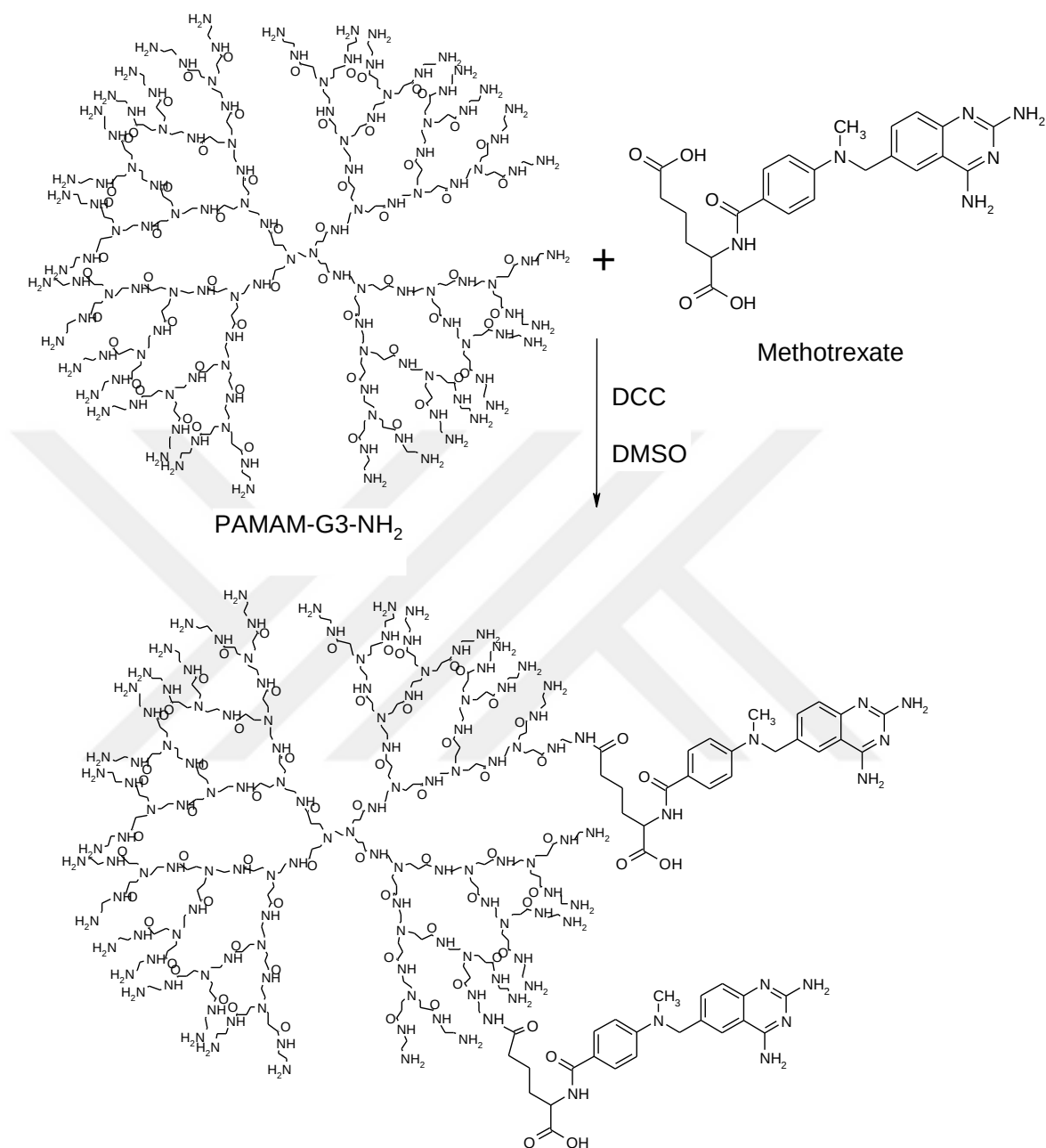


Figure 15. Synthesis scheme of PAMAM-G3-NH₂-MTX (Conjugate B)

2.3.2. SPACER LINKED CONJUGATES

2.3.2.i. Conjugation of MTX to PAMAM-G3-NH₂ through spacer EHH:

a) MTX-EHH conjugate: To prepare the PAMAM-G3-NH₂-MTX conjugate, first

COOH group of MTX has been coupled with the –OH group of the spacer EHH to form an ester-linked conjugate of the drug with the spacer. MTX (139.8 mg, 0.3079 mmoles) and 10 times higher molar equivalent of EHH (493 mg, mw= 160.22, 3.079 mmoles) were dissolved in a minimal amount of anhydrous DMF (3 ml) in the presence of DCC (635.3 mg, 3.079 mmoles) as the coupling agent. After 3 days under continuous stirring at room temperature, the reaction was stopped and the mixture was dried. Mono/diesters of MTX were separated from unreacted MTX by extraction with dichloromethane (DCM) as esters of the drug were easily taken into the solution while unmodified MTX remained insoluble. After the evaporation of the solvent, unreacted spacer and DCC were extracted with diethyl ether (DEE). Mono/diesters of MTX were not soluble in DEE whereas EHH and triethylamine (TEA) were infinitely soluble. Dry powder product was obtained upon evaporation of the solvent at room temperature. Esters were characterized with ^1H NMR and ESI-MS spectroscopic techniques.

b) PAMAM-G3-NH₂-EHH-MTX conjugate: MTX-EHH intermediate was further conjugated to the 3rd generation PAMAM dendrimer. Conjugation occurred as a result of the cleavage of the ethyl group from the spacer to allow the ester unit to form an amide bond with the dendrimer's –NH₂ end group. Equimolar amounts of MTX-EHH (190.5 mg, 0.258 mmoles) and dendrimer (0.008066 mmoles, 55.73 mg, based on 32 end functional groups) were dissolved in anhydrous DMSO (3 ml) with the addition of 50% excess of TEA (0.387 mmoles, 39.16 mg). Following a 3-day reaction period, the mixture was dialyzed against an excess amount of DMSO to separate unreacted MTX-EHH,

DCC, and leaving ethyl groups. Characterization of the conjugate was performed by ^1H NMR and MALDI-TOF techniques. ^1H NMR spectrum for the conjugate (400 MHz, DMSO): δ (ppm) 1.06-3.98 (m, 484H, CH protons of PAMAM overlapping with the aliphatic singlets of MTX, 9H, and CH_2 multiplets of the spacer, 12H), 6.62 (d, 2H, aromatic NH_2 of MTX), 6.76 (d, 2H, aromatic CH of MTX), 7.66 (d, 2H, aromatic CH of MTX), 4.74 (s, 2H, CH_2 of MTX), 8.51 (s, 1H, aromatic CH of MTX).

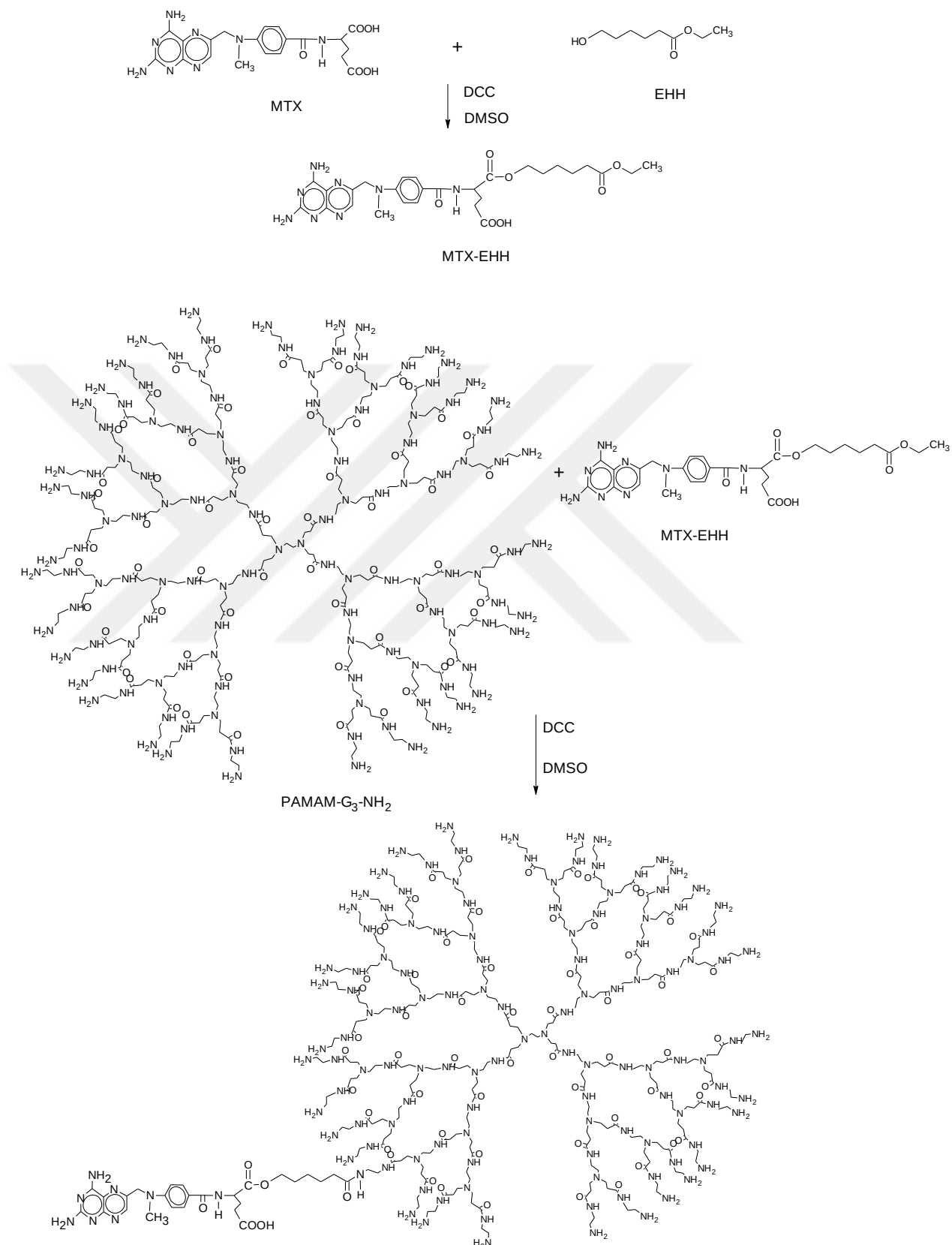


Figure 16. Synthesis scheme of PAMAM-G₃-NH₂-EHH-MTX conjugate

2.3.2.ii. Conjugation of MTX to PAMAM-G2.5-COOH through spacer

pentanol: a) MTX-pentanol conjugate: To afford spacer-linked conjugate of MTX with the dendrimer having both ester and amide bonds first we synthesized the intermediate MTX-pentanol conjugate in a reaction as follows: -COOH group of MTX (138.9 mg, 0.3059 mmoles) has been coupled to -OH of pentanol (623.5 mg, 3.059 mmoles) in a DCC-mediated (630.6 mg, 3.056 mmoles) condensation reaction where the reactants were fed at 1:10:10 molar ratios, respectively, dissolved in 3 ml anhydrous DMSO. The reaction mixture was stirred for 3 days at room temperature, after which the product was dried to obtain the esters of MTX, protected at the glutamate side of the molecule. Protected mono/diesters of MTX were separated from unreacted MTX by solvent extraction using DCM as esters of the drug were easily dissolved in the solvent while unmodified MTX remained insoluble. After the evaporation of the solvent, unreacted spacer and DCC were removed by extracting into DEE. Similarly to esters of MTX with EHH, mono/diesters of MTX with pentanol were not soluble in the solvent whereas *boc*-pentanol and DCC were instantly soluble. Dry powder product was obtained upon evaporation of the solvent to complete dryness at room temperature. Protecting group was removed by vigorously stirring the protected MTX dissolved in 50% Trifluoroacetic acid (TFA) in DCM for 45 minutes. The successful removal of the *boc*-protecting group from the spacer was demonstrated with ^1H NMR before and after the protection. Formation of mono/diesters was investigated with the ESI-MS spectroscopic technique.

b) PAMAM-G2.5-COOH-pentanol-MTX conjugate: Deprotected MTX-pentanol was directly conjugated to PAMAM-G2.5-COOH dendrimer in a DCC-mediated coupling reaction resulting in an amide bond between the spacer and the dendrimer. Briefly, equimolar amounts of deprotected MTX-pentanol (76.6 mg, 0.1419 mmoles), DCC (29.3 mg, 0.1419 mmoles), and DMAP (17.3 mg, 0.1419 mmoles) were mixed with the molar equivalent of PAMAM-G2.5-COOH dendrimer (27.8 mg, 4.44 μ moles, based on 32 end functional groups) in anhydrous DMSO (3 ml). Similar purification protocols were applied to separate the conjugate from unreacted MTX-pentanol and DCC, which was followed by drying to obtain the powder product. ^1H NMR spectrum for the conjugate (400 MHz, DMSO): δ (ppm) 1.22-3.37 (m, 356H, CH protons of PAMAM overlapping with the aliphatic singlets of MTX, 9H, and aliphatic CH_2 multiplets of spacer, 10H), 6.78 (d, 2H, aromatic CH of MTX), 7.70 (d, 2H, aromatic CH of MTX).

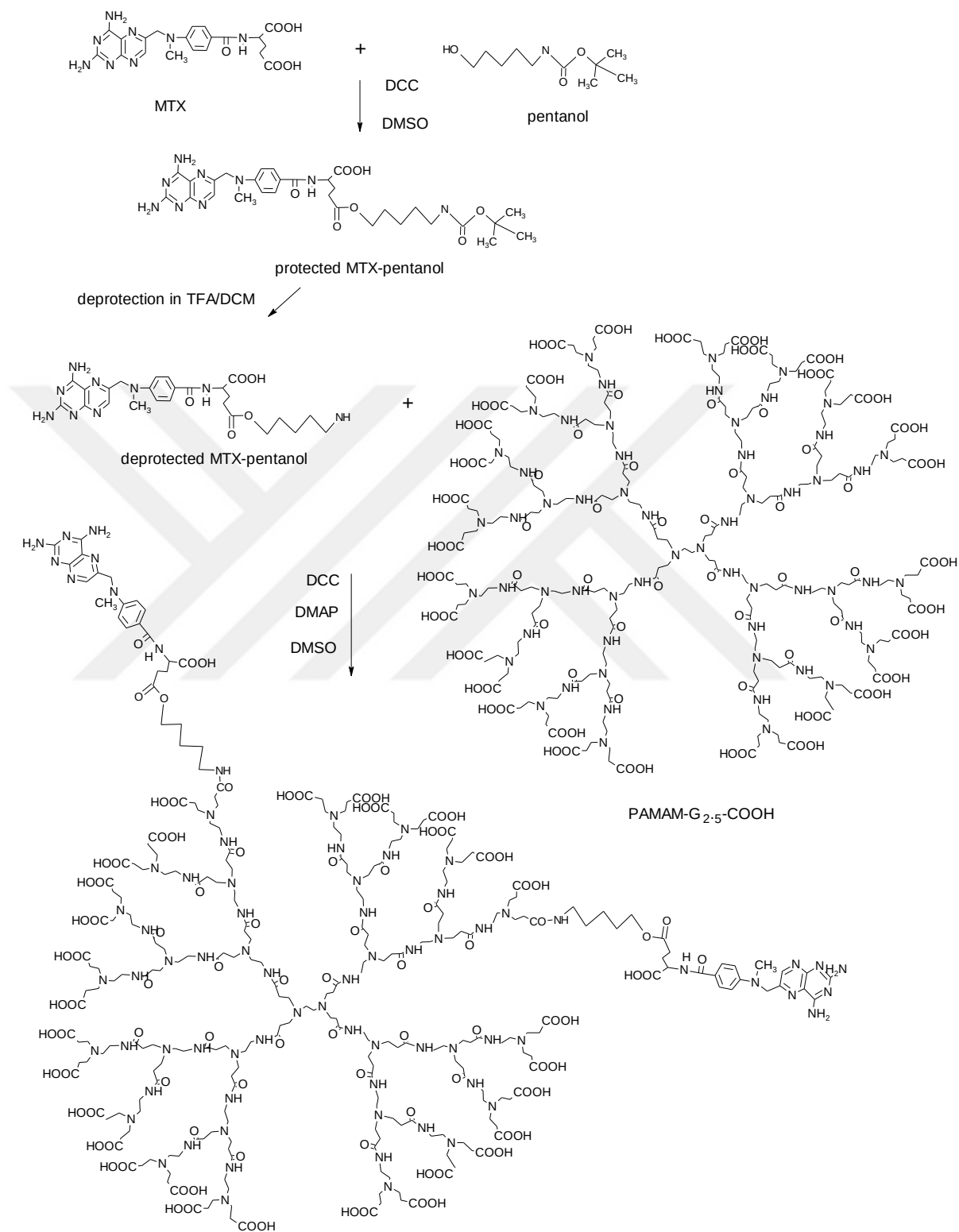


Figure 17. Synthesis scheme of PAMAM-G_{2.5}-COOH-pentanol-MTX conjugate

2.3.3. PROTECTION STRATEGIES

As mentioned earlier, other than traditionally created drug-polymer conjugates, we wanted to develop more elaborately designed structures, which can not only target but also present the targeting ligand to the receptors on the cells in such a way that interaction would occur at higher propensity and ease. At the same time, blocking of the reactive groups of the drug intended to be left unmodified during the synthetic protocol will increase the yield due to reduced chance of cross-coupling of the drug while conjugating with the dendrimer.

The following will describe the three different strategies employed in this respect for MTX and folic acid (FA) as the targeting ligand.

2.3.3.i. Protection of MTX/FA with ETFA: For the -NH₂ group protection of either FA or MTX, 10 times molar excess of ETFA (1.134 mmoles, 0.1429 g), coupling agent DCC (1.134 mmoles, 0.2339 g), and TEA (1.134 mmoles, 0.1147 g) were added to FA (50 mg, 0.1134 mmoles) dissolved in 3 ml of anhydrous DMSO. Following a 3-day reaction period, the reaction mixture was vacuum dried to complete dryness. The dry product was extensively washed with DEE to remove unreacted DCC, TEA, ETFA, and ethyl group of ETFA formed after the cleavage. Figure 18 shows the synthetic protocol followed for -NH₂ protection of FA as an example.

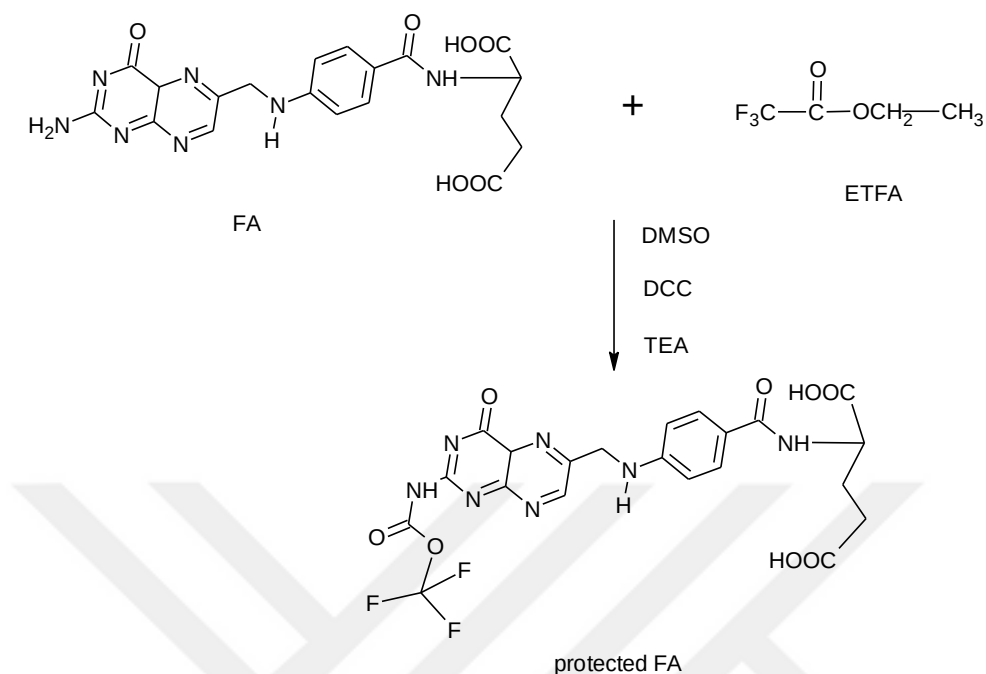


Figure 18. -NH_2 group protection of FA with ETFA

2.3.3.ii. Protection of MTX/FA with *t*-*boc*: Secondly, the commonly used amine protecting agent *t*-*boc* was used in a similar protocol to introduce protection at the amine group of MTX/FA. To a solution of MTX (40 mg, 0.0881 mmoles) and *t*-*boc* (0.1921 g, 0.881 mmoles) dissolved in DMF (3 ml), TEA (0.0891 g, 0.881 mmoles) predissolved in the same solvent was added to achieve the molar concentrations of the reactants in the final mixture at 1:10:10. Since nonaqueous condition was selected, the pH of the reaction solution was kept basic with the use of anhydrous DMF as the solvent without the need for the basic salts such as NaHCO_3 . After a 3-day reaction period, a similar purification protocol was used; drying of the product was followed by the removal of unreacted *t*-*boc* and TEA carried out by washing extensively with DEE, which is not the solvent for the protected MTX.

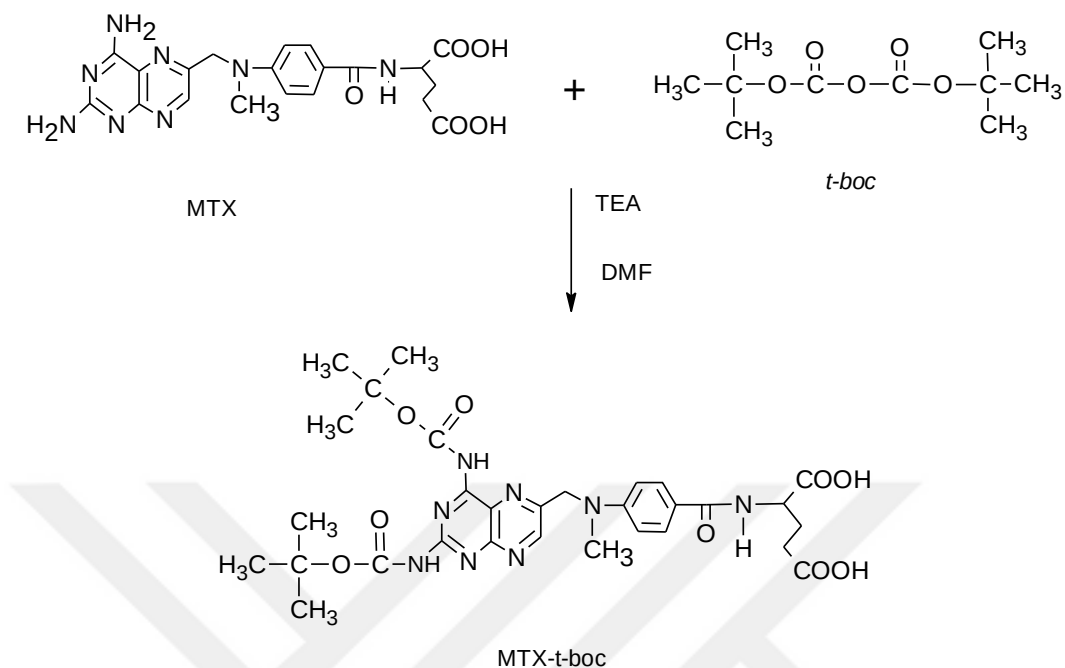


Figure 19. -NH_2 group protection of MTX with *t*-boc (shown as diprotected MTX species)

2.3.3.iii. Protection of MTX/FA with Fmoc: Another common amine protecting group, 9-fluorenylmethyl-*N*-succinimidyl (Fmoc), was also utilized to form Fmoc-MTX/FA. A total of 50 mg of MTX (0.1101 mmoles), 371mg (10.999 mmoles) of Fmoc, and 12 mg (0.1101 mmoles) of TEA were dissolved in anhydrous DMSO (3 ml). Under basic conditions due to the solvent choice, the reaction was stirred for 3 days at room temperature, at the end of which DOWEX 50W resin was added to the reaction mixture to neutralize the pH. The MTX species with the unprotected amine groups will adsorb onto the resin strongly while those with protected amine groups can be extracted easily into the solvent. Therefore, the resin was washed with DMSO to extract Fmoc-MTX species

adsorbed onto the beads. Then, the solvent DMSO was evaporated to complete dryness to obtain protected MTX/FA.

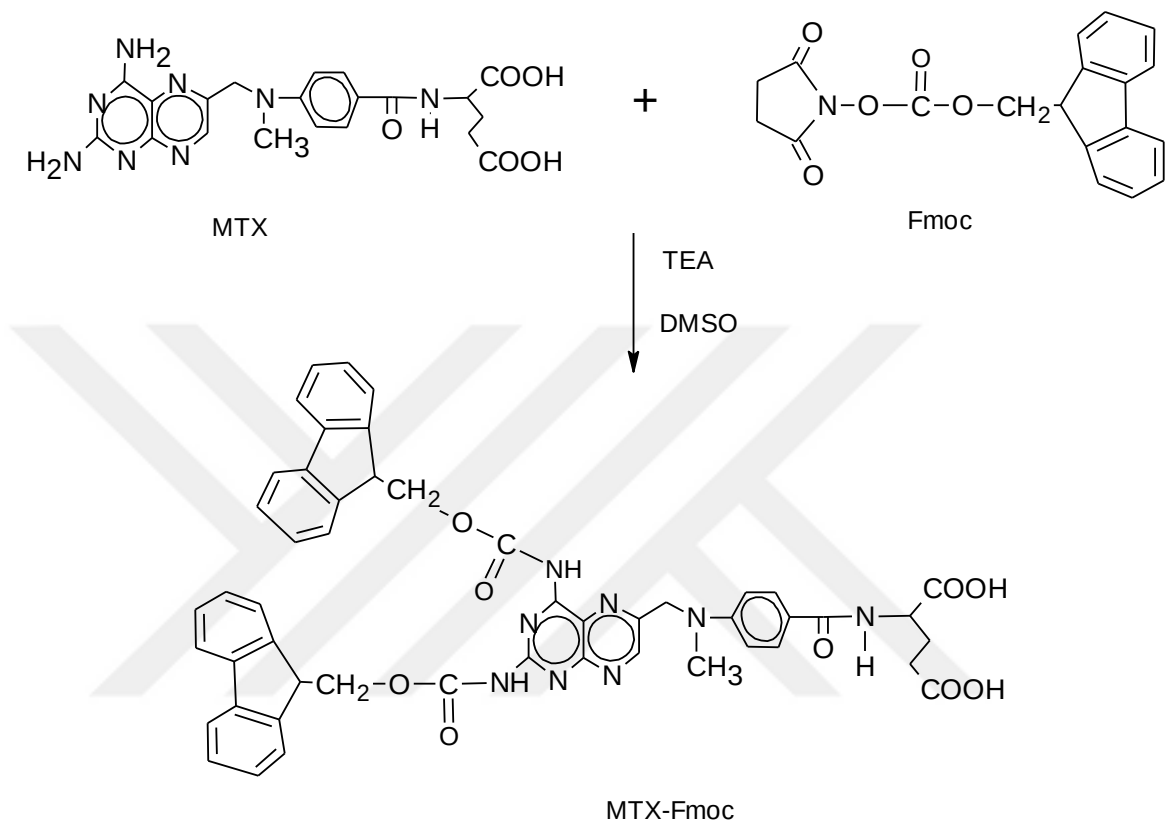


Figure 20. -NH_2 group protection of MTX with Fmoc (shown as diprotected MTX species)

2.3.4. CONJUGATION OF FITC TO PAMAM-G2.5-COOH

A similar protocol was used for the preparation of the conjugate of the dendrimer with fluorescent label FITC. Briefly, a given volume of FITC in anhydrous DMSO solution (34.2 mg/ml) was added to the dendrimer (55 mg, 8.776 μmoles) at a molar ratio of 10/1, respectively. Accordingly, coupling agent DCC was added to the reaction mixture at a ratio of 3 times the FITC-molar

equivalent in total. Due to relatively low reactivity of the –OH group in the FITC molecule, the reaction was allowed to go for 7 days in dark. Purification steps were similar to those used for the dendrimer-drug conjugates as explained before.

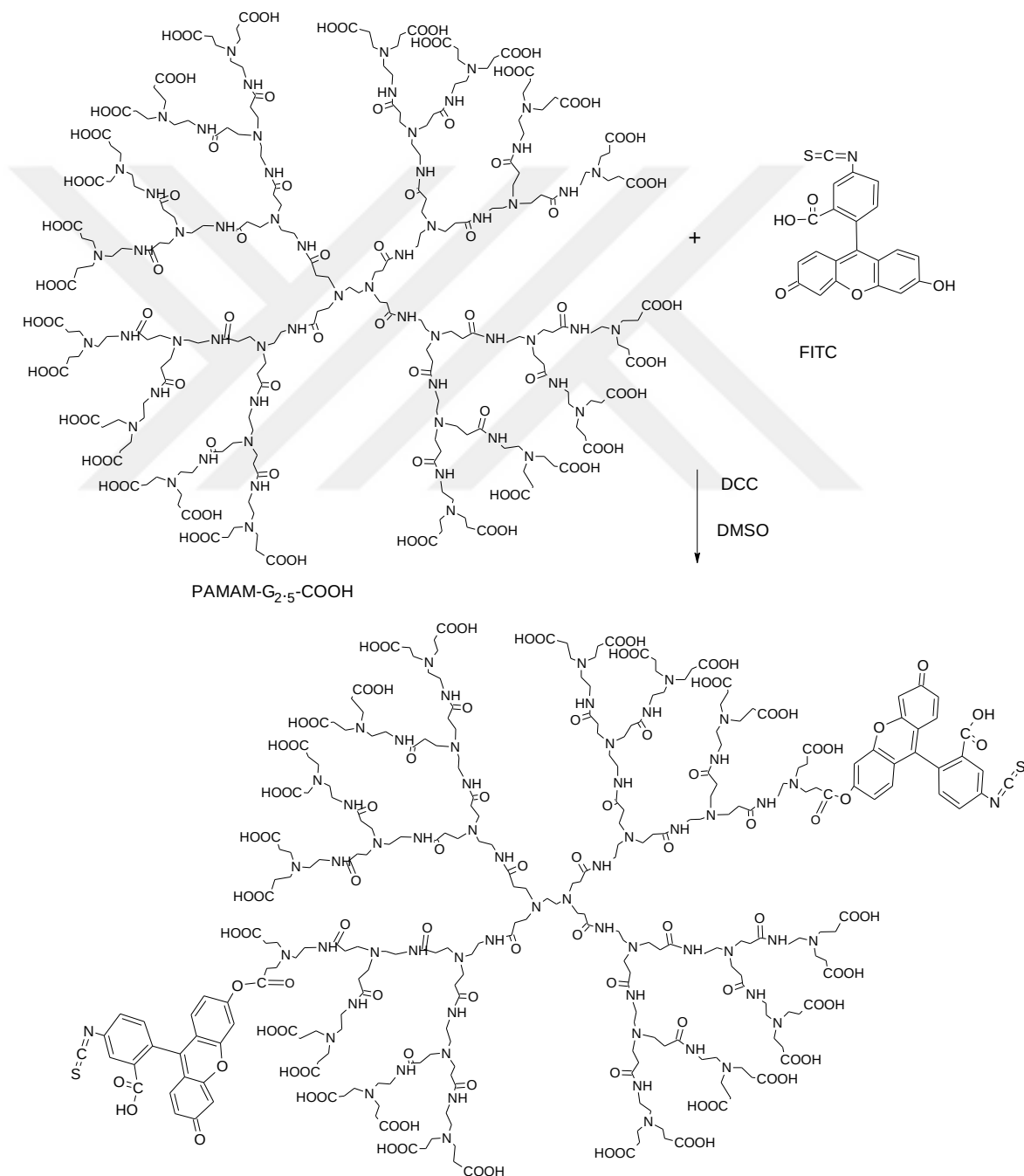


Figure 21. Synthesis scheme of PAMAM-G2.5-COOH-FITC conjugate

2.4. CHARACTERIZATION OF CONJUGATES

^1H NMR spectra were obtained in Varian 400 MHz spectrometer as the main characterization method for all the conjugates where the shifts from the reference tetramethylsilane (TMS) are indicated in ppm. Samples for the analysis were dissolved at the highest concentration possible in deuterated DMSO unless noted.

MALDI-TOF mass spectrometry was performed using Bruker Daltonics Ultraflex matrix-assisted laser desorption/ionization mass spectrometer with time-of-flight mass analyzer (MALDI-TOF) for further characterization of the conjugates. Linear/reflection operation mode was selected for pure dendrimers and the conjugates. A solution of the matrix 2,5-dihydroxybenzoic acid (20-200 mg/ml) was prepared with acetonitrile (ACN)/ H_2O (v/v = 50:50) while dendrimer/conjugate was dissolved in the same solvent/DMSO, respectively, at a concentration of 1-2 mg/ml. Equal volumes of matrix and analyte solution were mixed and 1 μl of the mixture was spotted on the target.

Electron-spray ionization mass spectroscopic (ESI-MS) technique was employed for the characterization of the intermediate products such as spacer-MTX conjugates. For that, conjugate was dissolved in methanol and under vacuum the solution was sprayed onto two closely spaced electrodes of Waters ZQ2000 single quadrupole mass spectrometer with electrospray ionization source. The analyte was then analyzed to assess the success of the reaction protocol used.

Dendrimers and the conjugates were additionally characterized by gel permeation chromatography in a Shimadzu LC-10ADVP liquid chromatograph equipped with CTO-10ASVP Shimadzu column oven and MesoPore 300×7.5 mm column (Polymer Laboratories) using HPLC grade DMSO (OmniSolv methylsulfoxide) as the eluent at a flow rate of 0.5 ml/min at 35°C.

Also, reverse phase high performance liquid chromatography (RP-HPLC) technique was exploited to further characterize the conjugates. Analysis was performed using a 3.9X150 mm Waters RP-C18 column at 303 nm as the detection wavelength with a mobile phase of 0.1 M KH_2PO_4 + 0.04 M TMAC and ACN (v/v = 80:20) pH = 2.5.

2.5. RESULTS

2.5.1. DIRECT PAMAM-METHOTREXATE CONJUGATES

MTX is a tetrafunctional molecule with α - and γ -COOH groups in the glutamate moiety as well as two $-\text{NH}_2$ groups at the 2nd and 4th positions in the 2, 4-diamino-6-pteridinyl group as shown in Figure 1, which are of interest in the synthesis scheme of direct conjugates. We prepared the conjugates of MTX not only through the $-\text{COOH}$ terminal group of the drug but also through the $-\text{NH}_2$ functionality which has been the first report in literature to best of our knowledge. Even though, one might argue that gelatin, a difunctional molecule with $-\text{COOH}$ and $-\text{NH}_2$ end groups, has been conjugated to MTX at the drug's $-\text{NH}_2$ group, there is no conclusive evidence for the modification done to MTX at that

particular site other than hypothesis (89, 90). Furthermore, it has been suggested that the presence of a free α -carboxyl group is crucial for the *in vitro* activity of the drug as first recognized by Rosowsky (91, 92) whom was later followed by Pignatello et al. (93-96) and Riebeseel et al. (97), describing the group as the region of significant bulk tolerance. Therefore, conjugating the drug at its $-\text{NH}_2$ group at the pteridine ring was envisaged worthwhile to test the earlier observations by leaving the glutamate moiety completely free. Even though one might argue that in DCC coupling reactions in which two carboxylic acids can come together to form acid anhydride as shown over long-chain fatty acids, this formation is highly unlikely in the presence of competitive reactants, as the $-\text{NH}_2$ supplying molecule, MTX in this case (98). Eventually, amide bonded conjugate of the generation 2.5 PAMAM dendrimer and the drug will form. The ^1H NMR spectra of the Conjugate A and Conjugate B are given in Figures 22 and 23.

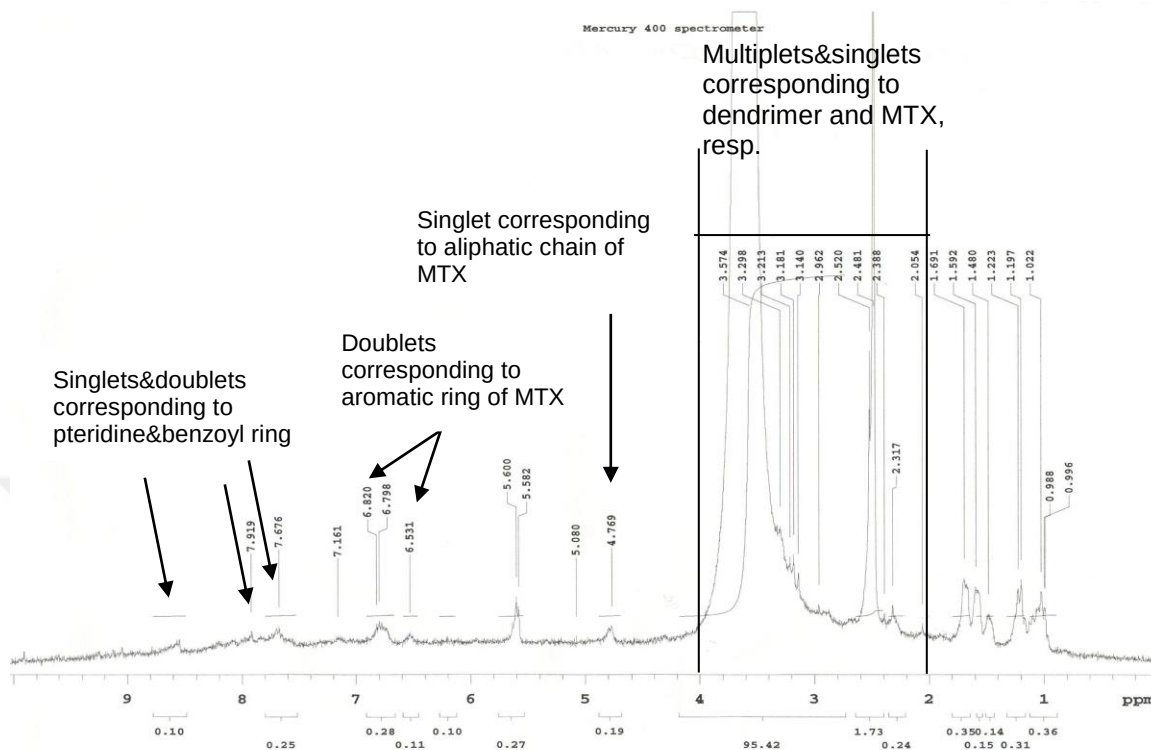


Figure 22. ^1H NMR of Conjugate A

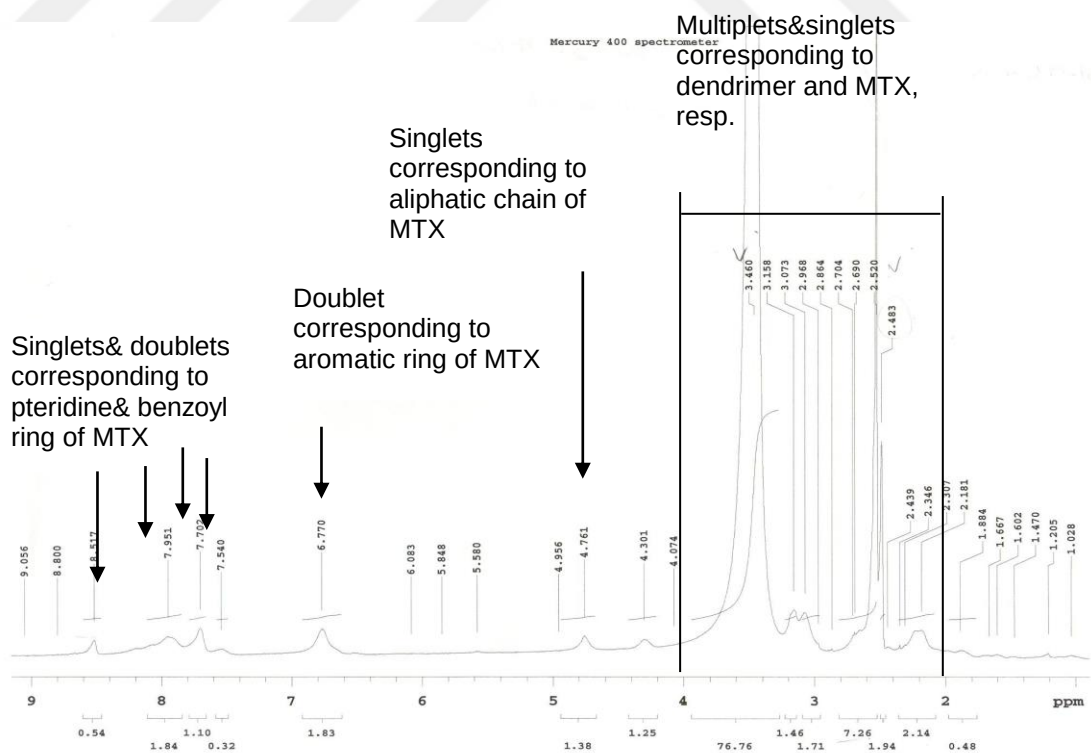


Figure 23. ^1H NMR of Conjugate B

The conjugation ratio of MTX to the dendrimer in each conjugate was estimated by ^1H NMR with the proton integration method: The integration ratio of MTX to dendrimer was calculated by taking the characteristic peaks of MTX and the PAMAM dendrimer into account as explained elsewhere given previously in Section 2.1.2.1. (49, 56, 67, 93, 99, 100). ^1H NMR of Conjugate A in DMSO represents characteristic proton signals of MTX and the PAMAM-G2.5-COOH dendrimer. The peaks at $\delta = 2.02\text{--}3.44$ ppm in Figure 22 were attributed to the total of 356 protons of PAMAM-G2.5-COOH dendrimer overlapping with characteristic peaks of MTX in the same region assigned to 9 protons. On the other hand, 6.8 and 7.9 ppm peaks were characteristics of the total of 4 protons of the benzoyl group in MTX. Taking these into consideration, we estimated the molar ratio of MTX to carboxyl-terminated PAMAM-G2.5 dendrimer (Conjugate A) to be 0.4. On the other hand, PAMAM-G3-NH₂ had slightly higher number of protons, 484 protons, due to half higher generation. Using a similar analogy, we estimated that there are 4.4 MTX molecules per one molecule of PAMAM-G3-NH₂ dendrimer (Conjugate B). However, the product might contain unreacted dendrimer along with the successfully conjugated fraction. Considering the fact that both of the conjugates have less than 5 MTX molecules per dendrimer molecule, the separation of the reacted and unreacted portion of the dendrimer is very difficult due to the limitations related to mainly molecular weight and altered physicochemical characteristics of the dendrimer upon conjugation.

On the other hand, such a high difference in the conjugation ratios of MTX to the dendrimers could be indicative of the differences in the reactivities of the -

COOH and -NH₂ groups of MTX towards the -NH₂ and -COOH functional groups of each dendrimer, respectively. Furthermore, there are two -COOH groups on MTX readily available for the interaction with amines, which may improve the probability of reaction for Conjugate B. In contrast, due to steric stabilization of the drug molecule at the 4th position as well as that at the 2nd position such a readily availability of -NH₂ group for the coupling is mostly unlikely (92). Hence, the conjugation of the drug with the amine terminated dendrimer exploiting the -COOH group on MTX produced a conjugate with higher drug loading while COOH-terminated dendrimer had lower MTX payload due to lesser reactivity of sterically more stabilized -NH₂ groups in the 2, 4-diamino-6-pteridiny group of MTX towards the dendrimer surface functional groups.

The MTX payload in Conjugate B is comparable to the literature when the dendrimers are concerned (48, 51, 101). As of now, even though we did not confirm which of the two -COOH groups in glutamate moiety of MTX is interacting with -NH₂ groups of the dendrimer, it is most likely the carboxyl group at the γ -position, since Kono et al. showed that the conjugation of folic acid to hydrazide-terminated dendrimers occurred through its γ -COOH group, which is shown to be more reactive toward the amino groups in carbodiimide-mediated coupling reactions (48). Additionally, conjugation of MTX to human serum albumin (HSA) proceeds mainly upon the involvement of γ -COOH group of MTX (102). In contrast, when the conjugate A is considered, we do not know whether it is the -NH₂ at the 2nd or the 4th position in the pteridiny group used for conjugation, even though the amine group at the 2nd position is expected to be

involved in the coupling reaction to a higher extent due to lesser sterical crowding at the point.

On the other hand, with a similar protocol in a later preparation, we conjugated 15.6 MTX molecules with PAMAM-G3-NH₂ based on ¹H NMR spectrum as shown in Figure 24, which is the highest MTX payload on a dendrimer so far.

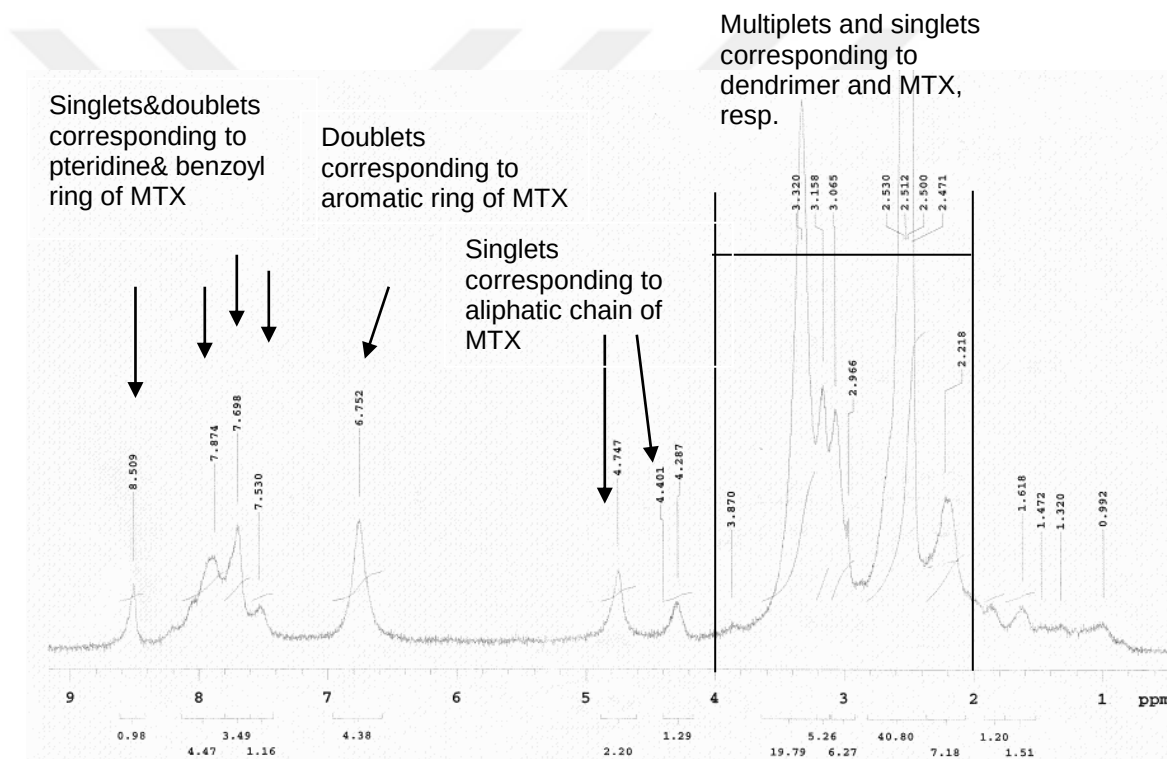


Figure 24. ¹H NMR spectrum of PAMAM-G3-NH₂-MTX conjugate

Relatively higher drug payload achieved on this particular conjugate could be reflecting the batch to batch inconsistency in the synthesis of either the dendrimer or the conjugate. However, on the upside such a high drug payload enabled us to characterize the conjugate with MALDI-TOF MS and GPC techniques to obtain the mass-related information of the particular species due to pronounced difference in the molecular weight from the parent cationic

dendrimer, PAMAM-G3-NH₂. In Figures 25 and 26, MALDI-TOF MS spectra for the dendrimer and the conjugate are given.

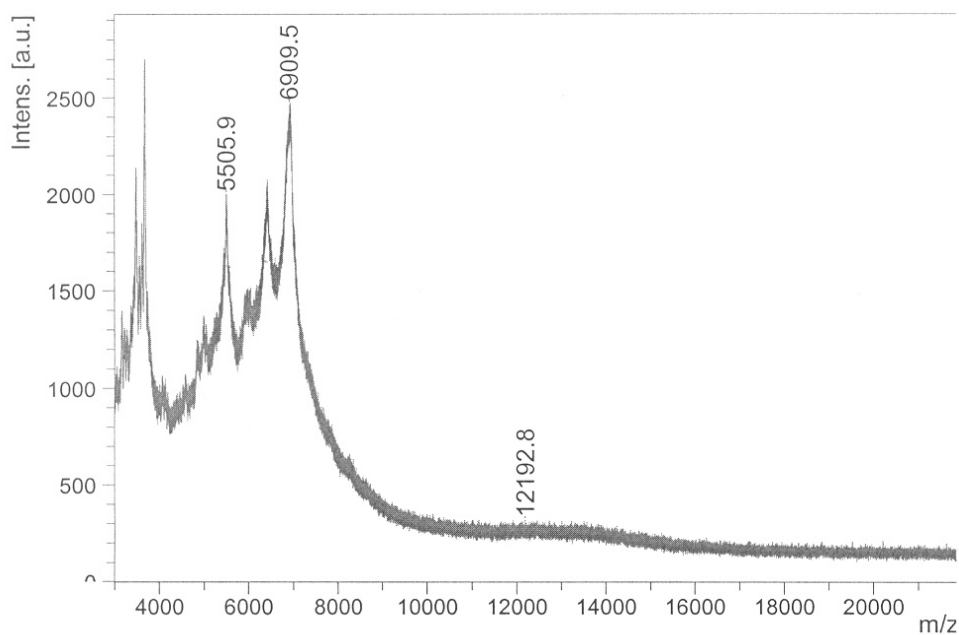


Figure 25. MALDI-TOF MS spectrum of PAMAM-G3-NH₂ dendrimer

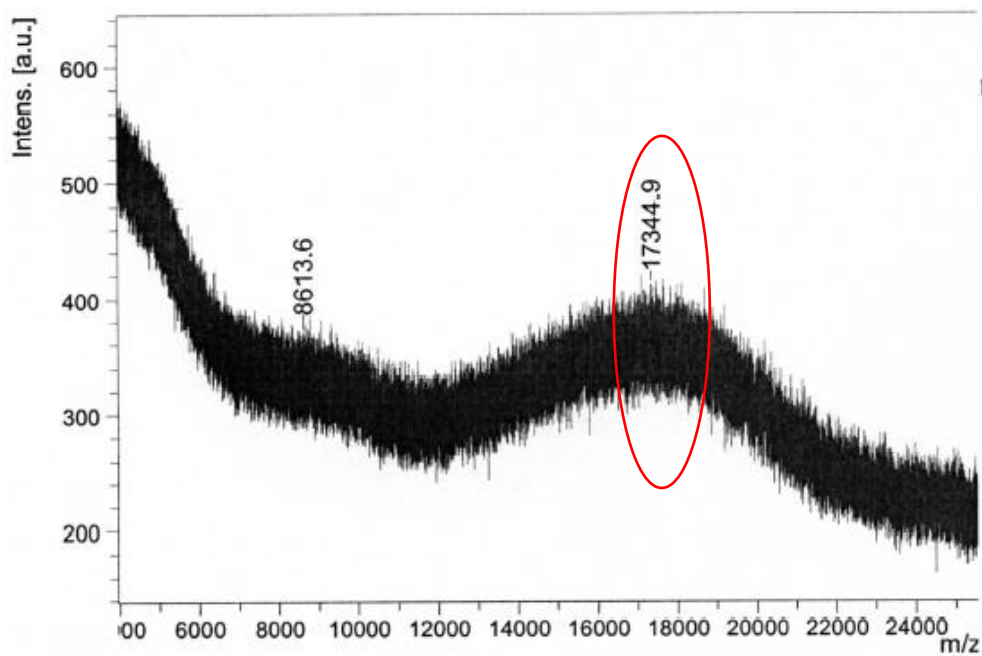


Figure 26. MALDI-TOF MS spectrum of PAMAM-G3-NH₂-MTX conjugate

The analyte prepared with DHB matrix at a conjugate to matrix ratio of 1:10 produced the expected peaks reflecting the shift towards the high molecular weight upon conjugation. On the other hand, the sample made with indoleacrylic acid (IAA) at various concentrations failed to show the expected molecular weight change (not shown). This could be due to relative inability of the matrix to absorb the laser beam which would lead to significant fragmentation in the molecular structure of the conjugate (103, 104). However, considering the molecular weight of the dendrimer shown in Figure 14 and that of the free drug, we calculated that there should be 19.6 MTX molecules per dendrimer molecule which differs from the ^1H NMR data, the difference can not be explained by the fragmentation produced by the inherent characteristics of the technique. This would be attributed to the calibration of the instrument for the particular molecular weights. Yet, a general molecular weight distribution was observed, suggesting that conjugate is monodisperse. The general character of the peak is comparable to what has been seen with the dendrimers upon modification of the end groups: as molecular weight increases the peak broadens up and becomes more blunt compared to smaller molecular weight objects, which produce sharp signal (103-106). Additionally, this technique was indicative of the absence of the free MTX since no peak attributable to the drug in the lower molecular weight frequency was observed.

As a third characterization method, we exploited the gel permeation chromatography (GPC) technique to further analyze the conjugate towards the search for the evidence of the formation of higher molecular weight object, which

is monodisperse and pure as suggested by MALDI-TOF MS. For that, conjugate and parent dendrimer were eluted with HPLC grade DMSO at 0.5 ml/min flow rate through a heated GPC column maintained at 35 °C. The elution profiles of cationic PAMAM-G3 dendrimer and the corresponding conjugate, measured using a light scattering detector, are displayed in Figure 27.

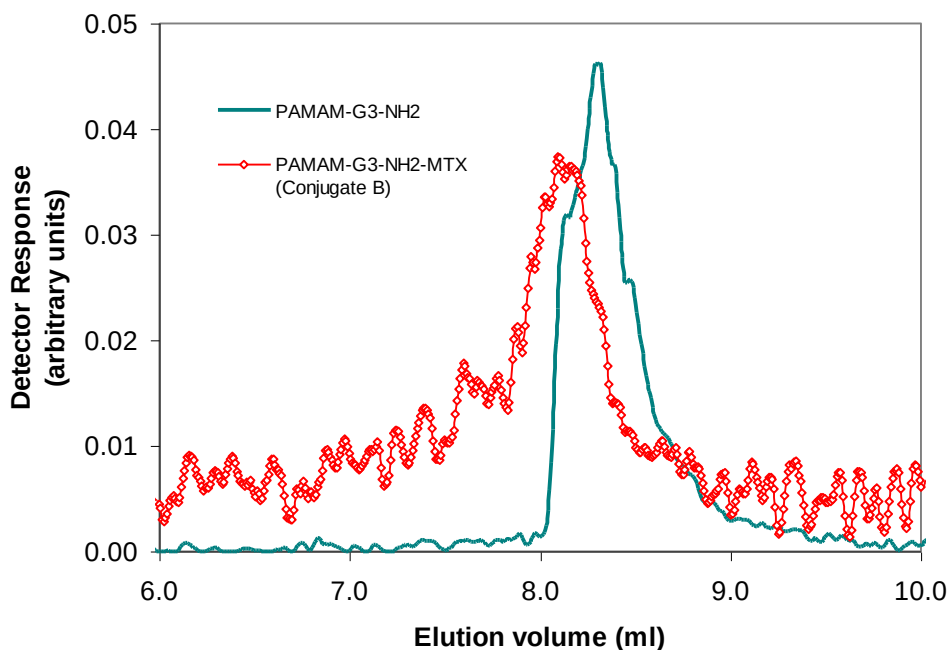


Figure 27. LC-GPC elution profiles of PAMAM-G3-NH₂ dendrimer and the corresponding conjugate with MTX

Peak elution volume of the pure dendrimer was observed as 8.32 ml whereas for the conjugate it was found to be 8.1 ml. The combination of monomodal nature and a clear shift towards lower elution volume/shorter elution times for the conjugate suggests the formation of a larger molecular weight object, which is monodisperse, confirming the findings of MALDI-TOF MS. However, the conjugate of MTX with anionic PAMAM-G2.5-COOH dendrimer did not yield any shift in the elution profiles since it has only 0.4 MTX molecules

attached to the dendrimer. Therefore, analysis method is insensitive to such small molecular weight changes in the analyte as might be expected.

Also, the cationic conjugate was further analyzed by HPLC on a RP C18 column for the presence of any free drug after the extensive dialysis. The samples taken from the dialysate at various time-points over a 45-h period were analyzed at 303 nm wavelength as the detection wavelength for free MTX.

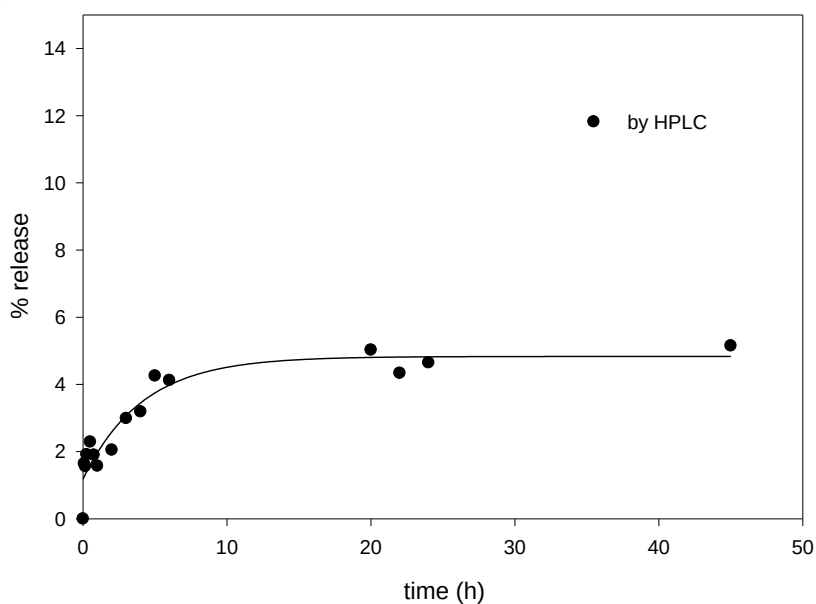


Figure 28. RP-HPLC analysis of G3-NH₂-MTX conjugate

As is shown, approximately within 20 h, it reaches the equilibrium value of 4.5% representing the free drug remained in the conjugate after 3 days of dialysis against extensive amount of DMSO. Yet, compared to the total amount of the drug included in the conjugate this is very small.

2.5.2. SPACER LINKED PAMAM-METHOTREXATE CONJUGATES

It has been a common unfortunate property of the prodrugs that almost exclusively a drug loses its potency despite the improved pharmacodynamics, mainly reduced nonspecific toxicity, a major factor in determining the scheduling and dosing of the regimen in cancer patients. This is due to total lack or impaired drug release from the conjugate (51, 53, 97, 107, 108). However, it has been also demonstrated that the placement of a spacer to extend the drug from the carrier surface for easier access of the proteolytic enzyme to the bond helps release the drug efficiently *in vitro*. For that reason, we wanted to synthesize two analogous conjugates with a spacer where the carrier will be either the anionic or the cationic PAMAM dendrimer. Since the locations of the amide and ester bonds are the same in both the conjugates it is unquestionably worthwhile to directly compare the likely differences *in vitro*.

2.5.2.i. PAMAM-G3-NH₂- EHH-MTX: Synthesis of EHH esters of MTX in a DCC-mediated reaction was carried out successfully with a yield of 23%. Esterification of the drug with the spacer proceeded rapidly at room temperature with the formation of dicyclohexylurea (DCU), a by-product of DCC-mediated reactions, only in a couple of hours. In the following figures (Figures 29 and 30), ¹H NMR spectra of MTX and MTX-EHH conjugate are given.

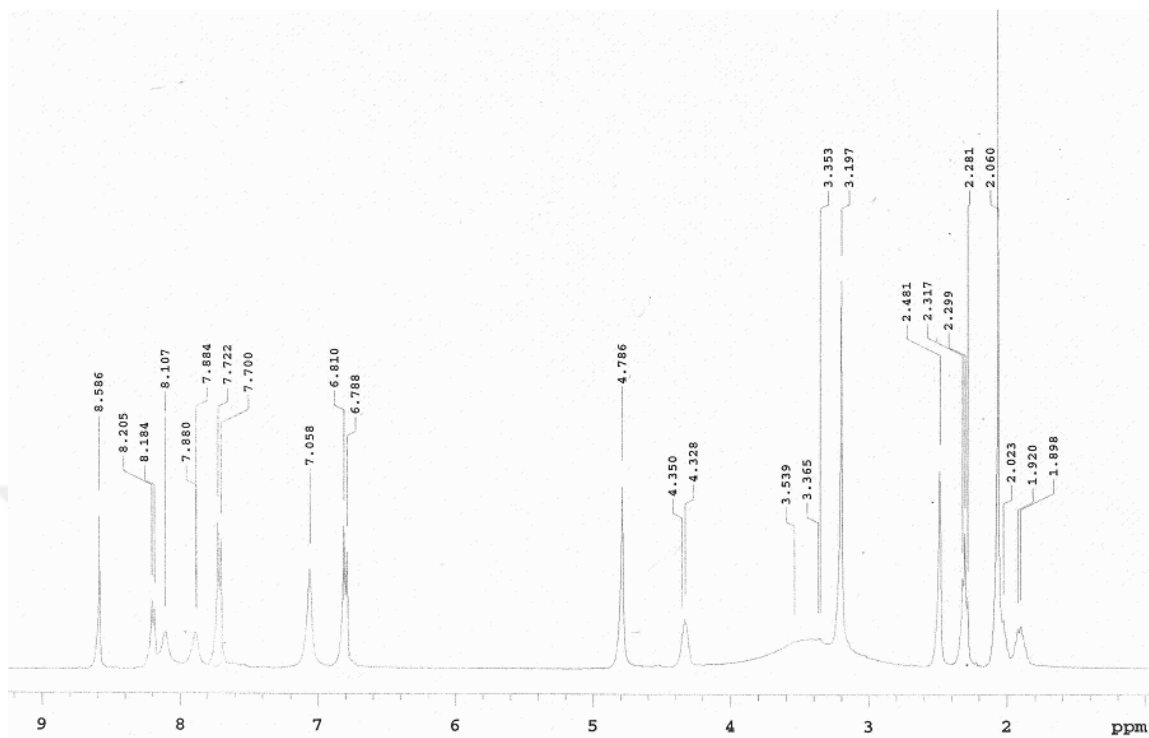


Figure 29. ^1H NMR spectrum of MTX

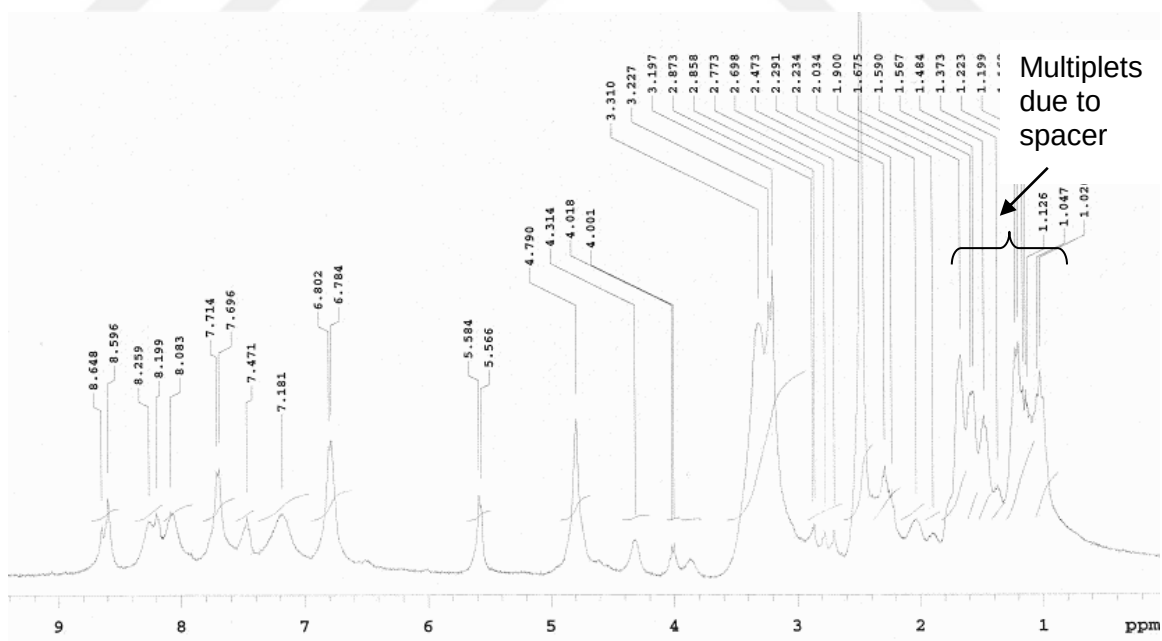


Figure 30. ^1H NMR spectrum of MTX-EHH

After excessive extraction with DEE and DCM, ester form of the drug was obtained as a pure product. The presence of the peaks from 1 ppm to 2 ppm

introduced by the spacer, which are absent in the ^1H NMR spectrum of the drug, reflects the successful attachment of the spacer to the $-\text{COOH}$ groups of MTX. Furthermore, by accounting for the total of 12 protons of the spacer in this region as opposed to 4 significant benzoyl protons of MTX at 6.8 and 7.8 ppm, we calculated that 1.93 or approximately 2 molecules of EHH are present in the intermediate MTX-EHH conjugate. Hence, it was concluded that the synthesis scheme led to the formation of α - and γ -esters of MTX where both of the $-\text{COOH}$ groups are condensed with the $-\text{OH}$ group of the spacer creating an ester linkage. Similar synthesis/purification protocol has been used for the preparation of lipophilic MTX by Rosowsky and Pignatello modifying the glutamate moiety of the drug by attaching long alkyl chains (91-96).

Further, conjugate was characterized by using ESI-MS technique.

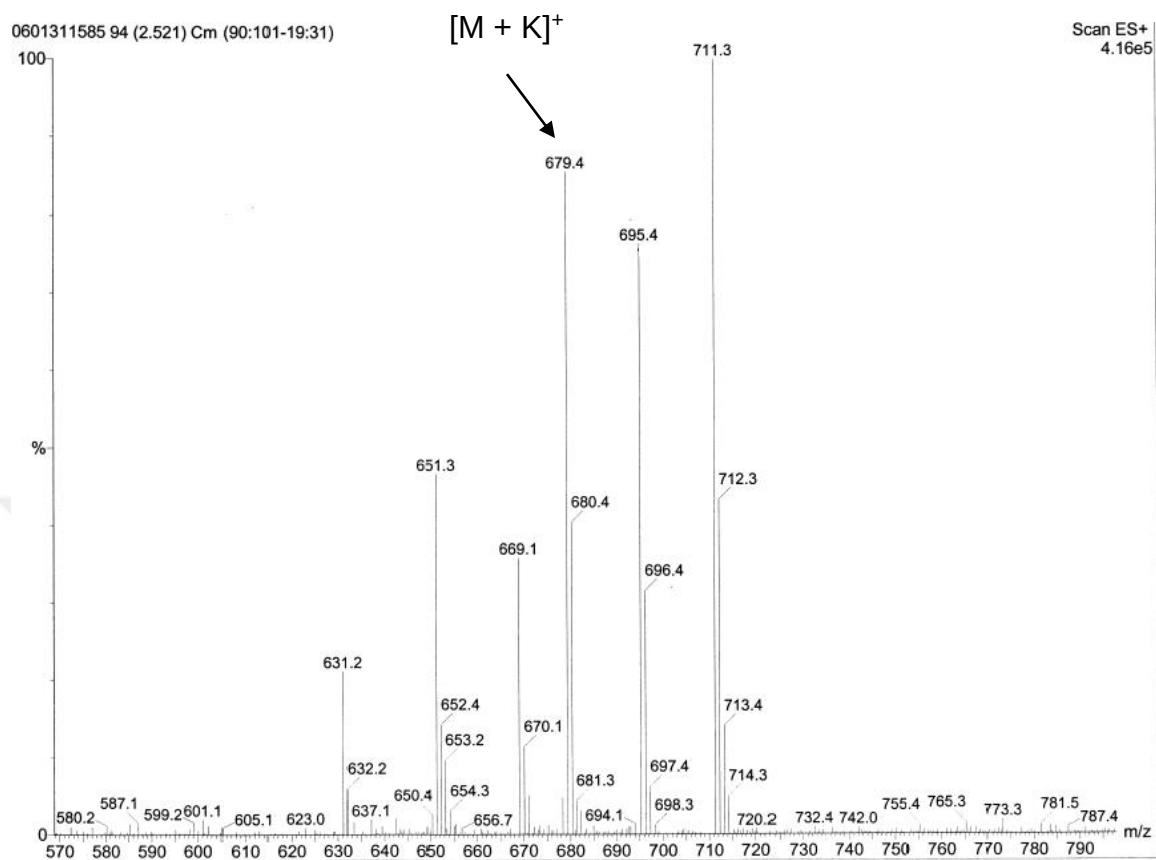


Figure 31. ESI-MS spectrum of MTX-EHH with KOAc

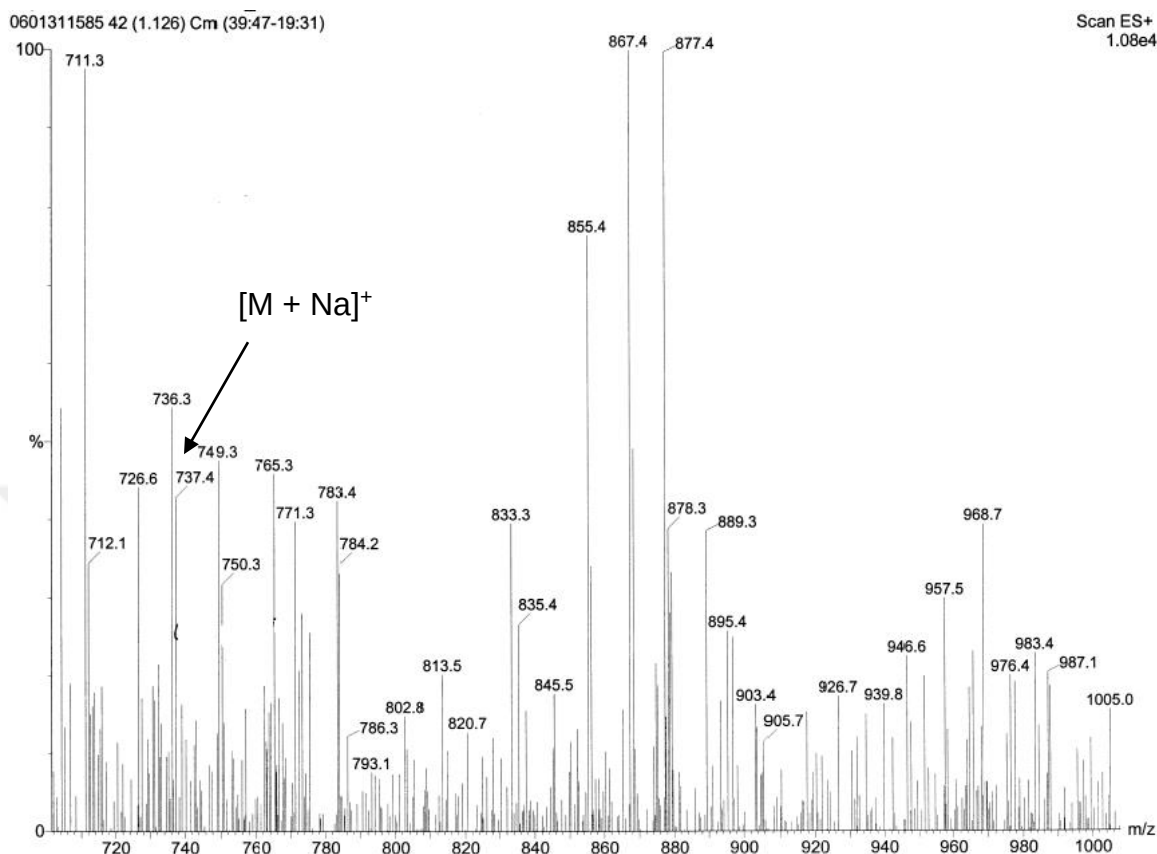


Figure 32. ESI-MS spectrum of MTX-EHH in MeOH

ESI-MS (m/z): 679.4 $[M + K]^+$ (87%), 737.4 $[M + Na]^+$ (45%)

The peaks at 679.4 and 737.4 are due to the K and Na adducts of the diester MTX species. Since the esterification of MTX was carried out successfully leading to exclusively the diesters of the drug, we further conjugated the intermediate with the cationic PAMAM-G3-NH₂ dendrimer in a similar preparation protocol as used for the directly linked conjugates. The estimation of the drug payload was done by using first proton integration method taking the characteristic peaks shown in Figure 33. Since apart from the previous conjugates, the glutamate moiety of MTX has been extended with the spacer, we had to consider all the corresponding peaks from 1 ppm to 4 ppm, with the

addition of 12 protons of the spacer to the total proton count from the dendrimer and the drug as discussed earlier. By doing a simple calculation, we found out that conjugate had 2.9 MTX or MTX-EHH molecules attached to one PAMAM-G3-NH₂ molecule.

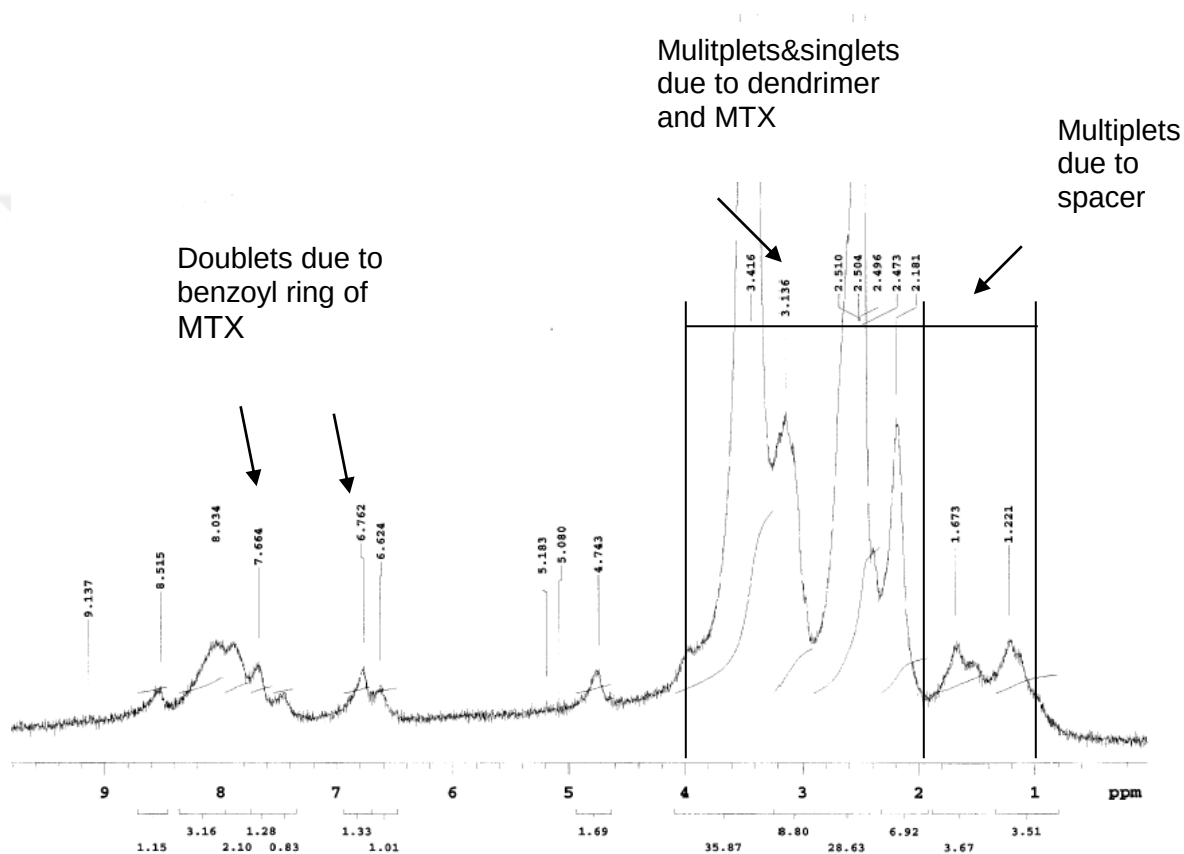


Figure 33. ¹H NMR spectrum of PAMAM-G3-NH₂-EHH-MTX

The relatively lower conjugation ratio obtained in this particular conjugate compared to the direct coupling reaction scheme despite the extension of the arms of MTX could be explained by the lower efficiency of the reaction as for the reaction to occur the ethyl group on the spacer has to fall off in the reaction catalyzed by TEA.

Further characterization of this conjugate was made with MALDI-TOF spectroscopic technique. Even though, in the other conjugates, the matrix (DHB) to conjugate ratio of 10:1 was the optimum, the analysis did not produce any significant peaks. Hence, we increased the concentration of the matrix by ten times to have a final matrix/conjugate ratio of 100.

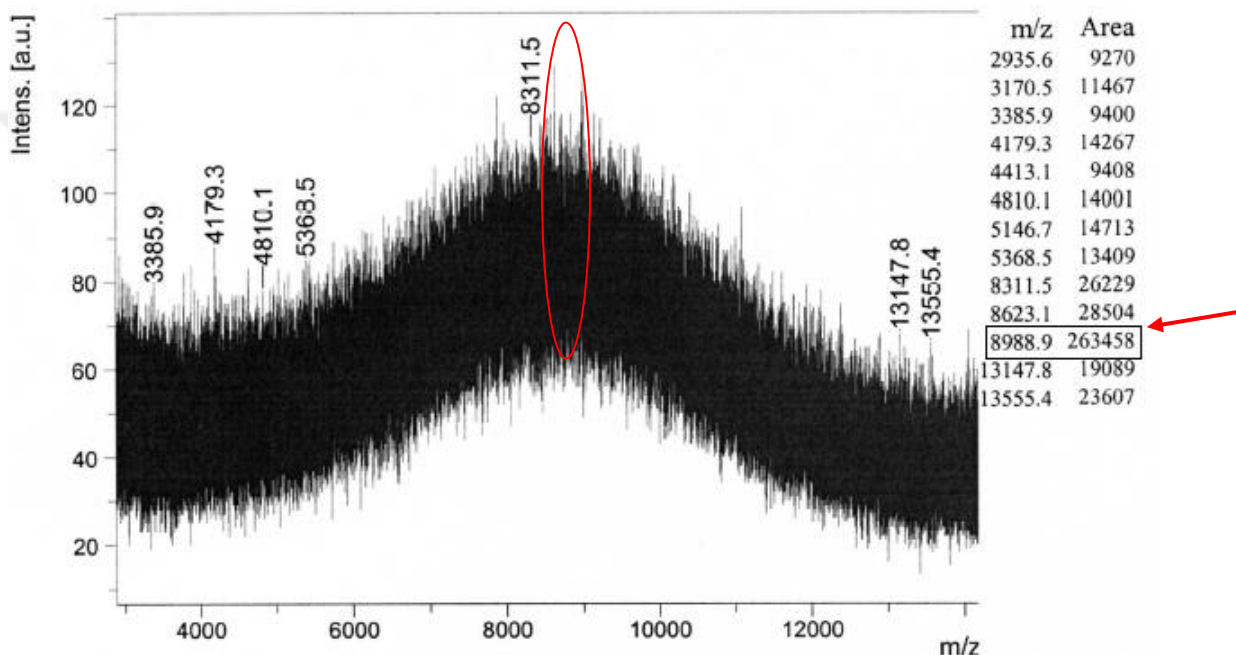


Figure 34. MALDI-TOF MS of PAMAM-G3-NH₂-EHH-MTX

Therefore, depending on the nature of the conjugate the composition of the analyte for the MALDI has to be re-optimized each time. By taking the molecular weight of MTX-EHH diester, 714, and that of dendrimer, 6909, into consideration we calculated that there would be 2.9 MTX or MTX-EHH molecules present in the conjugate, which is in agreement with the NMR data. In addition, the absence of the lower frequency peak indicates the successful removal of the MTX-EHH intermediate.

2.5.2.ii. PAMAM-G2.5-COOH-pentanol-MTX: Similarly, towards the synthesis of dendrimer-spacer-drug conjugate first we prepared protected MTX conjugate with *boc*-pentanol. The condensation of the --COOH group of MTX with the --OH group of the spacer yielded an ester bond between the drug and the spacer which could be easily broken up by the enzymatic reactions or simply by the changes in the pH of the environment as ester bonds are known to be relatively less stable compared to amide bonds. ^1H NMR spectrum of the conjugate right after the protection and removal of the *boc*-protecting group (deprotection) in the spacer which is far from the drug attachment site demonstrated that MTX has been indeed coupled with the spacer. In Figures 35 and 36 below related spectra are given for each.

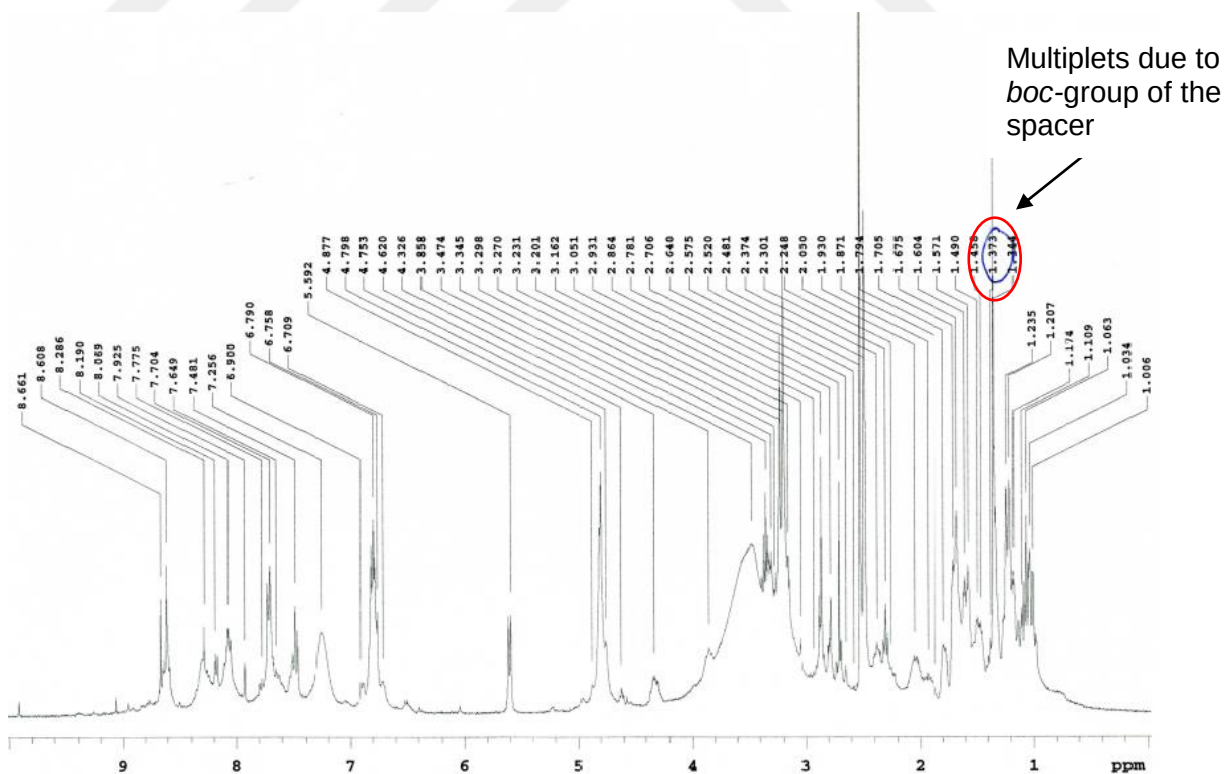


Figure 35. ^1H NMR spectrum of protected MTX-*boc*-pentanol

The peak at 1.373 ppm is attributed to the *tertiary* butyl protecting group of the spacer. Therefore, comparison of the spectrum with that of pure MTX shown in Figure 29 suggests that spacer has been successfully conjugated with the drug via an ester bond formation. On the other hand, as it can be seen in Figure 36 obtained after the deprotection of the *boc*-group with 50% (v/v) TFA in DCM, there was a total disappearance of the 1.373 ppm peak.

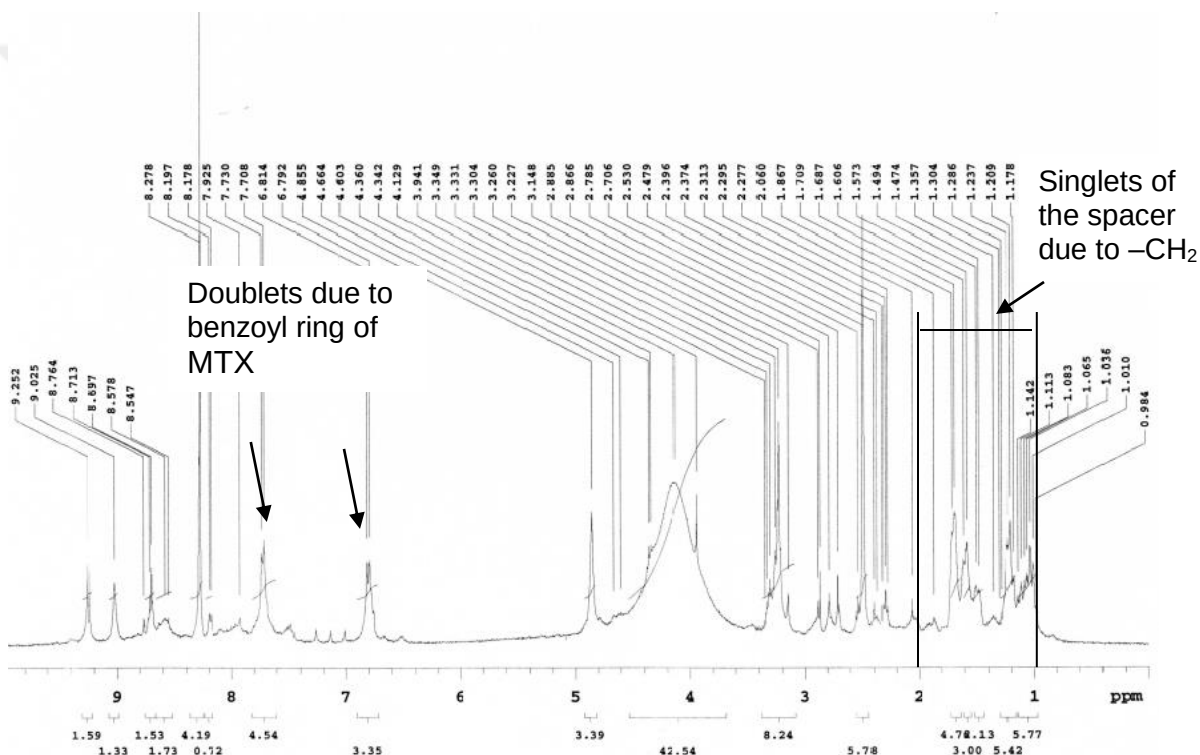


Figure 36. ^1H NMR spectrum of deprotected MTX-pentanol

To get an estimate on how many spacer molecules attached to MTX, we again used proton integration method. However, as has been observed before (93), the $-\text{NH}_2$ protons in the pteridine ring of MTX are not well resolved yet aromatic protons in the benzoyl ring at 6.8 ppm and 7.7 ppm showed the characteristics of the doublets. Therefore, calculations were made confidently accounting for 4 protons on the benzoyl ring. On the other hand, it can be seen

in the Figure 36, after the deprotection, the total count of $-\text{CH}_2$ protons of pentanol is only 10, which appeared in between 1 ppm and 2 ppm. Calculations suggested that for one MTX molecule, there is 1.1 pentanol molecule. Hence, it has been concluded that coupling of the spacer pentanol to MTX afforded monoester of the drug as opposed to diester obtained with the EHH spacer. Similarly, ESI-MS spectrum has been taken on the *boc*-protected MTX (Figure 37). Na and K adducts of the monoester products have been obtained with relatively low yields. ESI-MS (m/z): 645.2 $[\text{M} + \text{Na}]^+$ (<10%), 661.2 $[\text{M} + \text{K}]^+$ (21%), 549.1 $[\text{M}]^+$ (15%). Yet, the technique supplied additional evidence for the formation of monoester of the drug with the spacer.

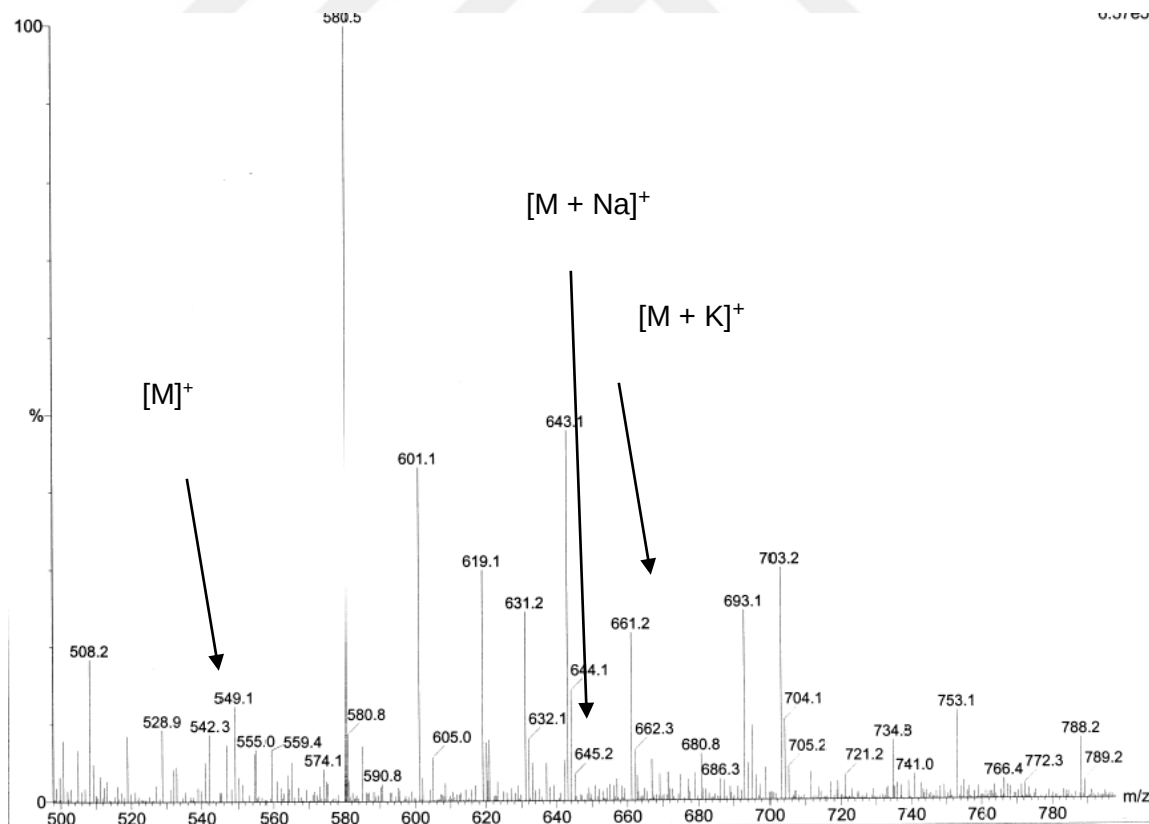


Figure 37. ESI-MS spectrum of MTX-*boc*-pentanol

After the total characterization of the intermediate MTX-pentanol we further coupled it with anionic PAMAM-G2.5-COOH dendrimer. However, as has been observed with the directly coupled MTX at the $-\text{NH}_2$ site, reaction proceeded slower and resulted in a lower drug payload.

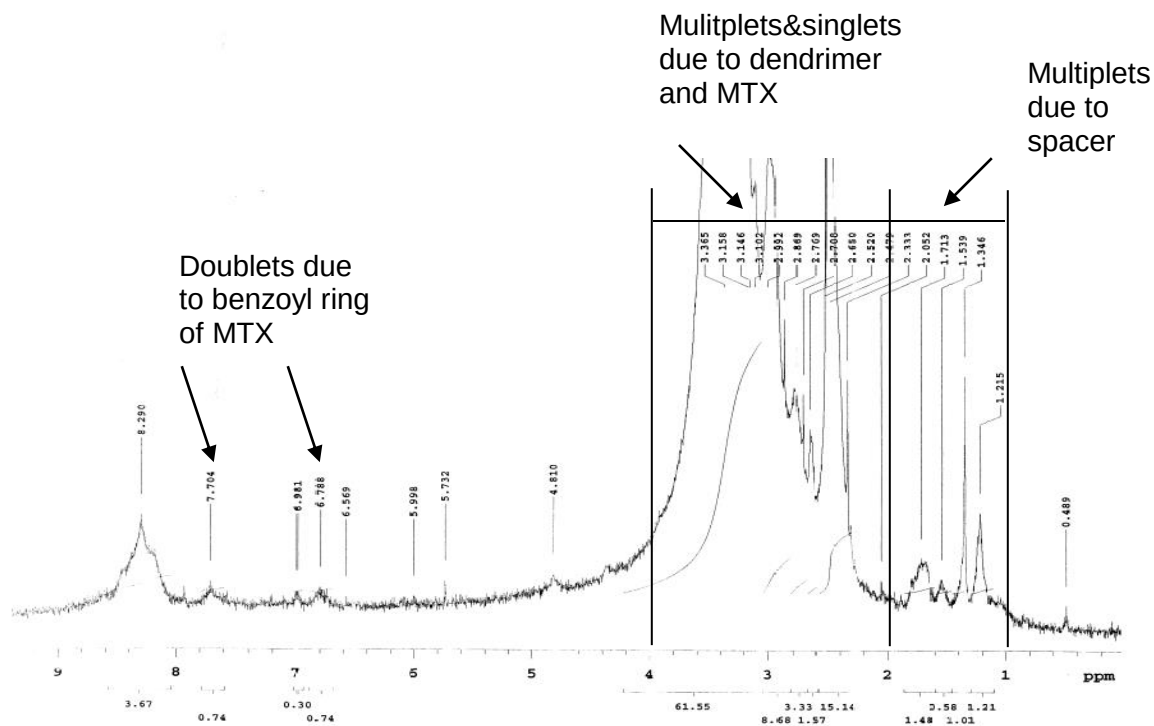


Figure 38. ^1H NMR spectrum of PAMAM-G2.5-COOH-pentanol-MTX

To estimate the drug payload on the dendrimer, we considered 10 multiplets from the $-\text{CH}_2$ of the spacer as well appearing well resolved in between 1-2 ppm. However, the $-\text{NH}$ proton of the intermediate species seems to be buried in the downfield peak at 8.29 ppm. Upon performing the proton integration calculation we found out that 1.82 MTX or MTX-pentanol molecules were attached to the $-\text{COOH}$ of the dendrimer through an amide bond. However, we could not perform a conclusive MALDI-TOF MS analysis on this particular conjugate to get further insight into the higher molecular weights created after

the conjugation. This is most probably due to the specific requirement by the conjugate for the higher concentrations of the matrix in the analyte, which could not be dissolved in the solvent mixture (50% [v/v] acetonitrile in water) at concentrations above 200 mg/ml.

2.5.3. PROTECTION OF METHOTREXATE/FOLIC ACID

As mentioned earlier, protection of the reactive groups in MTX and FA would serve means of achieving high reaction yields due to lower propensity of the cross-coupling between the same species. Furthermore, a conjugate where FA is presented at the periphery, attached to the dendrimer through MTX, would have a better association with the receptors on the target cells as suggested by Quintana et al. after extensive molecular simulations to optimize the location of the ligand (51). Schematic representation of such a conjugate with the anionic dendrimer is given as an example in Figure 39.

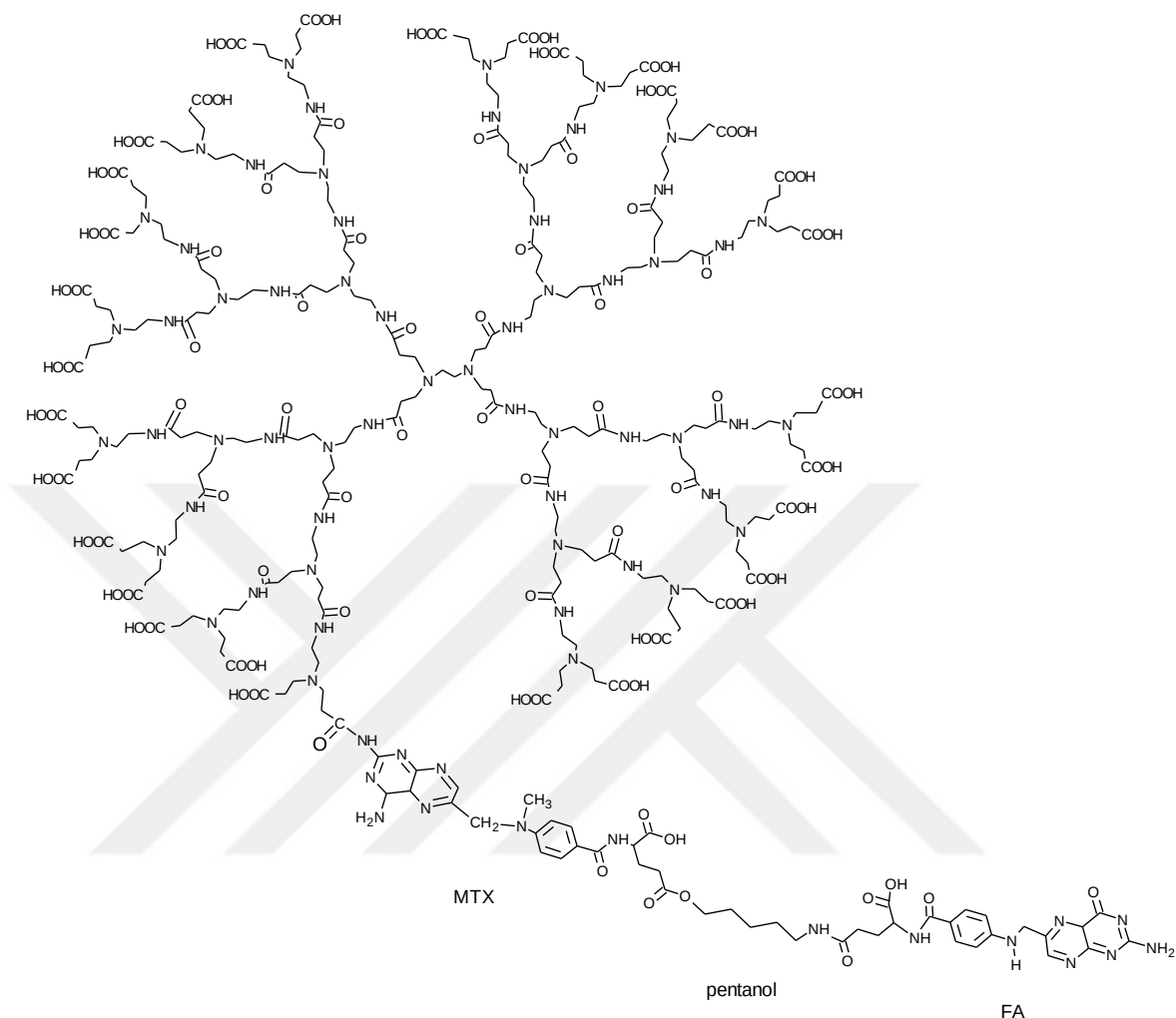


Figure 39. FA-targeted PAMAM-G2.5-COOH-pentanol-MTX conjugate

However, formation of such a construct requires rigorous protection/deprotection processes involving both MTX and FA prior to the conjugation with the dendrimer as shown in Figure 39. Towards this goal, as explained in the experimental procedures, we attempted to protect -NH_2 groups in both MTX and FA so that there will not be any crosslinking between MTX and FA since they have both -NH_2 and COOH reactive groups (Figure 40). Therefore, to allow the involvement of only the desired functional groups in the synthesis protocol, blocking the amine groups in each species is crucial. In

addition, as demonstrated earlier, MTX-pentanol construct has already been successfully synthesized. The following will summarize the findings of the second part of the synthetic protocols.



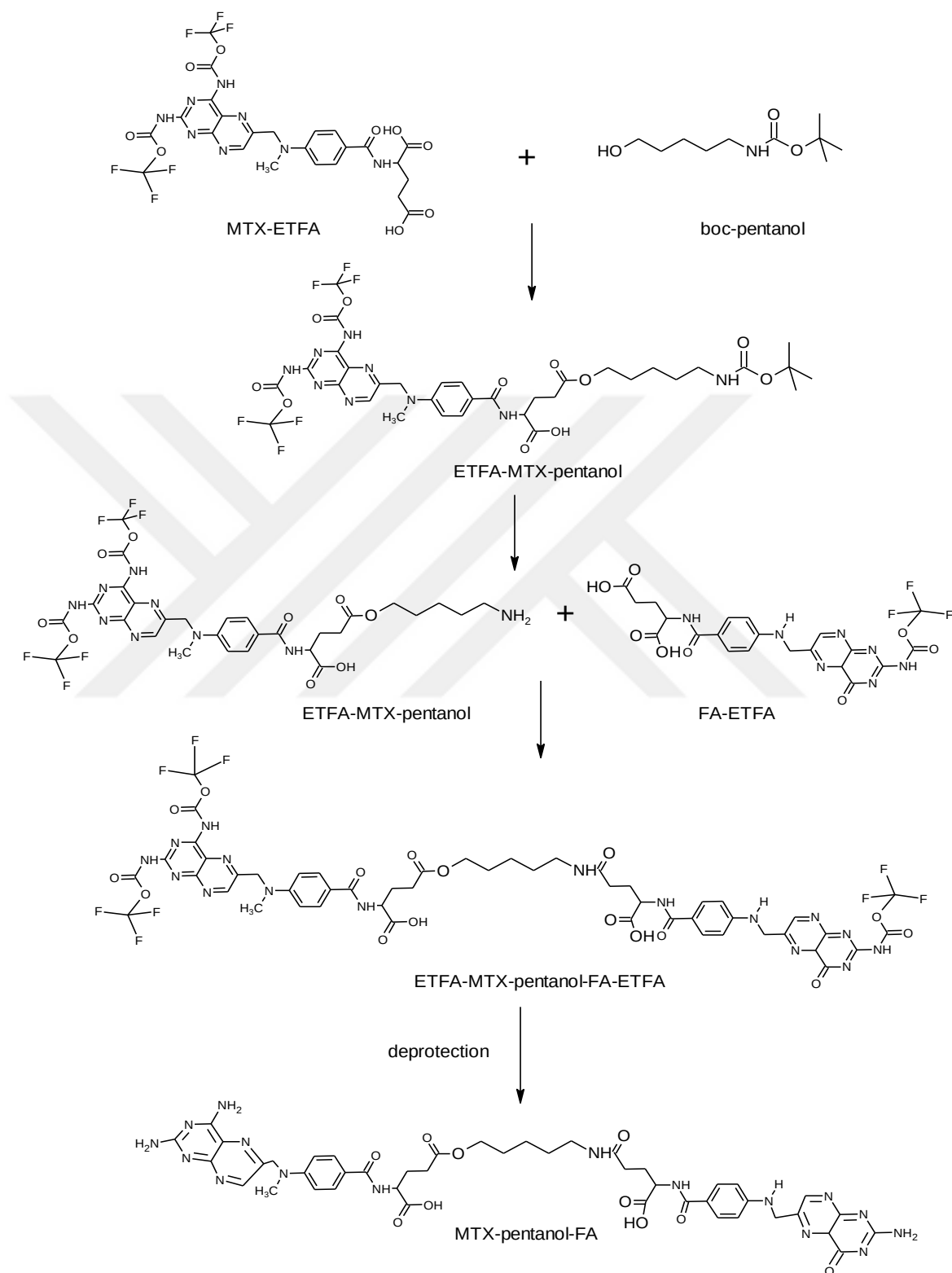


Figure 40. Synthesis of MTX-pentanol-FA in a protection/deprotection reaction scheme

2.5.3.i. Protection of MTX/FA with ETFA: Regioselective blocking of drug/ligand molecules has been studied with the help of ETFA, which can be easily cleaved in basic conditions so that the ester bond between the drug and the spacer pentanol will survive the deprotection step. ETFA was chosen since it is known to react selectively with the primary amines in the presence of secondary amines, present both in MTX and FA (Figure 40) (109). Since a similar protocol was used for both of the bioactive molecules, FA will be given as an example. The easiest method to prove the presence of $-\text{CF}_3$ groups in the new construct is to perform ^{19}F NMR which can identify newly introduced fluorine atoms to FA. The following figure shows the ^{19}F NMR spectrum for the FA-ETFA construct.

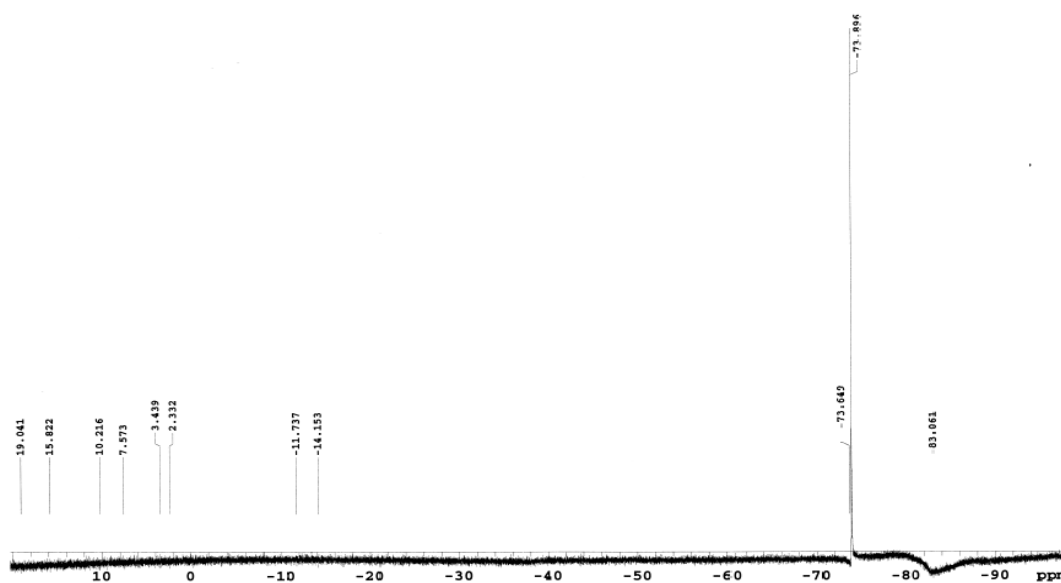


Figure 41. ^{19}F NMR of FA-ETFA

If there had been a reaction between FA and ETFA, there should have been a peak corresponding to multiplet fluorine at δ 65.13 ppm (110). However, other than sharp solvent peak at 73.89 ppm no peak was observed suggesting

that the reaction did not occur between FA and ETFA. On the other hand, due to the electron rich nature of the fluorine atom, ^{19}F NMR can be sometimes problematic as the protons are buried under a thick cloud of electrons, unlike the conditions encountered in ^1H NMR. Therefore, ESI-MS has been utilized to detect any possible higher molecular weight object, which ^{19}F NMR failed to prove due to the likely reason mentioned.

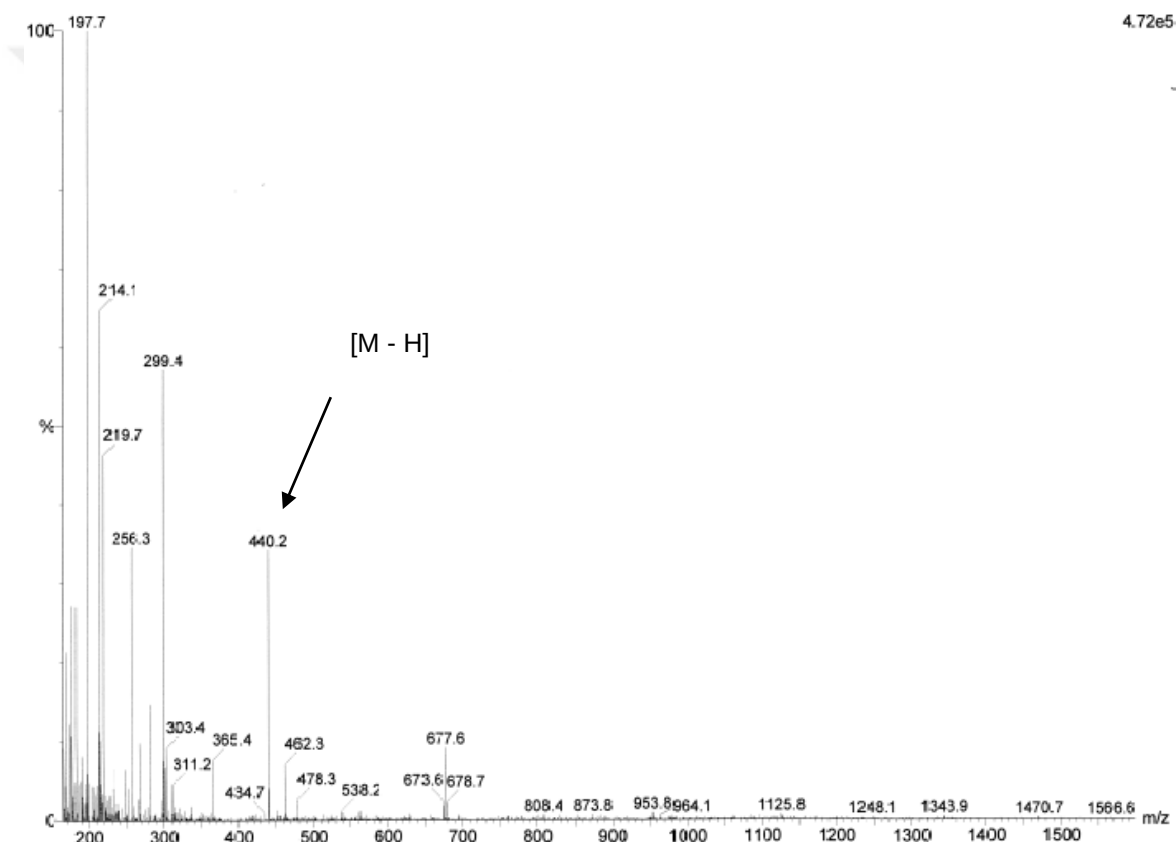


Figure 42. ESI-MS spectrum of FA-ETFA

The peaks at 440.2 [M-H] and 441.2 [M] m/z correspond to native FA molecule in the negative scan mode. The expected molecular weight for FA-ETFA was 541. The peaks at lower molecular masses represent the impurities due to unreacted ETFA and its fragments still present after the purification. However, no FA-ETFA species was observed in the spectrum, which further

revealed that the reaction did not yield the desired product. Since MTX produced a similar behavior, it was concluded that the amine group was not reactive toward the protecting group, ETFA.

2.5.3.ii. Protection of MTX/FA with *t*-*boc*: Since ETFA protection did not afford the desired protected species, alternate approaches were sought. One of these was traditional *boc* protection of the amines. In a coupling reaction under the basic conditions, we had expected to introduce the new alkyl chain of *t*-*boc* protection group into the structure of MTX/FA. In the following section, the findings for MTX-*t*-*boc* construct will be described.

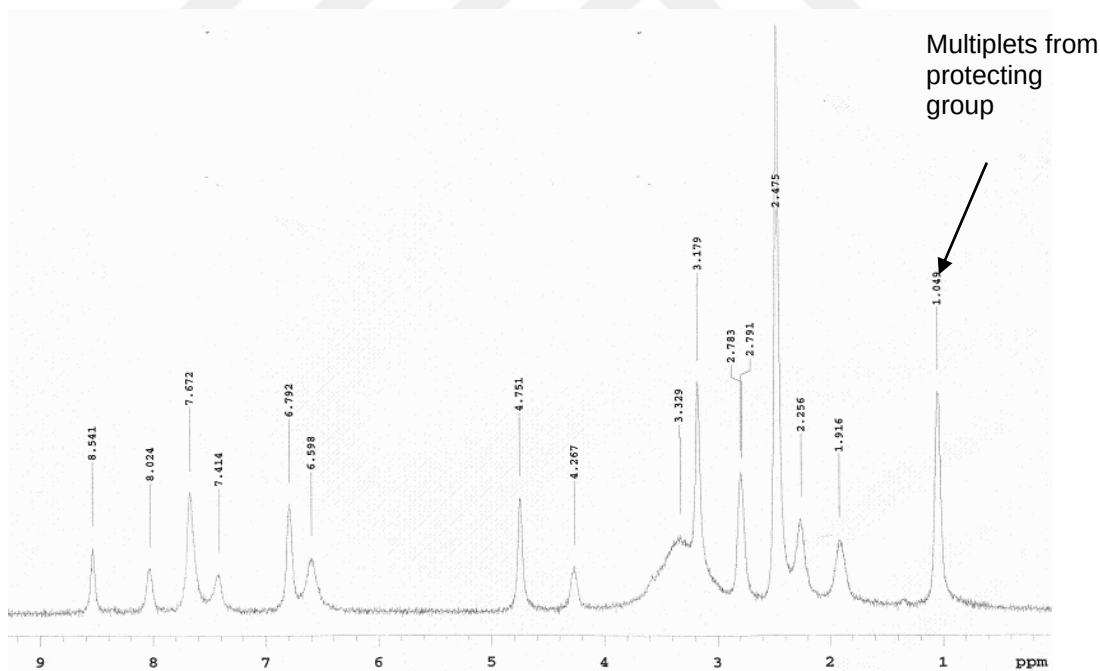


Figure 43. ¹H NMR spectrum of MTX-*t*-*boc*

The peak at 1.049 represents the multiplets from the -CH₃ group of the protecting agent. The rest of the spectrum shows all the characteristic peaks of MTX which is shown earlier in Figure 29. Therefore, ¹H NMR was suggestive of

the presence of the protecting group at the pteridiny group of MTX. Hence, we further sought to look for the molecular weight increase by ESI-MS upon the addition of the new species (Figure 44). The expected molecular weights for mono and di-substituted MTX are 554.4 and 654.4. The molecular weights were identified in the spectrum as follows: ESI-MS (m/z): 455.0 $[M + H]^+$ (100%), 456.1 $[M + 2H]^+$ (21%), 477.1 $[M + Na]^+$ (18%), 493.2 $[M + K]^+$ (15%). Even though the peak at 556.1 m/z might be due to monosubstituted MTX the intensity is too low compared to the other others. Similarly, in the higher molecular weight region there was no peak attributable to the disubstituted MTX. The use of FA in the synthetic protocol produced similar results to that of MTX. Therefore, we concluded that $-NH_2$ protection of MTX/FA with *t-boc* protection agent failed.

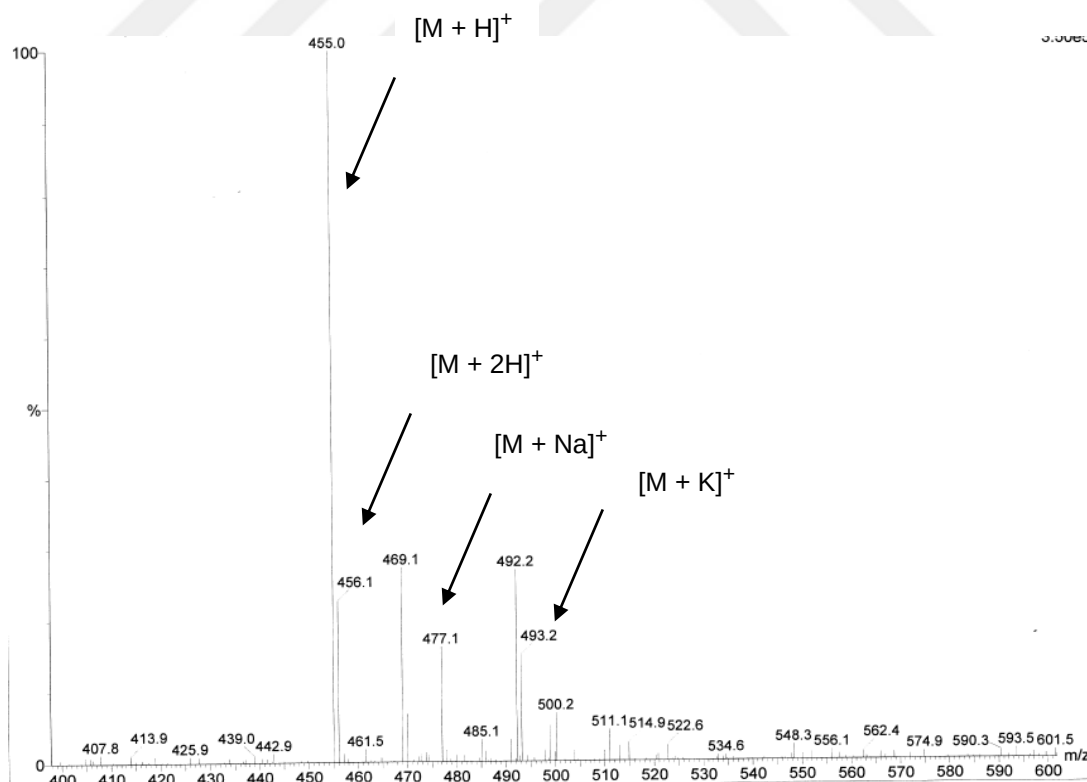


Figure 44. ESI-MS spectrum of MTX-*t-boc*

2.5.3.iii. Protection of MTX/FA with Fmoc: For the Fmoc protection of MTX/FA with a third agent, 9-fluorenmethyl-N-succinimidyl carbonate, an already established procedure for DOX was adopted (111, 112). Due to the similarities of the proton shifts of Fmoc and MTX, ^1H NMR spectrum was not conclusive. Therefore, ESI-MS analysis has been performed (Figure 45). The mono and di-substituted MTX would have molecular weights of 675.4 and 989.4, respectively. ESI-MS (m/z): 455.0 $[\text{M} + \text{H}]^+$ (57%), 493.0 $[\text{M} + \text{K}]^+$ (100%), 530.9 $[\text{M} + 2\text{K}]^+$ (78%).

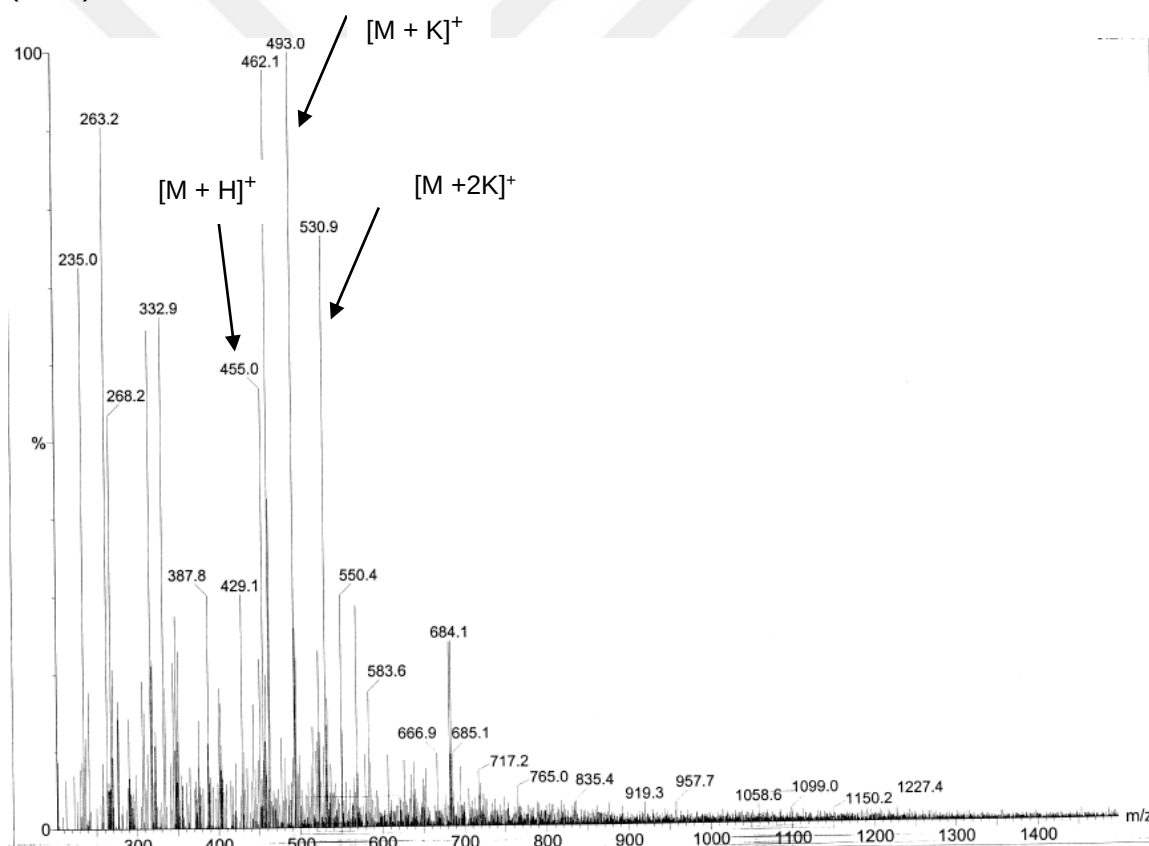


Figure 45. ESI-MS of MTX-Fmoc

Therefore, the absence of peaks between 600 and 900 m/z with the expected molecular weight was suggestive of the unsuccessful reaction protocol

to afford mono/di-substituted MTX with the protection group. Similar results have been obtained with FA as well.

Hence, we concluded that the -NH_2 groups of either MTX or FA are not reactive towards any of the three protection agents used in this report.

2.5.4. PAMAM-G2.5-COOH-FITC CONJUGATE

Conjugate of anionic dendrimer with fluorescence label FITC was performed in conventional DCC coupling reaction scheme. Characterization of this conjugation was done simply by proton integration method as explained before for directly coupled MTX conjugates. Figure 46 shows the ^1H NMR spectrum of PAMAM-G2.5-COOH-FITC conjugate taken in DMSO.

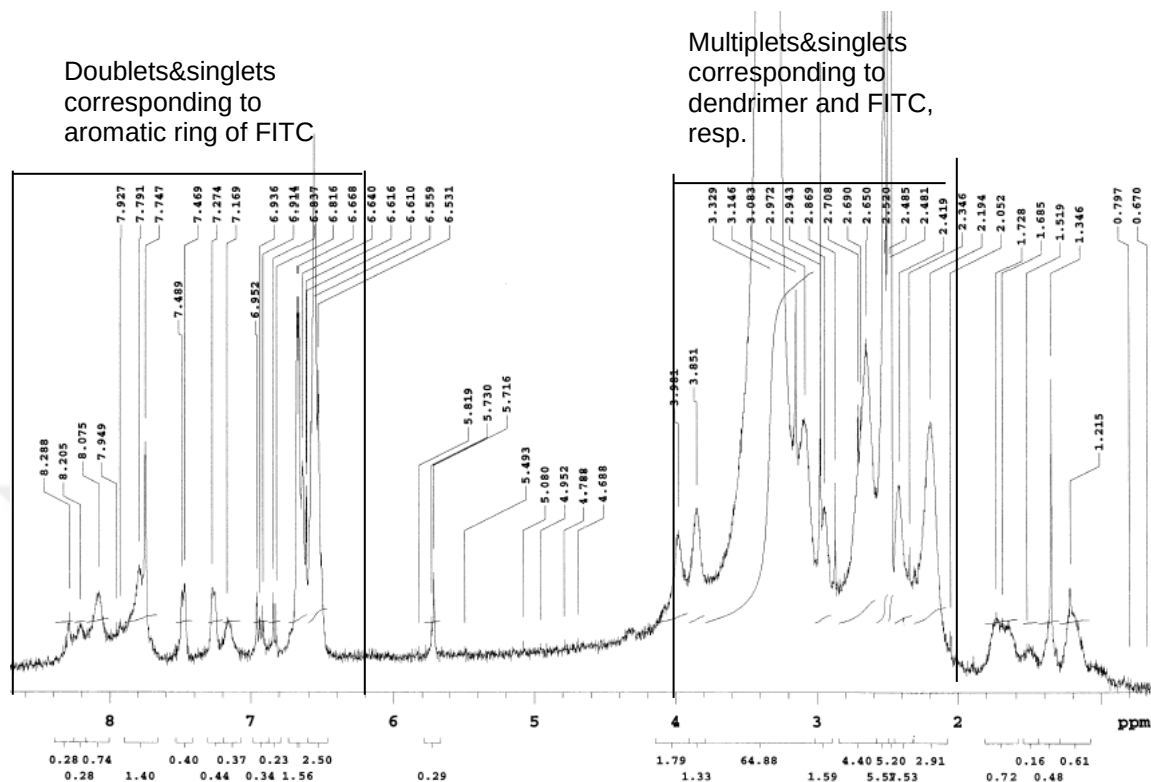


Figure 46. ^1H NMR spectrum of PAMAM-G2.5-COOH-FITC conjugate

Considering the 2 singlet protons of FITC between 2-4 ppm in addition to 356 multiplet protons of dendrimer and those at the downfield between 6-9 ppm corresponding to 9 protons of FITC, we calculated that there are 4.1 FITC molecules on each dendrimer molecule. This labeled conjugate was used in the flow cytometric measurement, which will be discussed in the next chapter.

CHAPTER 3

***IN VITRO* ACTIVITY OF PAMAM-METHOTREXATE CONJUGATES**

We tested directly linked PAMAM-MTX conjugates in terms of their growth inhibitory activities on various cell lines. This chapter deals with the *in vitro* evaluation of the conjugates and understanding of the action mechanism of the conjugated drug. In addition, the cellular entry profile of the anionic dendrimer will be demonstrated. First a brief discussion will be given on the pharmacology of MTX followed by the experimental work.

3.1. CELLULAR ACTION OF METHOTREXATE

As mentioned in section 1.1.1, various agents are used for the treatment of neoplastic diseases. Among those, folate antagonists are the first chemotherapeutic drugs. The classical folate antagonist aminopterin was first synthesized by Seeger et al. in 1947 and Farber et al. used it clinically in 1948 for remissions in childhood acute lymphoblastoid leukemia (ALL). However, severe systemic toxicity of the drug prompted the search for analogs with milder toxicity. One year later, Smith (American Cynamid Company) synthesized methotrexate (MTX) and soon it replaced aminopterin in clinical trials on account for its milder toxicity (113). As an antimetabolite, MTX (4-amino-4-deoxy-methylpteroylglutamic acid) shares a similar structure with folic acid, except for the 4-amino group in the pteridine ring and the methyl group at the N¹⁰ position.

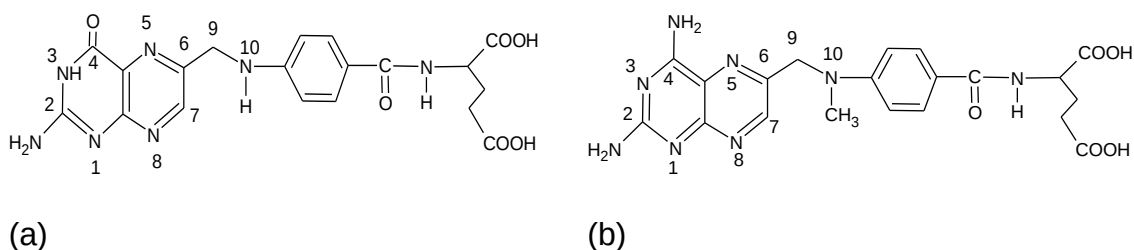


Figure 47. Molecular structures of FA (a) and MTX (b)

Therefore, the active site of the molecule appears to be pteridine ring portion via the interaction between the Asp27 site of the target DHFR enzyme and pteridine N¹ and N² positions upon its 180° rotational conformation (114, 115).

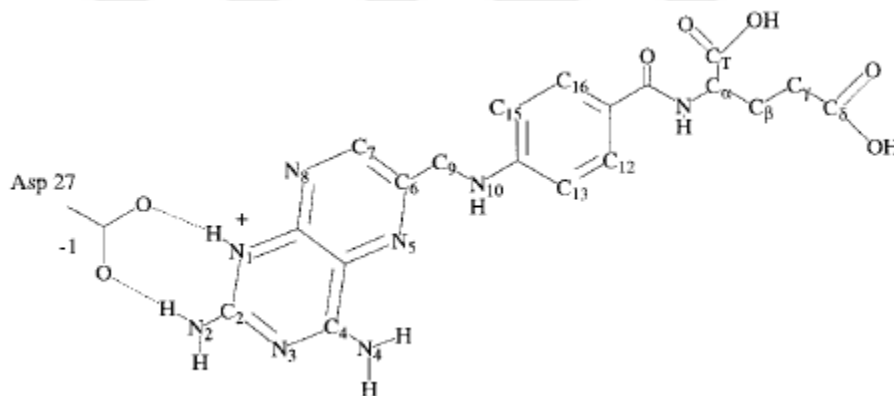


Figure 48. Binding of MTX to the target enzyme DHFR through interaction with enzyme active site [Adapted from (114)]

MTX is a widely used chemotherapeutic agent for the several neoplastic diseases such as non-Hodgkin's lymphoma, leukemia, choriocarcinoma, head and neck cancer, and breast cancer as well as for some autoimmune diseases like rheumatoid arthritis and psoriasis (116). In addition, it is exploited for the prevention of rejection in organ transplantations. Because of the high polarity of natural folates at neutral pH of biological fluids, i.e. two negatively charged

carboxyl groups in the glutamate moiety, they are highly hydrophilic and hardly cross the cellular membrane by passive diffusion (117, 118). Therefore, folates are transported into the cells by specific and high-affinity membrane transporters. In this respect, transport of folate compounds takes place by two different energy-dependent mechanisms occurring via folate receptor (FR) and reduced folate carrier (RFC). The first mechanism involves membrane associated folate-binding protein with nanomolar affinities, K_d for folic acid=1 nM and K_d for MTX=100 nM (119). The folate receptor (FR) is a 38000 Da glycosylphosphatidylinositol-linked (GPI) membrane protein and there are three forms of receptor subtypes in humans: membrane-anchored α - and β -subtypes with very high affinities for folic acid (~ 1 nM), and secretory form, γ -subtype (117, 120). According to the hypothesis, folates bind to GPI-anchored FR, which in turn moves into caveolae followed by the sealing of the mouths of the vesicle transiently. Then, vesicle is acidified thereby leading to the dissociation of FR-folate followed by the transport of the released folate into the cytoplasm while FR is recycled to the cell surface. This process is termed "potocytosis" (121). However, MTX is a poor substrate for FR as seen in the K_d value. Furthermore, the recycling rate of FR is only half of that of RFC. Therefore, FR can participate in the transport of the drug only when RFC function is very low or the drug has a relatively higher affinity for it (117). On the other hand, recently there has been a new argument about the triggering mechanisms of localization of GPI-anchored proteins to caveolae. It was suggested that the proteins can cluster into caveolae

selectively only after crosslinking. This has been shown with cross-linked FR- α localizing to caveolae (122).

The second route involves transmembrane RFC capable of transporting reduced folate analogues such as leucovorin and MTX with similar efficiencies. This carrier is not directly related to energy metabolism and even though it has a high affinity for the reduced folates, it poorly transports oxidized folates such as folic acid (123). Intracellular transport of MTX by RFC occurs in association with an anion gradient acting as a co-factor for this carrier. However, transport of MTX proceeds much below its maximal capacity in the presence of reduced folates at physiological conditions, 5-50 nM (124). RFC-mediated uptake shows saturable behavior at the concentrations of 1-5 μ M and is inhibited by anions (116, 125). Other than these two mechanisms, at high concentrations, MTX uses passive diffusion, which is the most important route for it in cells that do not have active transporters (126).

The main target of MTX is dihydrofolate-reductase (DHFR) enzyme, an intracellular enzyme, which reduces folic acid to tetrahydrofolate cofactors, which in turn, serve as key intermediates in several vital biochemical pathways such as de-novo biosynthesis of purines and thymidilate. Lack of reduced folates in addition to purines and thymine in actively proliferating cells like tumor cells leads to inhibition of DNA and RNA synthesis and eventual cell death (117). MTX is converted to polyglutamate forms by binding of two to five polyglutamate groups catalyzed by folylpolyglutamate synthetase (FPGS) prolonging the retention of polyglutamate derivatives in cells since they are not the substrates

for folate transport systems (117). The build up of polyglutamates in the cells blocks the intracellular target in a sustained manner contributing to the antineoplastic activity of the drug appreciably even after the extracellular antifolate concentration falls below their therapeutically effective level (116). These polyglutamate species also have significantly increased affinities for other folate-dependent-enzymes such as thymidylate synthase (TS) (117).

Since MTX inhibits a key cellular function, it is very cytotoxic especially against replicating cells. Therefore, it exerts activity not only towards neoplastic cells but also bone marrow. It also causes inflammation of upper gastrointestinal tract (mucositis) and severe gastrointestinal disturbance particularly in patients going under high dose therapy.

There are several reasons for the cells to pose resistance towards MTX:

1. Increased expression of DHFR: It has been shown that under physiological conditions, only 5% of the enzyme activity is required to meet the requirements for tetrahydrofolates (THF) (127). To achieve the same extent of enzyme inhibition in the cells with increased levels of DHFR calls for very high drug concentrations. Considering the severe limitation on concentrative transport of the drug, it is very difficult to meet this requirement.
2. Decreased affinity of DHFR for MTX: Alteration of the enzyme due to mutations decreasing K_i value for MTX preserves activity towards dihydrofolate (DHF) contributing to drug resistance.

3. Impaired membrane transport: RFC transport mechanism regulating the influx of the drug has a critical impact on the net level of free drug intracellularly. Alterations in membrane transport are a most common reason for drug resistance as has been shown (118, 128, 129).
4. Low polyglutamylation: This could be due to alterations in the expression of FPGS resulting in the lower levels of intracellular MTX polyglutamates.

Additionally, it has been demonstrated that multidrug resistance-associated protein (MRP) family an ATP-dependent folate export mechanism is responsible for the efflux of anionic drugs like MTX. Four MRPs have been identified in cell cultures showing resistance to the drug. However, since MRPs interact with the substrate at the cytosol-membrane boundary, they have a little effect on unidirectional drug influx. Apart from MRP, another efflux mechanism, the P-glycoprotein (Pgp) transporter, renders the cell resistance to the hydrophobic drugs. Pgp can recognize, capture, and eject the drugs as they travel across the cellular membrane before reaching the cytosol in contrast to MRPs. However, MTX is not considered as a Pgp substrate owing to its hydrophilicity and carrier-mediated transport, thus escaping from the interaction with the multidrug resistance (MDR) family at the cellular membrane. On the other hand, as mentioned before at high concentrations, MTX uses passive diffusion as a transport route so that the multidrug resistance family of proteins may confer resistance (117). Furthermore, recently the group led by Schneider

made extensive studies to investigate the role of another ATP-dependent efflux pump called breast cancer resistance protein (BCRP). It has been suggested that BCRPs can efflux not only MTX but also MTX-polyglutamates with decreasing affinity as glutamate chain increases (130, 131).

3.2. MACROMOLECULAR DELIVERY OF METHOTREXATE

MTX has been coupled with a variety of carriers from natural macromolecules such as albumin and dextran to synthetic polymers such as dendrimers and hydrogel. The versatility of dendrimers in regard to prodrugs of anticancer drugs including MTX has been discussed in section 1.6 (50, 51, 53). This chapter will give some examples to studies conducted on several other carriers.

Conjugates of MTX with synthetic molecules: As a water-soluble and biocompatible polymer PEG of MW 750 to 40000 has been conjugated with MTX (97). Formation of amide bond between the drug and the carrier led to a stable conjugate which did not release the free drug in either the cell conditioned medium or cell-free buffer resulting in 60-700 times lower *in vitro* activity of the drug in the conjugated form. Surprisingly, in cell-free DHFR inhibition activity assays, all the conjugates inhibited the activity of the target enzyme to a similar extent to free drug, which was the most active form. Therefore, it has been suggested that even the conjugated drug could be active without being released from the carrier (97).

Differential cytotoxic activities of free MTX and MTX conjugated to either poly-L-lysine (PLL) were evaluated against WI-L2 human lymphocytes, marrow and human tumor cells (132). Interestingly, conjugate formed between PLL and MTX showed more inhibitory activity against cell lines derived from solid human tumors than those obtained from human lymphocytes with approximately 10 times less IC_{50} value. The selective toxicity of MTX-PLL toward the human solid tumor cells derived either from the increased uptake of the analog or from the ability of these cells to convert MTX-PLL at relatively low concentrations either to free MTX or to an MTX derivative able to inhibit DHFR. In fact, the uptake of the conjugate by the T24A astrocytoma cells was 3-4 times higher than uptake by WI-L2 lymphocytes. Yet, this conjugate still required 2 to 40 times higher drug concentrations for 50% growth inhibition compared to the free drug. The cytotoxicity of MTX conjugated especially with PLL 60K on tumor cells was significantly decreased after the treatment with lysosomal enzyme inhibitors suggesting that proteolytic enzymes may play a critical role for the activity of the conjugate.

Recently, colloidal formulation of MTX for oral administration was performed by Moutardier et al. (133). The use of liposomes with a polymerized core rendered them less sensitive to external factors such as salts, temperature, and enzymes found in biological compartments. However, encapsulation of the drug inside the polymerized core liposome reduced the permeability by 4-fold in Caco-2 cell. On the other hand, the TC7 subclone of the wild type Caco-2 cells accumulated the encapsulated and free drug to comparable levels.

Biodegradable hydrogel preparations of MTX was made with the use of poly(ethyleneoxide)-block-poly(2-hydroxyethyl aspartamide) (PEO-b-PHAA) copolymers (134). Li and Kwon successfully attached MTX to PEO-b-PHAA at a substitution ratio of 20-45%. Attachment of water-insoluble MTX to poly(L-amino acid (PLAA) block as a hydrophobic chain of PEO-PLAA imparted amphilocity to the diblock copolymer and made the micelle self-assemble, which resulted in the stabilization of ester bonds in the nonpolar core of the micelles. This was suggested as the reason to high stability of the ester bond to hydrolysis at neutral and acidic pH 5 and 2.2, even after 65 h, releasing only about 20% of the drug in the latter case. Once the supramolecular core/shell structure of the micelles is disrupted by SDS, the ester bond is exposed to water and an eventual faster release was attained which was approximately 35% after 249 h. Similar release profiles were obtained by Kang et al. on self aggregates of MTX-poly(2-hydroxyethyl aspartamide) copolymer with pH-insensitive hydrolysis of the ester bond where the release at neutral and acidic pH values were quite similar (100).

Conjugates of MTX with natural macromolecules: Fibrinogen is a macromolecule that has a property of accumulating in tumors due to the likely relation between tumor invasion and the hemostatic system. Therefore, Boratynski et al. were encouraged to study role of fibrinogen as a carrier for MTX to improve the efficacy of the drug. The *in vitro* conjugate showed much less activity than free drug. Furthermore, the conjugate demonstrated lethal toxicity even at approximately 5 times lower MTX dosages compared to free MTX,

attributed to the accumulation of the conjugate in the spleen and liver. Therefore, a more elaborate design is necessitated (135).

With the earlier findings demonstrating the over-expression of insulin receptors on various malignant tumor cells, Ou et al. aimed to deliver MTX to hepatoma cells via insulin receptor-mediated endocytosis using insulin both as a carrier and targeting ligand (136). The MTX-insulin conjugate at a molar ratio of 2: 1 competed with free insulin for the binding sites in a concentration-dependent manner. Binding of 5 nM ^{125}I -insulin was reduced to 50% when co-incubated with ~ 100 nM conjugate. Furthermore, the *in vitro* MTX–insulin conjugate selectively targeted human BEL 7402 hepatoma cells over HL 7702 hepatocytes at 10-times higher ratios.

Overexpression of albumin binding proteins on human tumor cells such as a lymphoma cell line and those originating from breast, stomach, and leukemia motivated researchers to use albumin as drug carrier (137). Several groups conjugated MTX to human serum albumin (HSA), which even made it to the clinical trials. One advantage of using HSA is the fact that the plasma half-life of the conjugate is as long as that of native albumin, which was reported as 16-19 days, quite high compared to free drug. Hence, prolonged exposure to the drug can be achieved. Burger et al. reported superior *in vivo* activity of MTX-HSA to free MTX in three human tumor xenografts in nude mice developed from soft tissue sarcoma, prostate cancer, and osteosarcoma (138). The most encouraging effect was observed in the soft tissue sarcoma SXF 1301 where MTX-HSA caused complete remission after only a single injection whereas free

MTX led to short-lasting partial tumor regression. However, in the lung, bladder, and breast cancers no activity was observed with either forms of the drug.

One other group demonstrated the importance of the loading ratio in terms of targetability to the tumor. Stehle et al. reported that only the conjugates with a loading ratio less than 3 accumulated in the tumor tissue while the ones having more drug molecules were immunogenic and were cleared from the circulation by the macrophage system (139). In a similar conjugate system, Weigand et al. compared the activity of free drug and MTX-HSA conjugate with a payload of 5.7 on CCRF-CEM and its transport resistant phenotype CCRF-CEM/MTX cells (137). The conjugate showed both concentration and time-dependent toxicity profile. On CCRF-CEM cells after 96 hrs of exposure to 5 μ M, 10 μ M and 20 μ M of HSA-MTX caused 12%, 32%, and 81% cell death. On the other hand, at the two highest concentrations of the conjugate, CCRF-CEM/MTX cells showed responses to the conjugated drug up to 30-40% of the untreated control. It is suggested that albumin binding sites will be involved in the shuttling of the conjugate to the tumor cells as suggested earlier by Torres (140). In addition, high amounts of liberated MTX with lower polyglutamates is an indication of the different uptake mechanism of MTX-HSA and entry of MTX into the cytoplasm from the lysosomal compartment without accessing to the FPGS which would otherwise easily polyglutamate the drug internalized via RFC due to close association of the folylpolyglutamate synthetase with the plasma membrane or a channeling of the substrate from carrier to the enzyme. Wosikowski et al. supported the previous findings by investigating the uptake

kinetics of the conjugate in KB cells (102). It has been showed that [^3H]MTX and [^3H]MTX-HSA showed different uptake kinetics. However, when the cells were treated with ^{125}I -HSA and ^{125}I -MTX-HSA similar uptake kinetics was obtained suggesting that the conjugate enters the cells by albumin-mediated endocytosis facilitated by albumin binding proteins. In addition, the cytotoxic activity observed on the cells was circumvented with the use of lysosomal inhibitor methylamine, pointing out that growth inhibitory activity of the conjugate is dependent on the lysosomal processes. However, *in vitro* growth inhibitory activity of the conjugated MTX was approximately three orders of magnitude lower than the free drug as tested on 13 different human leukemia cell lines. On the other hand, conjugate accumulated in tumor tissue to yield local concentrations between 18 and 29 μM , sufficient enough to induce antiproliferative effect.

MTX-HSA conjugate has gone under Phase I and II clinical trials (141, 142). In a phase I trial of MTX-HSA to determine dose-limiting toxicity (DLT) and maximum tolerated dose (MTD) in a weekly regimen, 17 cancer patients who are not amenable to any other standard treatment were enrolled (141). Weekly injections of 20, 40, 50, and 60 mg/m^2 MTX –HSA were performed up to 8 weeks. Mainly stomatitis as DLT occurred; beginning at the dose of 50 mg/m^2 and in one case thrombocytopenia was observed. No significant leucopenia, nausea, or renal toxicity was observed, showing well tolerability of MTX-HSA. Additionally partial/minor tumor responses were seen in three different patients with renal carcinoma, pleural mesothelioma, and one another patient with renal cell carcinoma.

In a phase II clinical study with 17 patients with metastatic renal cell carcinoma who progressed after previous immunotherapy were enrolled in the study (142). It has been found that toxicity was manageable from mild to moderate. Ten patients out of 17 had grade 2/3 mucositis and only in one patient grade 4 thrombocytopenia was reported. However, no objective responses were observed in RCC patients. It is suggested that since the regimen is safely well tolerated, different investigational settings with different patient groups with less-advanced stages of diseases may be further studied.

Kim et al. reported the efficient liver and lung targeting ability of microspheres of different sizes containing MTX-HSA conjugate. The lungs and the liver contained highest amount of either free MTX or conjugated-MTX when male ICR mice were injected i.v. (143).

As has been observed in almost all prodrug systems, it is crucial that carrier releases the drug. Therefore, Chau et al. synthesized prodrugs of MTX with another natural macromolecule, dextran with different peptide sequences to tune the drug release upon the cleavage by matrix metalloproteinase (MMP) II and IX, which are involved in the promotion of tumor cell growth, upregulation of angiogenesis, and triggering tumor cell motility (144). Release of MTX from the peptide linker ProValGlyLeulleGly, which is susceptible to MMP, was compared with those without any linker and with the scrambled peptide linker, which is no longer recognized by the enzyme. Proper arrangement of the amino acids in the first peptide linker allowed the conjugate to release up to 68% of the drug at the end of 1 h which increased up to 90% after 24 h. On the other hand, conjugates

without any linker or with the scrambled linker did not release the drug to any appreciable extent. This proved the importance of the amino acid sequence in the linker for higher drug liberation. At the same time, the incubation of the conjugate either with the human fibroblast HT-1080 cells expressing the MMPs or with those lacking of the target enzymes, BT-20 human breast cancer cells, confirmed the specificity of the linker. In the former model, 14% cleavage was observed.

Hudecz et al. investigated the effect of structural features of branched polypeptides on the activity and biodistribution of MTX conjugates (145). The average molar substitution ratio changed from 5 to 72 depending on the molar mass of the peptide. Conjugation of MTX to amphoteric carrier resulted in a significant reduction in its cytotoxicity in 791T osteogenic sarcoma cells, ~2 orders of magnitude less active than free MTX. On the other hand, conjugation with polycationic carrier produced a compound with cytotoxicity only 60 times less potent than free MTX, relatively less hampered activity was attributed to the presence of Leu amino acid as the chain termini perhaps due to decreased steric hindrance posed by the peptide chain to the enzyme. On the other hand, body distribution of the conjugates was found to be similar, accumulating predominantly in the spleen and liver.

Impregnation of MTX onto activated carbon particles was envisaged as improving its antitumor effectiveness in BALB/c nude mice with human colon carcinoma xenografts LoVo (146). The mean rate of changes in the tumor volume in MTX-CH treated groups was the lowest among all after a 28 day

treatment period which was 192.6% whereas it was found to be over 400% in the MTX treated groups. Additionally, the mean MTX concentration in the tumors of MTX-CH treated groups was 100.82 nmol/g at 6 h after administration in comparison to 5.74 nmol/g in MTX treated group after 48 h. Hence, intratumoral administration of MTX-CH is found to be superior to MTX in terms of tumor growth suppression.

3.3. EXPERIMENTAL WORK

Significance of the work: The directly coupled conjugates of MTX with both anionic and cationic dendrimers, denoted as conjugate A and conjugate B, respectively, have been evaluated in terms of cytotoxicity against four different cell lines, two drug-sensitive and two drug-resistant cell lines. Even though there is some data available for the *in vitro* performance of the dendrimer-drug conjugates, they have not been tested on both sensitive and resistant-cell lines side by side. Furthermore, as mentioned before, with the use of two different dendritic carriers we were able to assess the effect of the net surface charge of the conjugate imparted by the dendrimer. The exploitation of rescuing strategies for circumventing the growth inhibitory effects of the conjugated-MTX to understand the mechanism gives uniqueness to the study.

3.4. MATERIALS

Methotrexate (4-amino-10-methylpteroylglutamic acid) (MTX) and leucovorin ((6R,S)5-formyl tetrahydrofolate) were obtained from National Cancer Institute (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis). Polyamidoamine (PAMAM) dendrimers (generation 2.5 with -COOH end groups and generation 3 with -NH₂ end groups) were purchased from Sigma Aldrich Chemical Company and Dendritech, MI. Other reagents were obtained from assorted vendors in the highest quality available, and include RPMI 1640 culture medium (Invitrogen), penicillin and streptomycin (Invitrogen), dialyzed fetal bovine serum (Invitrogen), CellTiter Blue cell viability kit (Invitrogen), α -MEM culture medium (Mediatech), Hank's balanced salt solution (Sigma), Dulbecco's phosphate buffered saline (PBS) (Sigma), Adenosine (Sigma), Thymidine (Sigma), iron supplemented defined bovine calf serum (HyClone), dihydrofolate reductase (DHFR) (Sigma), dihydrofolic acid (DHF) (Sigma), and β -nicotinamide adenosine dinucleotide phosphate, reduced form (β -NADPH) (Sigma). All the solvents used were purchased from Fisher Scientific.

3.5. *IN VITRO* CHARACTERIZATION

3.5.1. CELL CULTURES

The human lymphoblastoid leukemia wild-type CCRF-CEM cell line and its MTX-resistant counterpart CEM/MTX were maintained in RPMI 1640 containing iron supplemented fetal calf serum (10%) (Hyclone), glutamine (2mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37 °C in 5% CO₂ atmosphere. CEM/MTX cells were selected from the wild-type CCRF-CEM cells after continuous incubation with 500 nM MTX (128). In a second set of experiments, Chinese hamster ovary (CHO) cells were used including MTX-resistant MTXRIIOua^{R2-4}, designated RII, and RII cells transfected with the human RFC, denoted as PC43/10 (147). CHO cells were grown in similar conditions as described before except for the RPMI 1640 medium, which was replaced with α-MEM medium. All the other conditions were kept the same.

3.5.2. *IN VITRO* CYTOTOXICITY DETERMINATION

In the first set of *in vitro* cytotoxicity tests, both MTX-sensitive, CCRF-CEM, and MTX-resistant, CEM/MTX, cells were treated with either free MTX or with dendrimer-conjugated MTX. Cell viability was assessed directly by microscope counting on a hemacytometer. In each set, cells were seeded in 24-well plates at $1.5 \times 10^5/2$ ml/well in complete RPMI medium including 10% dialyzed fetal bovine serum and incubated for 96 h with serial dilutions of either

form of the drug. All experiments were done at least in duplicate. Additionally, both of the free dendrimers were tested in the cell lines to determine the likelihood of the nonspecific toxicity due to the carrier. The concentrations of the dendrimers used correspond to that present in the conjugate at that particular MTX concentration.

In the second set of experiments, a fluorimetric assay was used for measuring sensitiveness of the CHO cells to the two forms of the drug, namely free and conjugated MTX. Cells were plated in duplicate at 4000 cells/200 μ l/well in a 96-well culture plate in complete RPMI 1640 medium containing dialyzed serum and serial dilutions of either free MTX or conjugated MTX. Cells were grown in the incubation medium for 96 h and cell viabilities were determined with the CellTiter Blue assay kit. At the end of 96 h, medium was removed and cells were washed out with 100 μ l of Hank's balanced salt solution. Removal of the salt solution was followed by the addition of 100 μ l phosphate buffered saline (PBS). Cells were then incubated with 20 μ l of CellTiter Blue assay for 4 h. Incubation of the cells with resazurin led to the conversion into a fluorescent end product, resorufin, by the metabolically active viable cells. The number of viable cells in each well was determined by measuring the fluorescence at 560 nm (ex) and 590 nm (em) on a 96-well fluorescent plate reader. Experiments were performed three times. Cell viability is expressed as the percentage of that obtained for untreated control cells in parallel.

For understanding of the mechanistic aspects of the cytotoxicity of the conjugates, in additional experiments, cells were coincubated with nucleoside

protection agents (a combination of 60 μ M adenosine and 10 μ M thymidine) along with either free MTX or conjugated MTX in order to confirm the antifolate activities of the conjugates.

In all the experiments, the amount of MTX in the dendrimer-MTX conjugates was determined from the conjugation ratios estimated by ^1H NMR. The concentrations of conjugates in the incubations were adjusted to maintain equivalent molar amounts of MTX to that used in the runs for the free MTX.

For the determination of IC_{50} values for each species in the activity plots, SigmaPlot 2000 software was used to draw the best fitting curve to each individual data point. The selection of the type of curve depends upon only how well each data point falls into the fitted curve. No specific equation or whatsoever was used in this regard.

3.5.3. CELLULAR ENTRY OF FITC-LABELED DENDRIMER BY FLOW CYTOMETRY

RII-CHO cells grown in 60x15 mm culture dish in log phase with a density of 100000 cells/ml were washed with 5 ml of PBS and treated with 2 ml serum free RPMI 1640 medium. This was followed by the addition of 20 μ l of FITC-attached PAMAM-G2.5-COOH dendrimer solution to obtain a final concentration of 10 μ g/ml in the treatment medium. Cells were treated for various time periods from 0-4 hrs. At the end of 4 hrs, cells were washed with serum free RPMI 1640 medium once followed by two times washing with ice-cold 1X PBS buffer.

Afterwards, the cells were dislodged with trypsin, neutralized with 2 ml of growth medium and spun down twice for 5 minutes at 1500 rpm. The cell pellet was resuspended in 1 ml of PBS for flow cytometric analysis.

Light scattering and fluorescent properties of individual cells were determined using FACSCalibur flow cytometer equipped with Cellquest software. Cell gating was done using forward and side scatter histograms with a minimum of 10000 counts. Gated cells were analyzed in terms of green fluorescence (FL1-H).

3.5.4. DHFR INHIBITION ASSAY

The effect of either free MTX or conjugated MTX on the target enzyme, DHFR, was measured in a microplate type of assay where the oxidation of NADPH to NADP^+ was followed at 340 nm spectroscopically. Assay mixture contained 0.05 M Tris buffer with a pH of 7.5. 20 μl of test compound dissolved in 10 nM PBS to achieve final concentrations of 0-100 nM (or only the solvent for the control well), was added to a reaction mixture of 0.003 units of human recombinant DHFR enzyme expressed in *E.coli* and 87 μM NADPH. The reaction mixture was allowed to equilibrate for 5 minutes at room temperature, which was followed by the addition of 153 μM DHF, completing the final volume to 300 μl . The change in the absorbance was measured using kinetic mode with a reading interval of 20 sec for a duration of 20 minutes. A blank reaction rate was determined by processing the change in the absorbance due to the

oxidation of the substrate, NADPH, from 2 to 18 minutes. Comparison of linear decrease in the same time period in the test wells with the blank well revealed the DHFR inhibition activity of the test samples. Results were given as percent inhibition of the enzymatic activity in comparison to the control.

3.6. RESULTS

3.6.1. *IN VITRO* ACTIVITIES OF DENDRIMERS, METHOTREXATE, AND DENDRIMER-METHOTREXATE CONJUGATES

In vitro growth inhibition activities of free MTX, dendrimers, Conjugate A, and Conjugate B were investigated first with the CCRF-CEM and CEM/MTX lymphoblastic leukemia cell lines. Following these initial studies, MTX resistant and sensitive Chinese hamster ovary cells (designated RII and PC43/10) were exploited in a fluorescence-based cell viability assay. Previously, both the CEM/MTX and RII cells have been characterized by an almost complete loss of RFC-mediated MTX uptake (118, 128, 129, 147). The concentration of the test sample for each cell line was varied as will be seen in the corresponding figures depending on the previously known drug sensitiveness of the cells. In each set of cells, the concentration of the drug was kept constant regardless of the form of the drug.

3.6.1.i. *In vitro* Cytotoxicities of PAMAM Dendrimers: The CCRF-CEM and CEM/MTX human lymphoblastic leukemia cell lines were treated with pure

dendrimers at various concentrations corresponding to amount of the dendrimer in that particular conjugate (0-6667 nM for PAMAM-G2.5-COOH and 0-11112 nM for PAMAM-G3-NH₂) and growth inhibition was assessed as described earlier. In Figure 49, growth inhibition activities of both anionic and cationic dendrimers on CCRF-CEM and CEM/MTX cells were given.

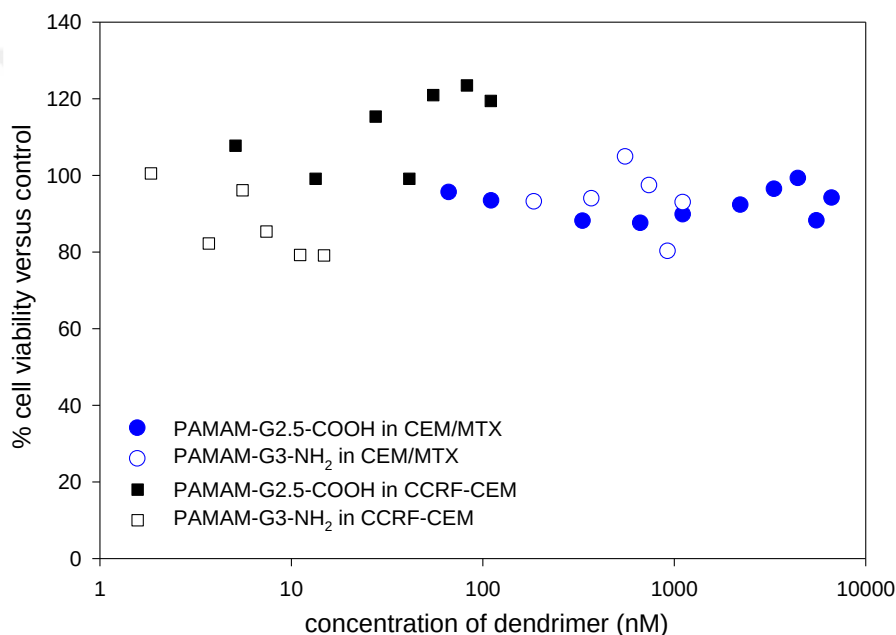


Figure 49. Growth inhibition profiles of PAMAM-G2.5-COOH and PAMAM-G3-NH₂ dendrimers

Neither of the dendrimers showed significant cell toxicity over a wide range of concentrations. For PAMAM-G2.5-COOH dendrimer, even at 6667 nM (0.042 mg/ml), more than 90% of the CEM/MTX cells were viable. The viability of the CEM/MTX cells treated with the cationic PAMAM-G3-NH₂ dendrimer ranged from 80-100% of the control. Similarly, the viability of CCRF-CEM cells treated with either one of the dendrimers was close to or above 80%. Anionic

nature of the dendrimer has been attributed to the nontoxic behavior in the tests. Furthermore, Malik and Duncan did not observe any toxicity of the same dendrimer in B16F10 melanoma cells up to the concentration of 2 mg/ml, which is much higher than the concentration we used (76). Therefore, the IC_{50} value for this particular dendrimer has been suggested as higher than 2 mg/ml. On the other hand, there is more data available for the cationic PAMAM dendrimers (76, 83, 84, 86). Regardless of the cell line, the IC_{50} value observed with the PAMAM-G3-NH₂ dendrimer was quite high compared to ours (76, 83, 84, 86). For example, the same researchers found out that the IC_{50} for PAMAM-G3-NH₂ toward B16F10 cells was 0.05 mg/ml (76). The highest concentration used in our studies was only 1112 nM (0.0077 mg/ml). Furthermore, several other studies reported the variations in the toxicity values for this dendrimer as ranging from 0.9 mg/ml (84) to >10 mg/ml (83, 88).

In addition, with CHO cells the anionic PAMAM-G2.5-COOH dendrimer has been tested in fluorimetric assay, shown in Figure 50. Relatively higher toxicity values have been observed in both the RII and PC43/10 cells upon incubation with the dendrimer. However, even at the highest concentration of 0.01 mg/ml, the cell viability of the RII cells was approximately 60%. Therefore, dendrimers have been evaluated to be relatively nontoxic to the cells under the test conditions.

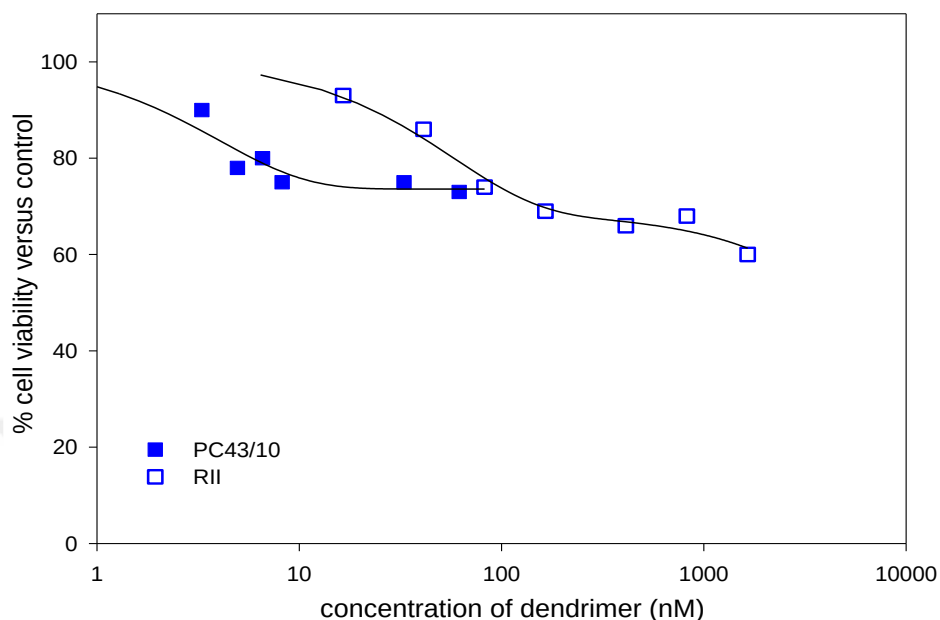


Figure 50. Growth inhibition profile of PAMAM-G2.5-COOH on PC43/10 and RII cell lines

3.6.1.ii. *In vitro* Cytotoxicities of free MTX and Dendrimer-MTX

Conjugates: The *in vitro* growth inhibitory activities of MTX and both the conjugates were determined in human leukemia and hamster ovary cell lines. First, the activities of free MTX and Conjugate A will be discussed. The IC_{50} values for free MTX in the MTX-sensitive CCRF-CEM and MTX-resistant CEM/MTX cell lines were approximately 20 nM and 1575 nM, respectively, comparable to the values reported in literature by several other groups in the same cell lines (93, 97, 118, 128, 129, 148). By comparison, the IC_{50} values for Conjugate A in the CCRF-CEM and CEM/MTX cell lines were found to be 6.8 and 206 nM, respectively. Thus, Conjugate A is approximately 3-fold more potent

than free MTX toward the MTX-sensitive CCRF-CEM cells, and 8-fold more potent toward the resistant CEM/MTX cell line.

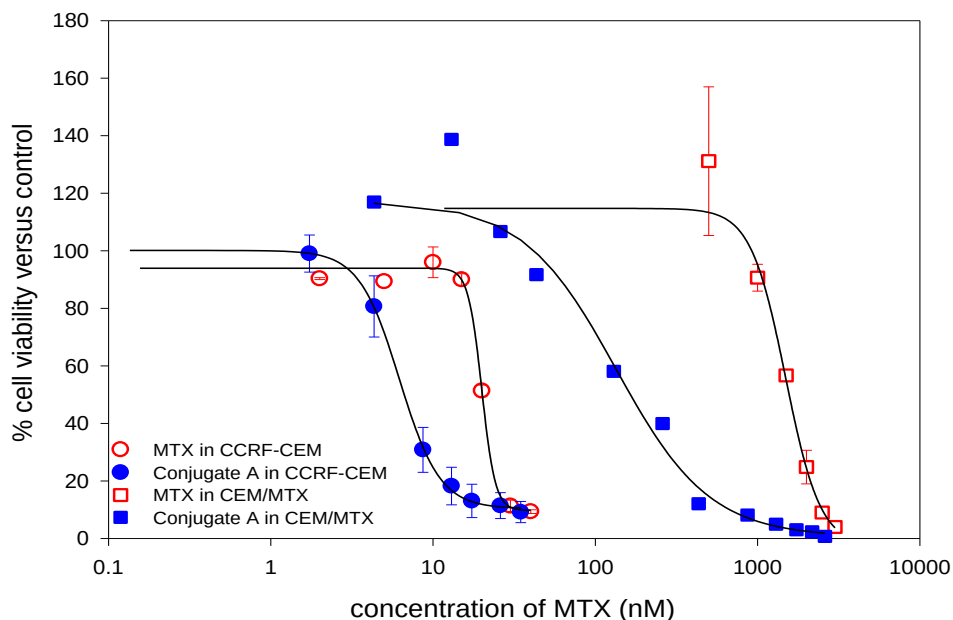


Figure 51. Growth inhibition profiles of MTX and Conjugate A in CCRF-CEM and CEM/MTX cell lines

Secondly, the species demonstrated similar *in vitro* activities on Chinese hamster ovary cells (CHO) as shown in Figure 52. The MTX-sensitive PC43/10 cells with high levels of human RFC expression showed an IC_{50} for free MTX of approximately 4 nM, similar to an earlier report (147). Similarly, the IC_{50} obtained on the MTX transport impaired RII cells, 420 nM, was comparable to the value previously reported by Wong et al. (147).

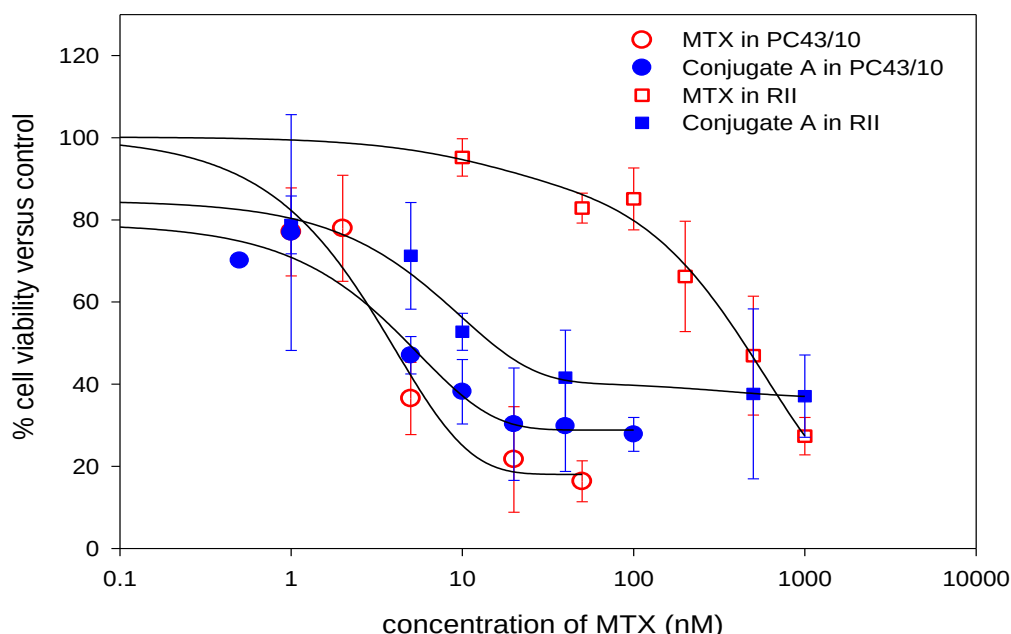


Figure 52. Growth inhibition profiles of MTX and Conjugate A in PC43/10 and RII cell lines

On the other hand, upon the incubation with the Conjugate A, a significant reduction in IC_{50} was observed such that it was 5 nM in PC43/10 cells, whereas a value of 17 nM was recorded in the MTX resistant RII cells, 24-fold reduction over the unconjugated MTX.

The conjugate of MTX with PAMAM-G3-NH₂ dendrimer (Conjugate B) was also evaluated in leukemia cells and CHO cells in terms of growth inhibition activity as shown in Figures 53 and 54.

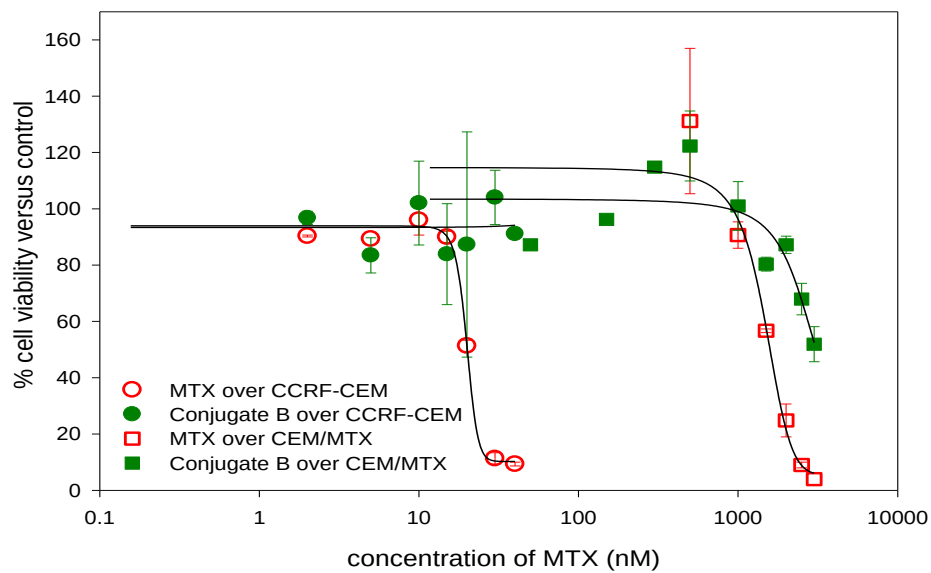


Figure 53. Growth inhibition profiles of MTX and Conjugate B in CCRF-CEM and CEM/MTX cell lines

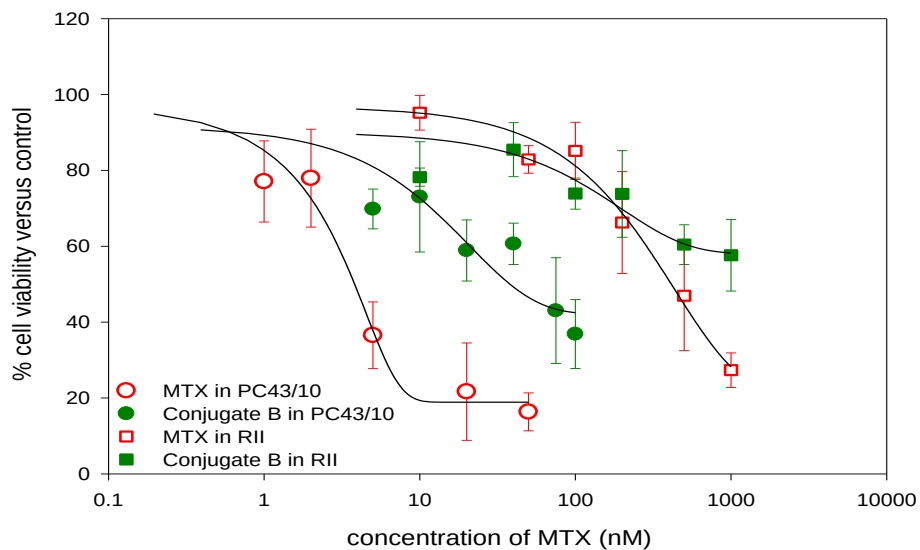


Figure 54. Growth inhibition profiles of MTX and Conjugate B in RII and PC43/10 cell lines

In all the cases, replacement of the anionic dendrimer with the cationic PAMAM-G3-NH₂ in the MTX conjugate led to a pronounced reduction in the *in vitro* activity of the drug. For example, on the CEM/MTX cells only at the highest concentration of 3000 nM, cell viability reduced down to 50% approximately whereas the conjugates was almost completely nontoxic to the CCRF-CEM cells (Figure 53). Similarly, relatively high IC₅₀ values were recorded in CHO cells as well (Figure 54). For PC43/10 cells, the IC₅₀ was increased to 40 nM from that for MTX by about a factor of 10. In RII cells, 65% of maximal growth was seen in the presence of 1000 nM of Conjugate B.

Therefore, the data suggests that conjugation of MTX with the anionic dendrimer (Conjugate A) through the -NH₂ group in the pteridine ring results in a more potent growth inhibition than either free MTX or the conjugate formed with the cationic dendrimer (Conjugate B) through coupling of the -COOH group on MTX. A high activity of the conjugate A was observed not only in drug sensitive cells but also in drug-resistant cell lines as well, bypassing the fact that these cells are impaired in terms of drug transport via the RFC mechanism.

3.6.1.iii. Cellular Rescue with Nucleosides: It has been suggested that inhibition of cell growth can be recovered with the addition of nucleosides such as adenosine, thymidine, guanosine, hypoxanthine, and inosine to the incubation medium in a noncompetitive manner despite the presence of MTX (147-150). Depending on the choice of the nucleosides, either individual or combinations of them can partially/totally circumvent the growth inhibitory effects of the drug.

Therefore, to gain insights into the possible mechanism that the Conjugate A is following to achieve such high activity, CHO cells were co-incubated with either form of the drug in the presence of a combination of 60 μ M adenosine and 10 μ M thymidine. In Figure 55, the cytotoxic effects of MTX and Conjugate A toward PC43/10 and RII cells are given with respect to the absence/presence of nucleosides.

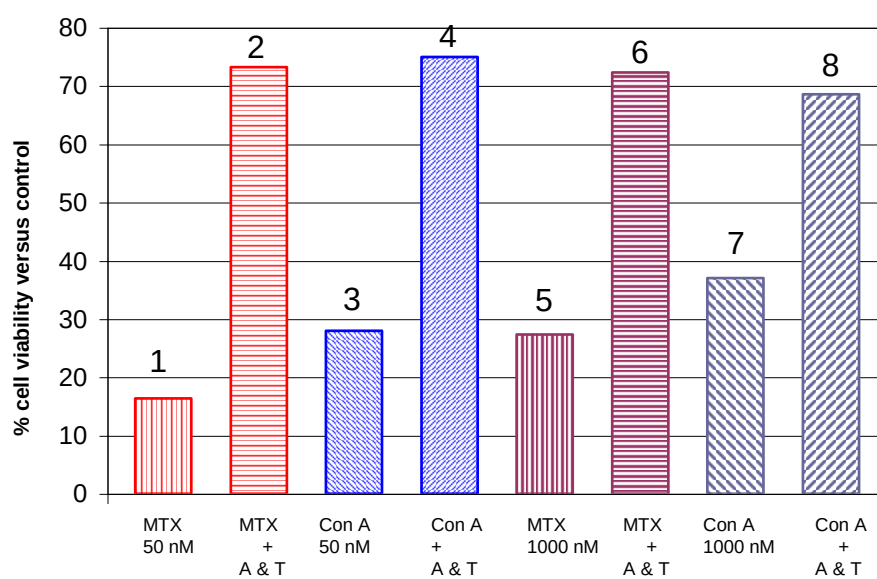


Figure 55. Comparative growth inhibition profiles of free MTX and Conjugate A with and without rescue agents (60 μ M adenosine + 10 μ M thymidine) in PC43/10 (Bars 1-4) and RII (Bars 5-8) cell lines

For easier visualization of the effect of nucleoside co-incubation, only one concentration point for each cell line is given, 50 nM for PC43/10 cells and 1000 nM for RII cells, which are even well above the IC_{50} values for each species. Significant protection was seen at 50 nM of either free MTX or Conjugate A for the sensitive cells, and at 1000 nM for the RII cells. In all the cases, cell

viabilities in the presence of the nucleosides were close to or above 70%. Therefore, it can be confidently said that conjugated MTX still relies on the inhibition of the target enzyme, DHFR, for the growth inhibition as an antimetabolite.

Conjugate B was also evaluated in cellular rescue experiments. As can be seen in Figure 56, viabilities of PC43/10 and RII cells were observed to be 70-92% of the control over the concentration range studied. Therefore, this further confirmed the DHFR inhibitory property of the conjugated MTX as the main mechanism of the antimetabolite action.

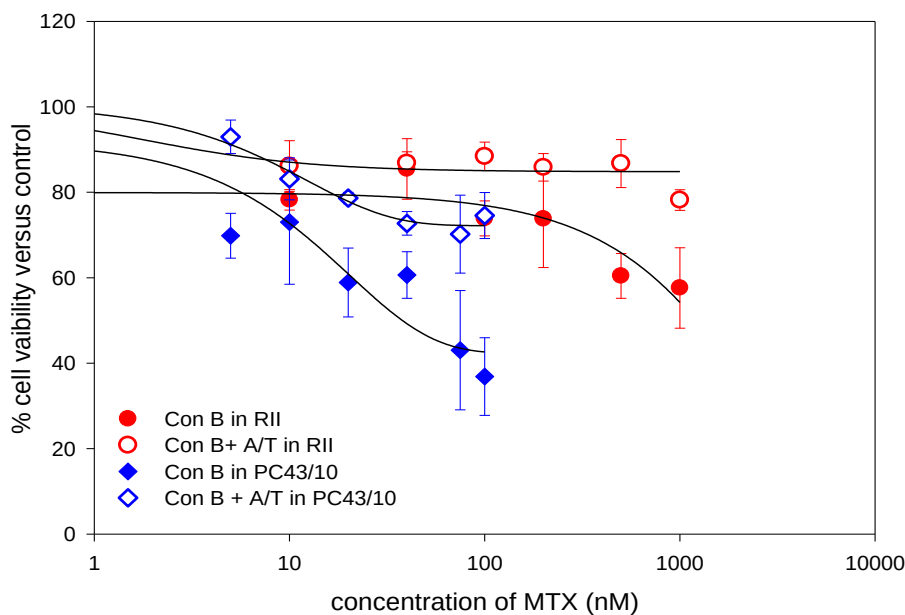


Figure 56. Growth inhibition profile of Conjugate B with and without rescue agents (60 μ M adenosine + 10 μ M thymidine) in PC43/10 and RII cell lines

3.6.1.iv. Cellular Rescue with Leucovorin: In addition to nucleoside rescue one other method is the treatment with leucovorin, which is used in high

dose chemotherapy with MTX. However, apart from nucleosides, leucovorin circumvents the growth inhibitory activity of the drug in a competitive manner since both are the substrates for the RFC system (117, 150). Furthermore, in addition to competition at the cellular membrane level, at high enough concentrations leucovorin can also prevent MTX from associating with DHFR and displace it from the enzyme binding site due to increased levels of intracellular DHF (127). Figure 57 shows how free MTX and both anionic and cationic conjugates performed in the absence and presence of leucovorin in CHO cells. Even though the experiments were done over a wide range of MTX concentrations as mentioned before, for better comparison the chart was prepared by extracting the cellular viabilities at only two particular drug concentrations; 50 nM for PC43/10 cells and 1000 nM for RII cells.

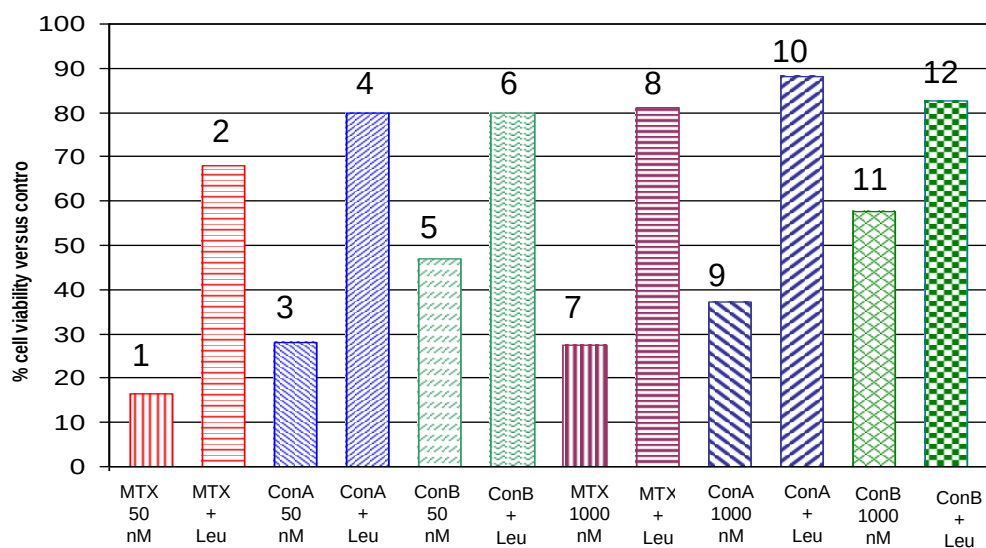


Figure 57. Comparative growth inhibition profiles of free MTX, Conjugate A, and Conjugate B with and without leucovorin (100 μ M) in PC43/10 (Bars 1-6) and RII (Bars 7-12) cell lines

Similar behavior has been observed upon co-incubation with leucovorin as expected. In all the cases, addition of leucovorin to the growth medium increased the cell viability up to 60-95% of the control. The recovered cell growth is attributed to the competition of the rescuing agent leucovorin with the intracellular MTX to bypass the blockage on the synthesis of tetrahydrofolates. The resumption of the conversion of DHF to THF enables the cells to synthesize purine and pyrimidine bases for the cellular replication despite MTX. Hence, co-incubation of the cells with leucovorin led to significantly increased cell viabilities confirming that conjugated MTX depends on the inhibition of the target enzyme DHFR as the mechanism of antimetabolite action.

3.6.2. CELLULAR ENTRY OF FITC-LABELED PAMAM-G2.5-COOH DENDRIMER

Cellular entry dynamics of the anionic dendrimer was studied on RII cells shown in Figure 58 as the log of fluorescence intensity against number of events. The dendrimer was starting to be internalized by the MTX-resistant RII cells only upon 5-minute incubation. Longer incubation times resulted in significant increase in the fluorescence intensity.

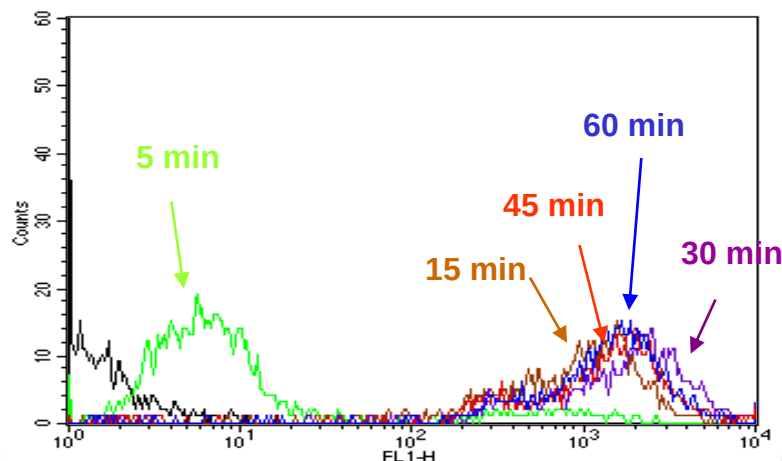


Figure 58. Fluorescence activated cell sorter analysis of cellular uptake of FITC-labeled anionic PAMAM-G2.5-COOH dendrimer by RII cells

From 5 minutes to 15 minutes, prominent enhancement in the fluorescence intensity was observed. The increase continued at 30 minutes and from then on no change was seen. Cellular entry of FITC-labeled PAMAM-G4-NH₂ dendrimer by A549 cells, as demonstrated by our group previously, was supportive of the findings of this study. The uptake of the PAMAM-G4-NH₂ dendrimer was reported to occur as fast as 5 minutes with most of the dendrimer was taken in only after 30 minutes (45). Even though we did not perform any quantitative assay to measure the amount of dendrimer taken inside the cells, in the same study involving FITC-labeled PAMAM-G4-NH₂ dendrimer it has been demonstrated that within 2 h, more than 90% of the dendrimer was internalized by A549 cells (45). We expect the anionic dendrimer to be taken up by the cells of interest at comparable levels. Therefore, the efficient internalization of the dendrimer by the RII cells would explain the significant decrease in the IC₅₀ value for the corresponding conjugate regardless of the functioning of the RFC system.

Hence, efficient transport of the anionic dendrimer on the RII cell line model has been successfully shown.

3.6.3. DHFR INHIBITION ACTIVITIES OF METHOTREXATE AND THE CONJUGATES

The DHFR enzyme inhibition activities of free MTX and the conjugated MTX were evaluated in a cell-free assay (Figure 59). As can be seen from the figure, all three forms of the drug showed concentration-dependent inhibitory activity against the target enzyme.

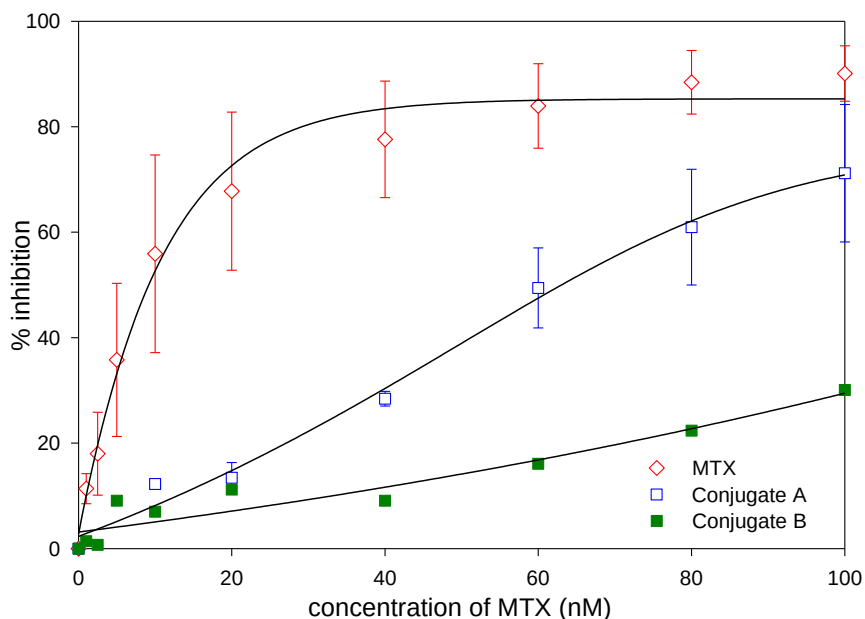


Figure 59. Comparative DHFR enzyme inhibition profiles of MTX, Conjugate A, and Conjugate B

Free MTX demonstrated an IC_{50} value of 7 nM, which agrees with the literature (93, 152, 153). On the other hand, both the conjugates showed

significantly lower affinity for the enzyme with significantly increased IC_{50} values. Conjugate A showed an IC_{50} value of 65 nM, while even at the highest concentration of 100 nM Conjugate B was able to circumvent only 30% of the enzyme activity. Such a serendipitous activity was not expected from the conjugates especially with Conjugate A inhibiting more of the enzyme activity than Conjugate B. If MTX were to show inhibitory activity in the conjugated form, one would expect Conjugate B more active in cell-free assay since the enzyme-binding site, namely the $-NH_2$ at the 2nd position in the pteridine ring of MTX is left free for the interaction with the target enzyme in Conjugate B during the synthesis (114, 115). However, in Conjugate A this site was occupied by the dendrimer. Therefore, in the intact form it is highly unlikely for MTX in Conjugate A to get in contact with the binding site in the enzyme and show even higher response than Conjugate B. The reason for this difference is not clear at present. However, still the conjugates showed less enzyme inhibition activity compared to free form of the drug as expected before.

The lower enzyme inhibition activity demonstrated by Conjugate A in this cell-free assay suggested that significantly improved *in vitro* activity can't be due to the intact conjugate. On the other hand, remarkably different cytotoxicity patterns of anionic and cationic conjugates points out the contribution of the surface properties of each dendrimer to the activity mainly as a result of the enhanced intracellular drug release as will be discussed later.

CHAPTER 4

***IN VITRO* RELEASE OF METHOTREXATE FROM THE CONJUGATES**

4.1. DRUG RELEASE MECHANISMS FROM PRODRUGS

The attachment of the drugs to macromolecules has the advantages of targeting the drug to diseased tissues and achieving improved pharmacodynamics. Since therapeutic activity depends on the free drug, the conjugate typically shows lower *in vitro* activity due to poor release characteristics. Even though some studies suggest that drug could show activity even in the conjugated form (97), from what has been found out so far one can say a bioactive agent needs to be cleaved from its carrier to yield the active form of the drug in the target.

In this respect, the lysosome sets the limits for the effectiveness of the macromolecular drug delivery constructs and represents a great opportunity to the scientists. Hence, the novel bioconjugate will have a linkage that can be cleaved as a result of either the pH changes in the lysosomes or the activity of the proteolytic enzymes in the compartment (154).

4.1.1. pH-DEPENDENT DRUG RELEASE

The macromolecular drug delivery devices relying on pH-dependent drug release utilize pH-labile bonds such as hydrazone and cis-aconityl linkages since

they can undergo hydrolysis at low pH more easily than ester bonds especially amide bonds. For example, conjugates of MTX with generation 5 PAMAM dendrimers with an ester bond released less than 5% of the drug after 2.5 h at physiological pH whereas amide bonded conjugate did not show any release (53). Poor release characteristics of a similar amide bond linked conjugate was reported by in a study involving a conjugate of cisplatin with anionic 3.5 generation PAMAM dendrimer (59). The authors demonstrated that even though the construct released more drug at pH 5.5 than that at pH 7.4, it still comprised less than 1% of the total drug loaded at the end of 74 h incubation period in various buffers.

Yang and Lopina synthesized an ester conjugate of venlafaxine, a novel third generation antidepressant, with generation 2.5 PAMAM dendrimer. The conjugate demonstrated a prolonged release profile over the period of 120 h with approximately half of the drug being released in the first 18 h. Therefore, PAMAM conjugation of the drug extended the time frame of antidepressant release for the better therapy of depression in terms of prolonging the dosing schedule and reducing the side effects in favor of the patients' compliance (155).

Even though hydrazone linkage is quite stable at neutral pH, it is easily cleaved at acidic conditions found in the lysosomes. Hence, until reaching the target conjugate will stay intact in the serum protecting the drug. To take the advantage of this feature, Padilla and coworkers covalently attached DOX to a three-armed PEO-based G-2 polyester dendron hybrid via a hydrazone bond. Drug release from the conjugate showed a strong dependence on pH such that

approximately 85% of the drug was released at pH 2.5 after 22 h (54). Release of ADR from HPMA copolymer bound with either cis-aconitic acid or enzymatically cleavable linkers has been studied, which will be discussed later. The higher *in vitro* activity of the pH-labile bonded conjugate was attributed to better release from this particular conjugate in comparison to that of the enzymatically cleavable linker (156).

4.1.2. ENZYMATIC DRUG RELEASE

It has been suggested that there is certain relationship between the activity of proteolytic enzymes especially Cathepsin B and carcinogenesis (157, 158). Therefore, a vast majority of the conjugates in the Phase I/II clinical trials as well as those in preclinical investigation include various peptidic spacers as the linkage between the drug and the polymeric material (159). Since most of the time the activity of the bioconjugate is restricted to its ability to release the drug, researchers have exploited the lysosomally cleavable linkers. Studies have been performed by several groups on poly(N-2-hydroxypropyl)methacrylamide (HPMA), PEG, and polyglutamic acid using various drugs such as DOX, camptothecin, cisplatin, 5-FU, daunorubicin, paclitaxel, and mitomycin C (25, 108, 160-164). This is also called lysosomotropic chemotherapy as the linkage is susceptible only to the lysosomal enzymes (164). Position of the linking site, enzyme-dependent hydrolysis rate, and composition and configuration of the oligopeptide chain for higher drug release have been studied.

Gianasi et al. showed the importance of the length of the peptidic spacer and the linkage strategy on both the drug release and the activity of the conjugate *in vitro* in B16F10 cells (108). *In vitro*, the conjugate with an amide bond relying only on the enzymatic cleavage, released little amount of the drug, <10% over the period of 72 h in the absence of the enzyme. On the other hand, under the same conditions, the corresponding conjugates with the carboxylate linkages showed significant drug liberation up to 80% in 24 h. Also, this conjugate showed high *in vivo* activity attributed to the accumulation of the carrier-attached drug selectively in the tumor tissue as a result of the increase in the molecular weight, as suggested in the sarcoma 180 model shown on carrier HPMA (25).

The significance of peptide configuration and the effect of the polymeric carriers, PEG, poly[N-(2-hydroxyethyl)-L-glutamine] (PHEG), and dextran, in terms of exposure of the linkage to various proteolytic enzymes such as collagenase, and cathepsin B and D to release the drug 5-FU was investigated (165). All of the enzymes showed similar affinities to the different peptidic sequences. It has been reported that tetrapeptidic sequence with Gly as the third amino acid after Gly-Phe was the best substrate for the cathepsin B. On the other hand, PEG, dextran, and PHEG conjugates having Gly-Phe-Gly-Gly as linker released free 5-FU with a rate determined by the polymeric carrier: PEG>PHEG>dextran. The least release from dextran was attributed to the steric hindrance posed by the large carrier molecule on the enzyme activity.

As a class of anticancer agents camptothecins pose many challenges to the scientists. First of all, the presence of lactone functionality in the structure is not only crucial for the anti-cancer activity but also confers a degree of instability of these agents in aqueous solutions. Furthermore, they have very low aqueous solubilities. Therefore, conjugation of camptothecin with folic acid where PEG served as a spacer rendered the native drug molecule stable protecting it from pH-dependent reversible inter-conversion between lactone and carboxylate forms (162, 163). Hydrolysis half-time of the camptothecin-PEG-folate conjugate with triglycine peptide was the lowest among the other oligopeptidic sequences. Pro-Ala and Pro-Gly sequences produced stable conjugates in PBS with 115 and 29 h as the half-lives, respectively, due to increasing steric hindrance of these amino acids in addition to hydrophobicity of the added amino acid chain which is a common problem with peptidic linkers. However, after just 1 h incubation with cathepsin B, the conjugate with tetrapeptidic sequence was degraded to mono and tetrapeptide degradation products, which in turn completely released the drug after 6.4 h (163). On the other hand, Daunorubicin conjugated with serum albumin directly or with only one leucine unit as an amino acid linker failed to release the drug upon incubation with the lysosomal enzymes (164).

Etrych et al. studied the relationship between the spacer structure and the rate of *in vitro* release from HPMA-DOX conjugates coupled through a pH-sensitive hydrazone bond susceptible to hydrolysis at pH ~ 5-6. The conjugate was formed by either with an aliphatic molecule, such as 6-aminohexanoic acid and 4-aminobenzoic acid, or with an oligopeptide, either a mono peptide or

oligopeptide (161). The highest DOX release in PBS pH 5.0, > 90% of the loaded drug, was obtained with the conjugate having 6-aminohexanoic acid as the spacer. However, when the conjugate either with Gly-DL-Phe-Leu-Gly or Gly-L-Leu-Gly was incubated with Cathepsin B, the release from the degradable spacer increased from 70-80% to ~98% in 48 h. On the other hand, under the same conditions nonbiodegradable aliphatic chain bonded conjugates did not display any difference in the release pattern. Since lysosomal release is relatively slow and would not be suitable for the use with antibodies due to likely aggregation of the antibody, researchers sought alternate ways to speed up the release yet keeping the biodegradable peptide chain in the structure to assure the specific drug liberation (160, 166). They have found out that when DOX is conjugated to the Z-Phe-Lys peptide chain through a self-immolative p-aminobenzylcarbonyl, PABC, drug is cleaved in only 8 min upon the enzymatic degradation by Cathepsin B while in the absence of PABC, no free drug was observed due to steric hindrance of substrate binding by DOX in the enzyme active site. The choice of peptidic sequence was based on the selectivity of the enzyme for strongly hydrogen bonding amino acids at the P_1 sites and hydrophobic ones at the P_2 sites (166). Later, this model peptidic sequence was further tested on additional drugs, paclitaxel and mitomycin C as well (160). Due to bulkiness of the paclitaxel, similar preparations with this drug led to a relatively very high half-life, ~ 9 h. On the other hand, mitomycin C was cleaved from the oligopeptide at a rate similar to that of DOX, 10 min, as it has comparably a smaller molecular structure than paclitaxel. The release of DOX was completely

abolished by the addition of the cathepsin inhibitor, E-64 to the lysosomal preparation, which implied that activity is restricted to cathepsins B, L, and H.

The DOX-HPMA conjugate with Gly-Phe-Leu-Gly amino acid sequence as the linker (PK1) shown in figure 60 progressed to Phase 1 clinical trials. When regimen was given once every 3 weeks, PK1 displayed greatly reduced toxicity with a MTD of 320 mg/m^2 , 4-5 times higher than the usual clinical dose of free DOX. Additionally, no polymer-induced toxicity was observed. Dose limiting toxicity in the absence of cardiotoxicity was otherwise typical of DOX and included mucositis and bone marrow suppression (167). Responses were seen in 4 of the 36 patients enrolled with non-small cell lung cancer, colorectal cancer, and anthracycline resistant breast cancer.

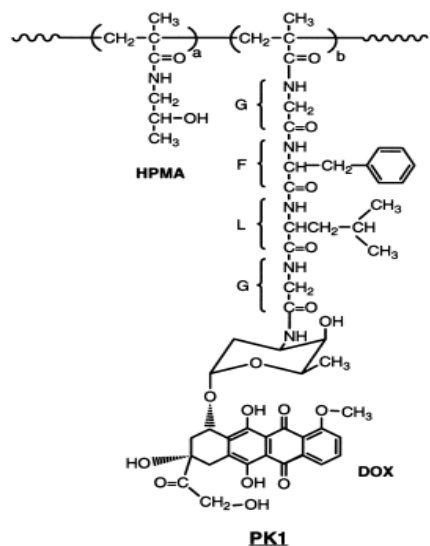


Figure 60. Structure of HPMA copolymer-doxorubicin (PK1) [Adapted from (8)]

Antitumor activity was observed in cancers resistant/refractory to conventional chemotherapy at low doses, $80\text{-}180 \text{ mg/m}^2$, which was indicative of the fact that an EPR effect might be playing a role. However, in a colorectal trial

no activity was demonstrated although breast and lung cancer responded to the treatment well (167).

Also, apart from enzyme-labile peptidic linkers, Wiwattanapatapee's 5-ASA conjugate with 3rd generation PAMAM dendrimers bonded with azo-linkers showed no drug release in the homogenates or stomach or phosphate buffer, pH 1.2 and 6.8. However, it released the drug upon incubation with the homogenates of the small intestine after 12 h thus showing site-specific drug liberation (67).

4.2. EXPERIMENTAL WORK

Significance of the work: The drug release characteristics of the conjugates can reveal a great deal about how the conjugate will perform *in vitro* and partly *in vivo*. In this chapter, I investigated the *in vitro* drug release profiles of the directly linked anionic and cationic conjugates whose cytotoxic activities were tested in the previous chapter. Any likely difference would provide insights to the already established significantly different activity patterns *in vitro*.

4.3. MATERIALS

Methotrexate (4-amino-10-methylpteroylglutamic acid) (MTX) ((6R,S)5-formyl tetrahydrofolate) was obtained from National Cancer Institute (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis). Polyamidoamine (PAMAM)

dendrimers (generation 2.5 with -COOH end groups and generation 3 with -NH₂ end groups) were purchased from Sigma Aldrich Chemical Company and Dendritech, MI. A regenerated cellulose dialysis bag of MW cutoff = 1000 was purchased from Spectrum Laboratories Inc. Other reagents were obtained from assorted vendors in the highest quality available and include 1,4-dithio-DL-threitol (DTT) (Sigma), ethylenediaminetetraacetic acid (EDTA) (Sigma), Cathepsin B (Sigma), 8-chlorothiophylline (Sigma), tetramethylammonium chloride (TMAC) (Sigma). All the solvents used were purchased from Fisher Scientific.

4.4. *IN VITRO* RELEASE FROM DENDRIMER-METHOTREXATE

CONJUGATES

4.4.1. FREE METHOTREXATE RELEASE

Free MTX (1 mg) predissolved in DMSO was diluted with 25 mM phosphate buffer, pH=5.5, to a concentration of 1 mM. The solution was transferred to a dialysis bag with a MW cutoff of 1000. Then, the dialysis membrane containing the analyte was put into a 50 ml centrifuge tube having 25 ml of the same buffer, which was then placed in a shaking water bath at 37 °C. At determined time intervals, 1 ml of the surrounding medium was withdrawn and replaced with fresh medium. Release samples were first analyzed with UV/Vis spectrometer at 370 nm as detection wavelength. RP-HPLC has been used for further characterization. For that, the samples were freeze-dried and

reconstituted in DMSO, with the addition of internal standard (8-chlorothiophylline), to get rid of the salts for the better performance of the column. Analytical conditions; a 3.9X150 mm Waters RP-C8 column at 303 nm with a mobile phase of 0.1 M KH_2PO_4 + 0.04 M TMAC and acetonitrile (ACN) (v/v = 80:20), pH=2.5, flow rate = 1 ml/min.

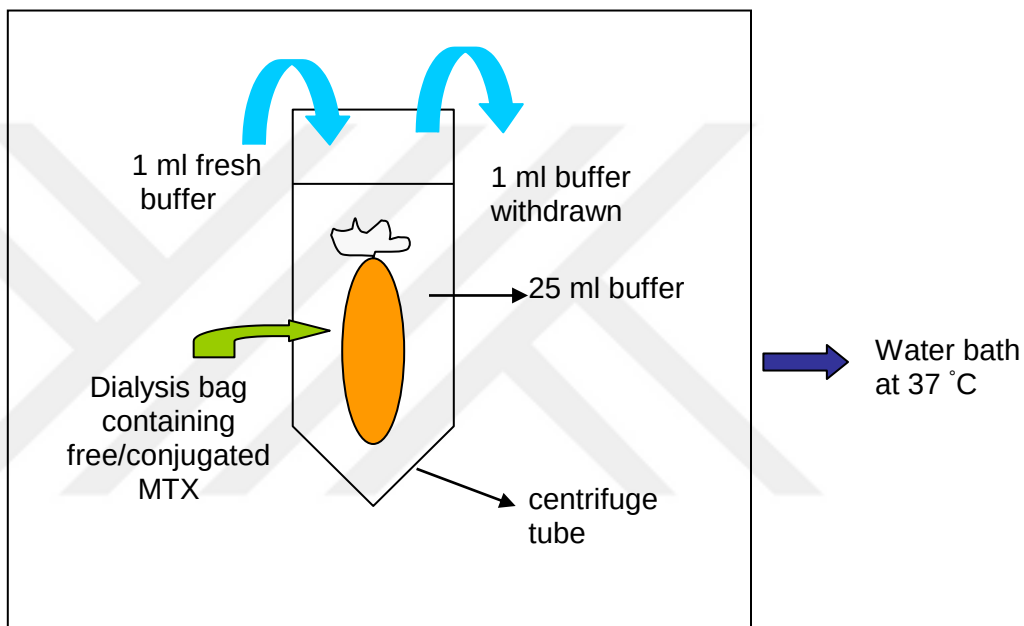


Figure 61. Schematic representation of setup used in release experiments

4.4.2. DRUG RELEASE FROM THE CONJUGATE

Conjugated MTX (0.5 mg) was dissolved in DMSO and diluted with 25 mM phosphate buffer, pH = 5.5, to a concentration of approximately 470 μM where the volumetric ratio of DMSO to buffer was kept at 9.5% in the final mixture in the dialysis bag, MW cutoff = 1000. In the case of enzymatic release, the amount of cathepsin B enzyme was adjusted based on the activity noted by the supplier and the literature (160, 166). On a molar basis, 60 mole of substrate

(conjugated MTX) was incubated with 1 mol of enzyme. The activation of enzyme was done in a mixture of 30 mM DTT/15 mM EDTA. The dialysis membrane containing the analyte (non-enzymatic drug release) or analyte and the enzyme (enzymatic drug release) was transferred to a 50 ml centrifuge tube having 25 ml of the same buffer, which was then placed in a shaking water bath at 37 °C. Similar sample collection and analysis methods were conducted as explained before.

4.5. RESULTS

4.5.1. FREE METHOTREXATE RELEASE

Diffusion profile of free MTX was studied over a period of 26 h. As early as 1 h, the color in permeate was visible to eye as the drug continuously diffuses out. Figure 62 shows the results of two independent runs of MTX release from the dialysis bag. As can be seen, release of the drug reaches equilibrium approximately within 4 h with 80% of the total drug being released. Furthermore, HPLC and UV/Vis spectroscopic techniques are in very good agreement with each other suggesting that detection methods, especially the conditions used for the HPLC analysis, are well established for the analysis of the samples for the presence of the drug.

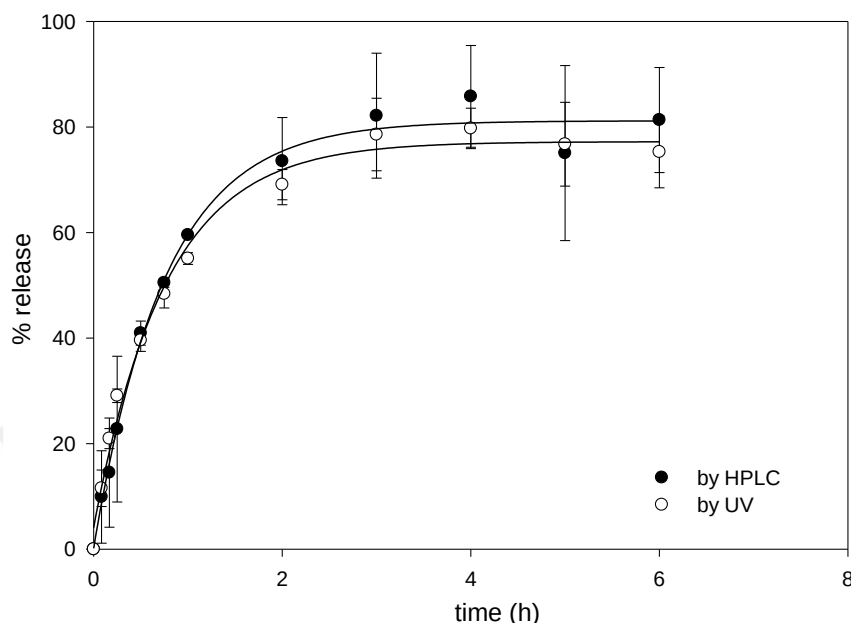


Figure 62. Free MTX release profiles by RP-HPLC and UV/Vis spectroscopy

Even though in an earlier study Patri et al. used a dialysis cassette with a molecular weight cutoff (MWCO) of 8 kD, since molecular weight of MTX is lower than the MWCO of the dialysis bags we used, we can make direct comparison. According to the authors, approximately 72% of the drug was released within 2.5 h in PBS as obtained by UV/Vis spectroscopy (53). Our results agree well with the findings of their study.

4.5.2. NON-ENZYMATIC RELEASE OF METHOTREXATE FROM CONJUGATE A

In the second stage of our efforts to understand the drug release from the conjugates under the conditions they encounter in the intracellular environment, a similar experiment has been done with the conjugate of MTX with anionic

dendrimer. This conjugate had slightly higher MTX payload, 2.5 MTX/dendrimer, than that used in the cytotoxicity experiments mentioned earlier. The diffusion profile of cleaved MTX from the conjugate was studied over a period of 26 h. As early as 1 h, the color in permeate became visible as the drug started to release/cleave from the conjugate. Figure 63 shows the results obtained by HPLC.

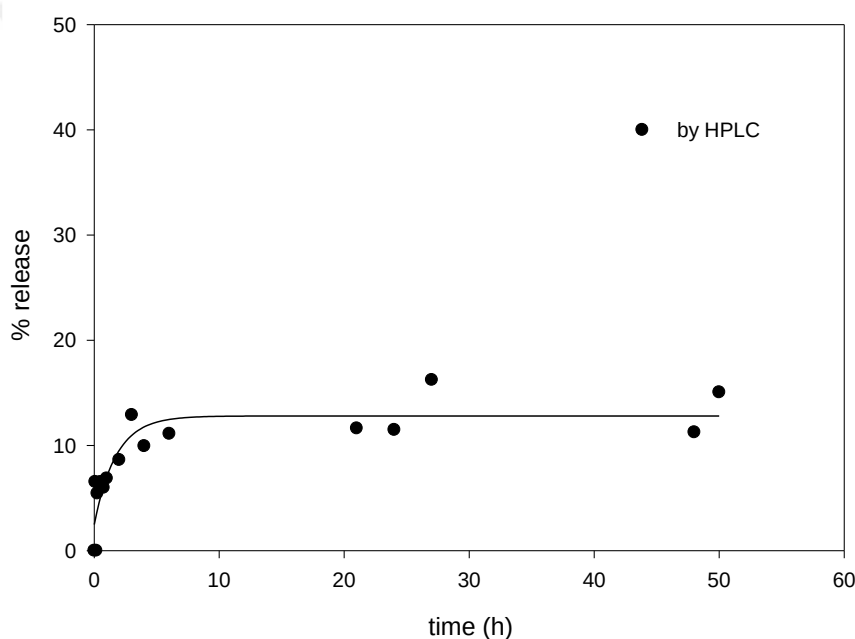


Figure 63. MTX release profiles from Conjugate A by RP-HPLC

As it can be seen from the figure, approximately in 10 h it reaches the equilibrium value of 12%. From then on no change was observed in the amount of MTX release over a period of 50 h. Therefore, we concluded that 12% of the loaded drug was released in the incubation medium. This could be either due to free MTX retained in the conjugate even after extensive dialysis or MTX nonspecifically cleaved from the conjugate. However, compared to the total amount of the drug in the conjugate this is very small.

4.5.3. ENZYMATIC RELEASE OF METHOTREXATE FROM CONJUGATE A

The likelihood of the cleavage of the bond leading to conjugate formation was studied in the presence of Cathepsin B (Figure 64, in comparison to non-enzymatic drug release).

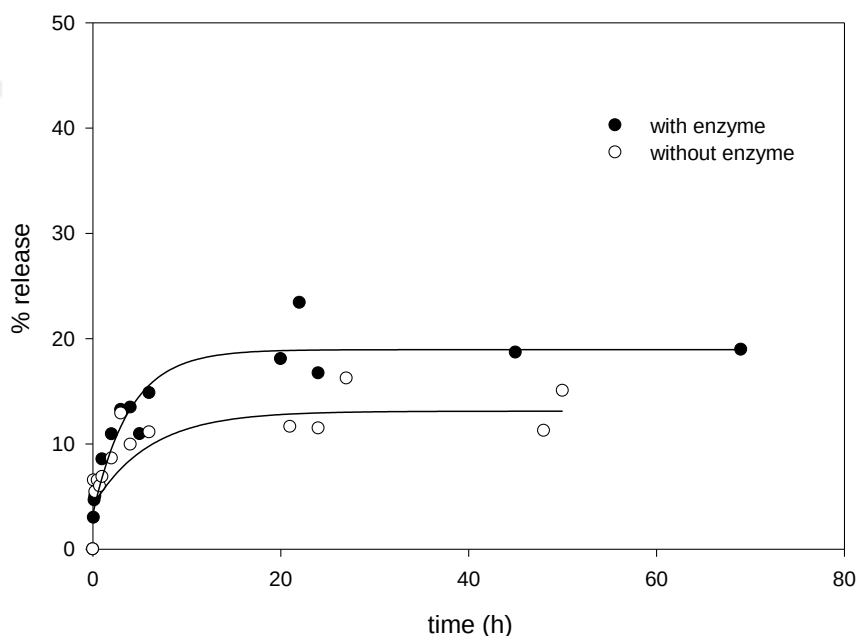


Figure 64. Comparative MTX release profiles from Conjugate A in the absence and presence of enzyme by RP-HPLC

The addition of the enzyme to the incubation medium slightly increased the amount of the released MTX found in the surrounding buffer. In the presence of the enzyme, equilibrium was achieved approximately at 19% MTX release. Considering the fact that 12% of the total would be from the nonspecific release from the conjugate/free drug, only 7% was possibly released as a result of enzymatic activity.

So far, on the dendrimer-conjugates with an amide bond, no release has been observed even though they did not resort the enzymatic cleavage (53, 59). The enzyme we used in our study specifically cleaves the amide bonds in the peptides such as those in the peptidic linkers at efficiencies depending on the type of the sequence as mentioned in section 4.1.2. Hence, further characterization is necessary to confirm the amount we obtained in our study is solely due to the enzymatic activity. One possibility is that the enzyme is not active after the very first few hours of the experiment. Therefore, to make sure that the enzyme had been successfully activated at the beginning of the experiment, activity of the enzyme in the release buffer should be established with an appropriate substrate known for Cathepsin B (160). The second possibility we need to consider is the likelihood of the need for more enzyme as time progresses. For that, we might have to determine the remaining amount of the enzyme in the dialysis bag at different time intervals using a protein assay. Hence, this would give an idea about if we need to add more enzyme as it is constantly consumed over the course of the experiment.

Also, we wanted to do similar experiments on cationic dendrimer conjugated MTX (conjugate B). Since the conjugate had very high drug content, 15.6 MTX molecules/dendrimer molecule, the conjugate predissolved in DMSO instantly precipitated in the release buffer similarly shown previously on a folic acid-dendrimer conjugate (48). Therefore, release from this particular conjugate could not be investigated. However, we will develop new formulation techniques

to solubilize this conjugate first in the buffer so that release studies can be performed.



CHAPTER 5

DISCUSSION

Dendrimers have been proven useful over many macromolecular carriers including linear/hyperbranched polymers, liposomes, and proteins due to their unique structural features. Their biological applications span from gene delivery to boron neutron capture therapy, diagnostics to burn treatment. In this thesis, we showed the versatility of PAMAM dendrimers for conjugating with MTX not only in direct coupling reactions but also in those involving various spacers between the dendrimer and the drug. The effect of the dendrimer end functional group on the *in vitro* activity of the conjugate was demonstrated in isogenic human lymphoblastoid leukemia and Chinese hamster ovary cells. As one of the oldest antimetabolites, MTX has been conjugated to many natural and synthetic carriers. However, conjugation of not only a drug but also any bioactive agent to a so called carrier affects the inherent activity of the bioactive molecule which is mostly shown to be a substantial decrease compared to the unmodified form. The eventual activity pattern of the conjugated drug is dictated by the combinatorial effect of both the nature of the carrier and the linking strategies pursued to form the new construct.

To this end, we selected two PAMAM dendrimers with comparable molecular weights but with different surface charges (anionic and cationic) to assess the importance of the charge on the *in vitro* activity. Furthermore, by

keeping the identity of the linkage the same in the conjugates, we were able to make a better judgement of the effect of the surface charge.

We synthesized two amide-bonded conjugates of MTX with anionic (Conjugate A) and cationic PAMAM (Conjugate B) dendrimers by utilizing either the $-NH_2$ or $-COOH$ group of the drug, respectively. Due to sterical hindrance on the amine groups of the drug, conjugation with the anionic carboxyl terminated dendrimer had lower MTX payload than the conjugate prepared with cationic counterpart where the drug is modified at the $-COOH$ group on the glutamate moiety. Even though the anionic Conjugate A had 11-times lower drug payload, it had superior *in vitro* activity on all of the cell lines not only compared to the cationic Conjugate B but also to the free drug. Especially, this activity enhancement was noteworthy in the MTX-resistant CEM/MTX and RII cells, with 8- and 24-fold reduction in the IC_{50} value, respectively.

Based on previous studies on similar dendrimer-MTX conjugates, we can safely say that we stand in a unique position in this respect. Previously, Quintana et al. reported that the amide bonded conjugate of MTX with 5th generation PAMAM dendrimer required a 2-times higher drug concentration to achieve the same effect as free drug in oral carcinoma KB cells even despite the fact that conjugate was targeted with folic acid moieties (51). On the other hand, a similar conjugate prepared with an ester bond resulted in 5-fold reduction in IC_{50} value in the same cell line. Furthermore, a similar conjugate of anionic 3.5 generation PAMAM dendrimer with cisplatin showed appreciably lower activity, 100 to 380-

fold increase in IC_{50} , than the free drug over several human cancer cell lines despite the relatively higher drug payload, 25% (w/w), than Conjugate A (59).

To address the substantial change of the drug's *in vitro* activity upon conjugation either with anionic or cationic dendrimer, we need to look from three different perspectives, which are solely related to the nature of the dendrimer. These are;

- ◆ Any difference in the cellular entry of the conjugates,
- ◆ Different modes of DHFR inhibition selected by each conjugate,
- ◆ Different intracellular MTX release from the conjugates.

Increased drug uptake: Even though high *in vitro* activity was demonstrated only by the Conjugate A, this strongly suggests that MTX is indeed taken up by the cells through a mechanism driven by the dendrimer. This is strengthened by the significantly bigger margin of the reduction reported in the IC_{50} values in the resistant cells which are characterized by the loss of RFC function (118, 128, 129, 147). It has been reported that MTX is taken up to only approximately 3% of the extracellular amount over a period of 4 h, which is due to the bivalent anion nature of the drug (127, 128, 168). Therefore, the change in the entry mode of the drug inside the cells will contribute to the *in vitro* activity in a positive way. The cellular entry of FITC-labeled anionic PAMAM dendrimer in RII cells demonstrated that the dendrimer is internalized efficiently by the cells, which was also reflected in the magnitude of the increase in the fluorescence cells treated for 15 and 30 minutes, 560 and 987-times increased fluorescence compared to control, respectively. The rate of cellular entry shown by the

dendrimer in our study is comparable to what has been observed previously in our group for -NH_2 and -OH terminated PAMAM dendrimers in A549 cells where a bigger portion of the dendrimer has been documented to enter the cells in as fast as 5 minutes (45, 46, 68). Therefore, we concluded that the observed much higher *in vitro* activity of the conjugate A in the drug-resistant cell lines compared to free drug is the result of the increased intracellular amount of the drug upon conjugation due to altered cellular uptake of the drug. On the other hand, the activity enhancement in the drug-sensitive CCRF-CEM and PC43/10 cells was lower since they already possess effectively functioning RFC system to create high enough intracellular MTX concentrations to lead to the association of at least 95% of the DHFR enzyme with the drug for growth inhibition (117, 127). As a consequence, in these cell lines, activity enhancement was about 3-fold.

Mode of DHFR inhibition: It has been an intriguing question whether the drug should be released from the conjugate or not to show activity intracellularly. So far, any macromolecular drug delivery vehicle or more specifically anticancer drug-dendrimer conjugates have shown lower drug activity since the drug is not cleaved from the carrier due to high stability of the bond (53, 59). However, it has been also shown on PEG-MTX systems that even though conjugate remains intact, they were able to inhibit the enzyme activity at levels comparable to free drug suggesting that drug can be active even in the conjugated form (97). Thus, we were prompted to perform a similar experimentation to test this possibility. In the cell-free DHFR inhibition assay, free MTX was the most active species in terms of inhibition of the enzymatic activity, where it had an IC_{50} value of 7 nM.

On the other hand, the conjugates showed a rather unexpected behavior such that the anionic conjugate demonstrated 50% inhibition of the enzyme activity at a concentration of 65 nM whereas for the cationic conjugate it was more than 100 nM. Considering the fact that the enzyme binding site, -NH_2 at the 2nd position of pteridiny ring, was hypothesized to be occupied by the dendrimer, the reason for the higher activity of the conjugate A was unclear. On the other hand, since cationic conjugate had that site open for the association with the enzyme, it should have been more active if the conjugated drug had been the active form. Therefore, it is not clear why conjugate A is more active than conjugate B at the present. Regardless of the underlying causes, the IC_{50} value of even conjugate A is a factor of 9 higher than that of free drug. On the other hand, since conjugate A is 8-24 times more active *in vitro* in the resistant cell lines, it strongly suggests that conjugated drug can't be the active form of the drug.

Further insight into the mechanism of action of the conjugates has been gained in experiments on CHO cells exploiting salvage pathways where either a combination of adenosine and thymidine, as purine and pyrimidine base sources in the presence of the antimetabolite, or leucovorin was used as rescue agents. Co-incubation of the cells with the nucleosides increased the cell viability close to or above 70% in the case of both free MTX and the conjugated MTX. Similarly, co-treatment of the cells with leucovorin led to recovered cell growth, reported to be 60-95% of the control over the range of concentrations studied. Thus, the classical mode of DHFR inhibition activity of the conjugates was well established.

Intracellular drug release: The third factor in establishing the *in vitro* activity of the conjugates would be attributed to the likely differences in the drug release. As mentioned earlier, due to the high stability of the bond between the drug and the carrier, especially the amide bonds, most of the traditional conjugates made so far did not release the drug leading to substantially ineffective conjugated drug (53, 59, 108). It is hypothesized that the differences in the lysosomal residence times of the dendrimers will determine the extent of the drug release from the conjugate. It has been documented for the cationic PAMAM dendrimers that they display proton-sponge effect due to convenient pKa values of tertiary and primary amines similarly to PEI, which makes them valuable gene delivery vehicles as they tend to escape from the endosome/lysosomes (87). This is known as lysosomotropic effect, which is also shown for small molecules as well as fluorescently labeled macromolecules (169-171). According to this hypothesis, any perturbation in the lysosomes leading to a pH increase, imparted by the highly cationic charge of the dendrimer, will translocate the contents of the lysosomes directly to the cytoplasm. Therefore, conjugate made with cationic dendrimer will spend less time in the lysosomes without being able to release sufficient amount of its cargo, conjugated MTX. On the other hand, in the conjugate A, the cationic charge of tertiary amines are shielded by the –COOH groups at the periphery imparting anionic charge to the conjugates giving it a higher chance to stay in the lysosomes to liberate enough drug for efficient growth inhibition. Thus, the activity obtained with conjugate A is much higher than that of conjugate B.

The involvement of protease enzymes in the drug release from especially linear polymer-drug conjugates has been well established as discussed before. However, generally amide-bonded conjugates do not release the drug or release only very small quantities (53, 59). To imitate the intracellular conditions that the conjugates encounter we performed preliminary enzymatic *in vitro* drug release from the anionic conjugate with a slightly higher drug payload, 2.5 MTX molecules/dendrimer molecule, than that used in the cytotoxicity measurements. By first analyzing the diffusion profile of the free drug with both RP-HPLC and UV/Vis spectroscopy we made sure that we were able to produce reproducible and consistent data with both of the techniques. Similar experimentation was done with the conjugated MTX as well in the absence and presence of the enzyme. It has been shown that the run involving the enzyme led to 7% more drug release from the conjugate compared to that where no enzyme used as the control. Therefore, the role of the enzyme needs to be investigated more elaborately to determine whether the drug is releasing indeed due to the activity of the enzyme. Yet, the data is quite interesting considering the fact that cathepsin B is specifically digesting the amide bond in the peptides. Additionally, the attachment of the drug to a relatively large macromolecule might have played a key role in the released amount of the drug. The attachment of anticancer drug 5-FU to large dextran molecule led to approximately 2-times lower drug release compared to smaller linear polymers even in the case of the best known peptidic substrate for cathepsin B (165). This could have been the case with the conjugate studied here such that large dendrimer molecule might have shielded

the linking site from the enzyme action. On the other hand, we were not able to perform similar experiments due to very high drug payload of a new set of cationic conjugate B, which precipitated immediately in the release medium. This is in accordance with the findings of Kono et al. in a study involving a folic acid-polyether dendrimer conjugate. Even though the conjugate had lesser folic acid molecules than ours, 12.6 vs 15.6, the precipitation took place at low pH values (48). Additionally, the amount of free drug observed in the conjugate in the drug release experiments could be envisaged as the effect of the solvent. Contrary to DMSO used for the purification of the conjugates, the use of PBS buffer in the release experiments will trigger and facilitate the removal of the drug encapsulated in the intra-domain of the dendrimer.

The interestingly high activity of the anionic conjugate encouraged us to synthesize an array of MTX-conjugates of both of the dendrimers with a spacer where the drug is attached to the dendrimer through a linking moiety keeping the identity of the bond on either side of the drug the same. Elaborately designed synthesis schemes enabled us to rationalize these spacer-linked conjugates, which are the first spacer linked dendrimer-MTX conjugates in the literature. It has been shown that the exploitation of even the synthetic molecules as spacers enhances the drug release from the drug-dendrimer conjugates, up to 57%, upon the enzymatic cleavage (67). In addition, with the fact that the newly synthesized conjugates have an ester bond, facilitated drug release from the conjugate *in vitro* can be obtained simply by the change of the pH of the buffer

while still remaining intact at neutral pH. This would lead to even further decrease in the IC₅₀ value observed for the anionic dendrimer.

Since drug release is a key issue for the *in vitro* activity of the conjugate, we synthesized PAMAM-MTX conjugates with various spacers as discussed before, from which we expect to achieve better drug release to have higher *in vitro* activity in turn. The *in vitro* activity test of the spacer-linked cationic conjugate is under progress. However, regioselective protection efforts to synthesize folic acid-targeted PAMAM-MTX conjugates where the folic acid moiety is intended to be protruding for easier FR association was not successful. In addition, the protection of the amine groups in the drug would have led to increased reaction yield to due to reduced propensity of cross-linking of the same species, MTX/FA, which is commonly observed in the DCC-mediated coupling reactions (13). The unsuccessful synthesis trials might be due to several reasons such as the choice of protection agent or reaction conditions. However, to make this particular conjugate possible several other protecting groups could be tested.

CONCLUSIONS

We investigated the conjugation of MTX with two different, anionic and cationic, PAMAM dendrimers in a DCC-mediated coupling scheme yielding an amide bond. The choice of the dendrimer not only determined the eventual drug payload but also played a major role in the *in vitro* activity of the conjugates as well.

- ◆ Due to relatively lower reactivity of the -NH_2 group of MTX, in both directly and spacer-linked conjugates, the anionic conjugate had slightly lower drug payload than the cationic conjugate prepared using the -COOH group of the drug.
- ◆ Regardless of the lower drug payload, the anionic conjugate showed substantially higher *in vitro* activity compared not only to its cationic counterpart but also to the free MTX.
- ◆ Anionic conjugate produced up to 8 to 24-times better *in vitro* response to the drug by the MTX-resistant CEM/MTX and RII cells, respectively, than that of free drug.
- ◆ The remarkable IC_{50} reduction in both of the MTX-resistant cell lines suggests the efficient uptake of the drug through the cell membrane by a mechanism dictated by the dendrimer, which is called fluid-phase endocytosis.
- ◆ The recovered cell growth upon co-incubation either with the nucleosides or leucovorin pointed out that the inhibition of the DHFR enzyme still prevails as the sole mode of action of the conjugates.
- ◆ Preliminary *in vitro* release experiments on an analogous anionic conjugate in the presence of the cathepsin B enzyme suggested that there is 7% increase in the amount of liberated drug compared to that with no enzyme in the incubation medium as control.
- ◆ The amount of drug release is worth more investigation while trying to address the differences in the *in vitro* activity pattern of the conjugates.

Further experimentation is necessary especially in terms of the cationic conjugate.

- ◆ In addition to the directly-linked PAMAM-MTX conjugates, spacer-linked conjugates of both dendrimers have been synthesized at comparable payloads. The realization of the spacer-linked conjugates of MTX with PAMAM dendrimers is unique in this area.

- ◆ The presence of the spacer is envisaged to lead to higher *in vitro* drug release due to both the extension of the drug molecules from the sterically crowded dendrimer surface and the presence of the hydrolysable ester linkage.

- ◆ On the other hand, protection strategies for the blocking of the amine group of MTX/FA need to be optimized in terms of the choice of the protecting group and the reaction conditions.

FUTURE WORK

We have demonstrated the efficient synthesis of both directly and spacer-linked PAMAM-MTX conjugates. *In vitro* cellular characterization of the directly-linked conjugates has proved that not only the type of the bond, as suggested by many bioconjugates in literature, but also the nature of the carrier is a key factor in dictating the eventual behavior of the bioconjugate *in vitro* and later *in vivo*.

Therefore, we aimed to gain further insights into the role of the building blocks of the conjugate by utilizing both the dendrimers individually again in different conjugates.

Synthesis: We have already prepared the conjugates MTX with anionic and cationic PAMAM dendrimers where a spacer is used to form both ester and amide bonds on the same conjugate. Since enzymatic drug release would require longer times depending on the linking strategy than simple hydrolysis processes, the use of an ester bond will facilitate the cleavage of the drug from the carrier. As ester bonds being readily labile to the pH changes, one would expect to have as efficient drug release from the cationic conjugates as that from the anionic conjugate thereby eliminating the dominant effect of the lysosomal residence times. At the same time, higher drug release is envisaged to lead to a higher *in vitro* activity.

As has been the focus of attention to researchers, the drug release can be made even easier from the bioconjugate with the use of various spacer molecules. One can attach MTX to the dendrimer through a peptidic spacer, which can be easily recognized by the target enzymes such as cathepsin family, especially cathepsin B. Hence, the elaborate optimization of the sequence for easy enzyme association would lead to increased drug activity. Furthermore, a targeting moiety such as folic acid could be integrated into the structure of PAMAM-peptide-MTX molecule to afford targeted constructs. While hydrolytically/enzymatically labile bonding is desirable for the attachment of the drug, stable linking of the targeting agent to the nanodevice is essential so that until internalization, the device will stay intact. At the same time, it has been suggested that the exposure of the targeting moiety to the cell surface plays a key role in the transport of the molecule (49, 51). The placement of the folic acid

away from the dendrimer surface through a long spacer such as linear PEG molecules will mask the steric crowding effect of the dendrimer surface without drastically changing the molecular weight of the whole molecule (Figure 65).

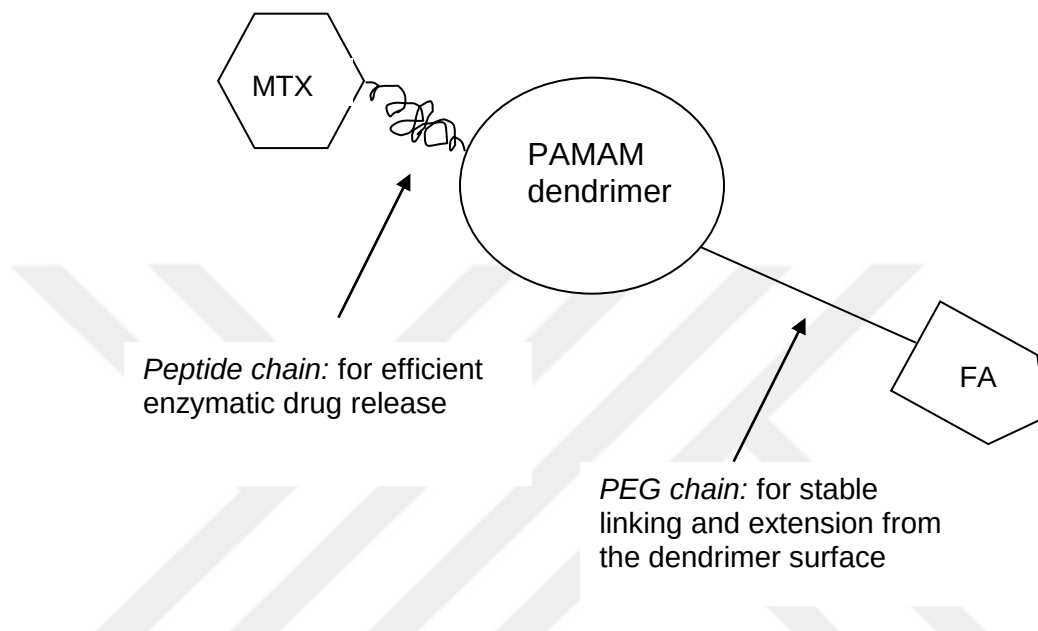


Figure 65. FA-targeted conjugate of PAMAM-MTX

We also started the synthesis of fluorescently-labeled MTX species, which will help in various ways: The detection of intracellular amount of MTX is difficult unless tritiated MTX or HPLC-fluorometry approaches for the quantitation of MTX polyglutamates (MTXPGs) is used (172). However, the use of tritiated MTX for the macromolecular conjugation with PAMAM dendrimers is not feasible considering the yield of our reactions. On the other hand, conversion of the MTXPGs to fluorescent analytes requires extra equipment to make the photo-oxidation reaction possible. Therefore, one simple solution to easily detectable MTX species would be the synthesis of FITC-labeled MTX (Figure 66) (173-175). The replacement of MTX with FITC-MTX in the synthesis scheme will enable us to trace the internalization and intracellular release pattern of the conjugates.

However, it should be born in mind that the *in vitro* activity of the FITC-attached MTX molecules as well as the main transport mechanism is different from MTX alone (174-176). Therefore, direct comparison of the FITC-MTX linked dendrimer conjugates should be made with FITC-MTX as control.

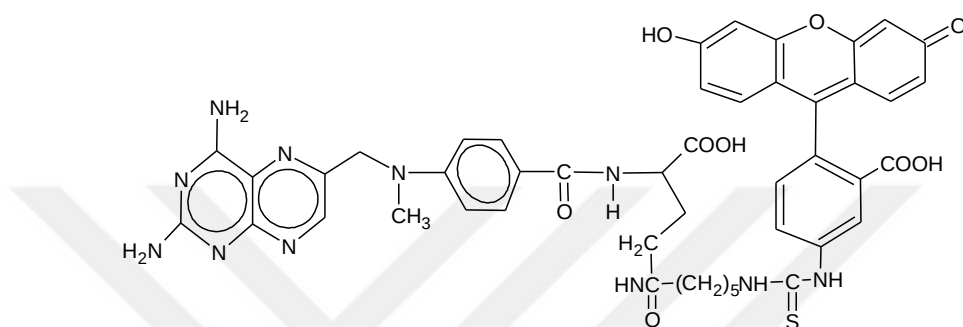


Figure 66. Molecular structure of FITC-MTX conjugate

In vitro characterization: The question of how long the anionic or cationic conjugates/dendrimers stay in the lysosomes still remains. We have shown that the entry of the anionic dendrimer can be as efficient as cationic dendrimers, shown previously by our group on A549 cells. The time-dependent intracellular lysosomal localization of either PAMAM-MTX-FITC construct where the fluorescent label is directly attached to the drug or MTX-PAMAM-FITC construct with FITC being linked to the dendrimer will answer the question. The use of lysosomal markers specific for organelles with low pH values in the confocal microscopic technique will supply quite valuable information.

The second stage of the cellular characterization of the conjugates would involve the spacer-linked with/without the targeting agent conjugates as mentioned before where both the increased drug release and specific internalization could be achieved in case of the targeted construct.

In vitro drug release: Our preliminary drug release data showed that there is 7% drug release upon the incubation with the enzyme. However, this low amount of drug release could be due to several reasons: (a) cathepsin B enzyme was not active even after the activation step, (b) the amount of the enzyme was not enough, (c) other enzymes are necessary such as other cathepsins or (d) a mixture of proteolytic enzymes like tritosomes rather than using just one isolated enzyme. The first two possibilities can be easily ruled out after thorough optimization for the amount and activation procedure of the enzyme. As has been mentioned earlier different linkers are recognized better by one or the other enzyme (165). Therefore, a wider range of enzymes could be screened.

In vivo characterization: Due to low aqueous solubility of the conjugates, we were not able to perform an *in vivo* test in the already established tumor xenografts created with CEM/MTX cells. Recently, we have started the synthesis of quarternary salts of the conjugates, which is quite promising in terms of the observed water solubility (177, 178). Additionally, it has been suggested that quarternization of the 4th generation cationic PAMAM dendrimer reduced the toxicity on cells (177). Even though it requires rigorous effort to determine how many iodine species are necessary to solubilize high enough concentrations of the conjugates without compromising the molecular weight to a great extent, it is very encouraging which can enable us to take a start on *in vivo* trials of the conjugates.

APPENDIX

a

ACN: acetonitrile

ADR: adriamycin

Ara-C: cytosine arabinoside

Ara-U: 1- β -D-Arabinofuranosyluracil

Ara-UMP: Uracil- β -D-arabinofuranoside

b

β -NADPH: β -nicotinamide adenosine dinucleotide phosphate

BCRP: breast cancer resistance protein

c

CH: activated carbon

d

DAB: diaminobutane

DAE: diaminoethane

DCC: N-N-dicyclohexyl carbodiimide

DEE: diethylether

DHF: dihydrofolic acid

DHFR: dihydrofolate reductase

DMAP: 4-dimethylamino pyridine

DMC: dichloromethane

DOX: doxorubicin

e

EGFR: epidermal growth factor receptor

EHH: ethyl-6-hydroxy-hexanoate

ESI-MS: electrospray ionization mass spectroscopy

ETFA: ethyltrifluoroacetate

f

FA: folic acid

5-ASA: 5-aminosalicylic acid

5-FU: fluorouracil

FITC: fluorescein isothiocyanate

Fmoc: 9-fluorenylmethyl-*N*-succinimidyl carbonate

FR: folate receptor

h

HPMA: poly(*N*-2-hydroxypropyl)methacrylamide

HSA: human serum albumin

i

Ibu: Ibuprofen

l

LDH: lactate dehydrogenase

m

MALDI-TOF: matrix-assisted laser desorption/ionization mass spectrometer with time-of-flight analyzer

MTX: methotrexate

MP: methylpredisone

MDR: multidrug resistance

MMP: matrix metalloproteinase

MRP: multidrug resistance-associated protein

p

PABA: p-aminobenzoic acid

PABC: p-aminobenzoylcarbonyl

PAH: p-aminohiipuric acid

PAMAM: poly(amidoamine)

PBS: phosphate buffered saline

PEG: polyethyleneglycol

PEO: poly(ethylene)oxide

PEO-*b*-PHAA: poly(ethyleneoxide)-block-poly(2-hydroxyethyl aspartamide)

Pgp: P-glycoprotein

PHEG: poly-N-(2-hydroxyethyl)-L-glutamine

PK1: poly(N-2-hydroxypropyl)methacrylamide–doxorubicin

PLAA: poly-L-amino acid

PLL: poly-L-lysine

PPI: poly(propylenimine)

r

RES: reticuloendothelial system

RFC: reduced folate carrier

RP-HPLC: reverse phase high performance liquid chromatography

s

6-TAMRA: 6-carboxytetramethylrhodamine

SMANCS: poly(styrene-co-maleic acid n-butylester)-conjugated

neocarzinostatin

t

t-boc: di-*tert*-butyl dicarbonate

TEA: triethylamine

TFA: trifluoroacetic acid

TMAC: tetramethylammonium chloride

TMS: tetramethylsilane

u

UV/Vis: ultraviolet visible spectroscopy

v

VEGF: vascular endothelial growth factor

VPF: vascular permeability factor

REFERENCES

- (1) Parkin M.D., B., F., Pisani P. (2005) Global Cancer Statistics, 2002. *Cancer Journal for Clinicians* 55, 74-108.
- (2) American Cancer Society. (2005) Cancer Facts & Figures 2006.
- (3) Feng, S.-S., and Chien, S. (2003) Chemotherapeutic engineering: application and further development of chemical engineering principles for chemotherapy of cancer and other diseases. *Chemical Engineering Science* 58, 4087-4114.
- (4) King, R. J. B. (1996) *Cancer Biology*, Addison Wesley Longman Limited, Essex.
- (5) Kupchella, C. E. (1987) *Dimensions of Cancer*, Wadsworth Publishing Company, Belmont.
- (6) Groninger, E., Proost, J. H., and de Graaf, S. S. N. (2004) Pharmacokinetic studies in children with cancer. *Critical reviews in oncology/hematology* 52, 173-97.
- (7) Haag, R. (2004) Supramolecular drug-delivery systems based on polymeric core-shell architectures. *Angewandte Chemie, International Edition* 43, 278-282.
- (8) Kopecek, J., Kopeckova, P., Minko, T., and Lu, Z. R. (2000) HPMA copolymer-anticancer drug conjugates: design, activity, and mechanism of action. *European Journal of Pharmaceutics and Biopharmaceutics* 50, 61-81.

- (9) Peppas, L.-B. (1997) Polymers in controlled drug delivery. *Medical Plastics and Biomaterials Magazine* Nov, 1-16.
- (10) Saltzman, W. M. (2001) *Drug Delivery: Engineering, Principles for Drug Therapy*.
- (11) Silber, B. M., Bialer M, Yacobi A. (1987) *Pharmacokinetic/Pharmacodynamic Basis of COntrolled Drug Delivery*, Vol. 29, Second edition ed., Marcell Dekker, New York.
- (12) Langer, R. (1995) Biomaterials and biomedical engineering. *Chemical Engineering Science* 50, 4109-21.
- (13) Veronese, F. M., Morpurgo M. (1999) Bioconjugation in pharmaceutical chemistry. *IL Farmoco* 54, 497-516.
- (14) Duncan, R. (2002) Polymer-drug conjugates: targeting cancer. *Biomedical Aspects of Drug Targeting*, 193-209.
- (15) Jorge, H. (1987) *Use of Polymers in Controlled Release of Active Agents*, Vol. 29, Second Edition ed., Marcel Dekker, Inc., New York.
- (16) Langer, R. (1998) Drug delivery and targeting. *Nature (London)* 392, 5-10.
- (17) Torchilin, V. P. (2000) Drug targeting. *European Journal of Pharmaceutical Sciences* 11, S81-S91.
- (18) Maeda, H. (2001) The enhanced permeability and retention (EPR) effect in tumor vasculature: The key role of tumor-selective macromolecular drug targeting. *Advances in Enzyme Regulation* 41, 189-207.

- (19) Maeda, H. (2002) Enhanced permeability and retention (EPR) effect: basis for drug targeting to tumor. *Biomedical Aspects of Drug Targeting*, 211-228.
- (20) Maeda, H., Fang, J., Inutsuka, T., and Kitamoto, Y. (2003) Vascular permeability enhancement in solid tumor: various factors, mechanisms involved and its implications. *International Immunopharmacology* 3, 319-328.
- (21) Maeda, H., Seymour, L. W., and Miyamoto, Y. (1992) Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. *Bioconjugate Chemistry* 3, 351-62.
- (22) Maeda, H., Wu, J., Sawa, T., Matsumura, Y., and Hori, K. (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics. A review. *Journal of Controlled Release* 65, 271-284.
- (23) Gillies, E. R., and Frechet, J. M. J. (2002) Designing Macromolecules for Therapeutic Applications: Polyester Dendrimer-Poly(ethylene oxide) \"Bow-Tie\" Hybrids with Tunable Molecular Weight and Architecture. *Journal of the American Chemical Society* 124, 14137-14146.
- (24) Maeda, H. (1991) SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. *Advanced Drug Delivery Reviews* 6, 181-202.
- (25) Seymour, L. W., Miyamoto, Y., Maeda, H., Brereton, M., Strohalm, J., Ulbrich, K., and Duncan, R. (1995) Influence of molecular weight on

passive tumor accumulation of a soluble macromolecular drug carrier.
Eur. J. Cancer, Part A 31A, 766-70.

- (26) Duncan, R. (1999) Polymer conjugates for tumor targeting and intracytoplasmic delivery. The EPR effect as a common gateway? *Pharmaceutical Science & Technology Today* 2, 441-449.
- (27) Boas, U., and Heegaard, P. M. H. (2004) Dendrimers in drug research. *Chemical Society Reviews* 33, 43-63.
- (28) Esfand, R., and Tomalia, D. A. (2001) Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. *Drug Discovery Today* 6, 427-436.
- (29) Wooley, K. L., Hawker C.J., Frechet, J.M.J. (1991) hyperbranched macomolecules via a novel double-stage convergent growth approach. *Journal of American Chemical Society* 113, 4252-4261.
- (30) Aulenta, F., Hayes, W., and Rannard, S. (2003) Dendrimers: a new class of nanoscopic containers and delivery devices. *European Polymer Journal* 39, 1741-1771.
- (31) Patri, A. K., Majoros I.S., Baker J.R.B. (2002) Dendritic polymer macromolecular carriers for drug delivery. *Current Opinion in Chemical Biology* 6, 466-471.
- (32) Liu, M., and Frechet, J. M. J. (1999) Designing dendrimers for drug delivery. *Pharmaceutical Science & Technology Today* 2, 393-401.
- (33) Gillies, E. R., Frechet J.M.J. (2005) Dendrimers and dendritic polymers in drug delivery. *Drug Discovery Today* 10, 35-43.

- (34) Sampathkumar, S.-G., and Yarema, K. J. (2005) Targeting Cancer Cells with Dendrimers. *Chemistry & Biology* 12, 5-6.
- (35) Duncan, R., and Izzo, L. (2005) Dendrimer biocompatibility and toxicity. *Advanced Drug Delivery Reviews* 57, 2215-2237.
- (36) Uhrich, K. E., Cannizzaro, S. M., Langer, R. S., and Shakesheff, K. M. (1999) Polymeric Systems for Controlled Drug Release. *Chemical Reviews (Washington, D. C.)* 99, 3181-3198.
- (37) Beezer, A. E., King, A. S. H., Martin, I. K., Mitchel, J. C., Twyman, L. J., and Wain, C. F. (2003) Dendrimers as potential drug carriers; encapsulation of acidic hydrophobes within water soluble PAMAM derivatives. *Tetrahedron* 59, 3873-3880.
- (38) Jansen, J. F. G. A., Meijer, E.W. . (1995) The dendritic box: shape-selective liberation of encapsulated guests. *Journal of American Chemical Society* 117, 4417-4418.
- (39) Stevelsman, S., Hest, J.C.M., Jansen, J.F.G.A., Boxtel, D.A.F.J., Meijer, E.W. (1996) Synthesis, characterization, and guest-host properties of inverted unimolecular dendritic micelles. *Journal of American Chemical Society* 118, 7398-7399.
- (40) Twyman, L. J., Beezer, A. E., Esfand, R., Hardy, M. J., and Mitchell, J. C. (1999) The synthesis of water soluble dendrimers, and their application as possible drug delivery systems. *Tetrahedron Letters* 40, 1743-1746.

- (41) Liu, M., Kono, K., and Frechet, J. M. J. (2000) Water-soluble dendritic unimolecular micelles: Their potential as drug delivery agents. *Journal of Controlled Release* 65, 121-131.
- (42) Kojima, C., Kono, K., Maruyama, K., and Takagishi, T. (2000) Synthesis of Polyamidoamine Dendrimers Having Poly(ethylene glycol) Grafts and Their Ability To Encapsulate Anticancer Drugs. *Bioconjugate Chemistry* 11, 910-917.
- (43) Bhadra, D., Bhadra, S., Jain, S., and Jain, N. K. (2003) A PEGylated dendritic nanoparticulate carrier of fluorouracil. *International Journal of Pharmaceutics* 257, 111-124.
- (44) Pan, G., Lemmouchi, Y., Akala, E. O., and Bakare, O. (2005) Studies on PEGylated and drug-loaded PAMAM dendrimers. [Erratum to document cited in CA143:199535]. *Journal of Bioactive and Compatible Polymers* 20, 503.
- (45) Kannan, S., Kolhe, P., Raykova, V., Glibatec, M., Kannan, R. M., Lieh-Lai, M., and Bassett, D. (2004) Dynamics of cellular entry and drug delivery by dendritic polymers into human lung epithelial carcinoma cells. *Journal of Biomaterials Science, Polymer Edition* 15, 311-330.
- (46) Kolhe, P., Misra, E., Kannan, R. M., Kannan, S., and Lieh-Lai, M. (2003) Drug complexation, in vitro release and cellular entry of dendrimers and hyperbranched polymers. *International Journal of Pharmaceutics* 259, 143-160.

- (47) Gillies, E. R., Jonsson, T. B., and Frechet, J. M. J. (2004) Stimuli-Responsive Supramolecular Assemblies of Linear-Dendritic Copolymers. *Journal of the American Chemical Society* 126, 11936-11943.
- (48) Kono, K., Liu, M., and Frechet, J. M. J. (1999) Design of Dendritic Macromolecules Containing Folate or Methotrexate Residues. *Bioconjugate Chemistry* 10, 1115-1121.
- (49) Choi, Y., Thomas, T., Kotlyar, A., Islam, M. T., and Baker, J. R., Jr. (2005) Synthesis and Functional Evaluation of DNA-Assembled Polyamidoamine Dendrimer Clusters for Cancer Cell-Specific Targeting. *Chemistry & Biology* 12, 35-43.
- (50) Kukowska-Latallo, J. F., Candido, K. A., Cao, Z., Nigavekar, S. S., Majoros, I. J., Thomas, T. P., Balogh, L. P., Khan, M. K., and Baker, J. R., Jr. (2005) Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. *Cancer Research* 65, 5317-5324.
- (51) Quintana, A., Raczka, E., Piehler, L., Lee, I., Myc, A., Majoros, I., Patri, A. K., Thomas, T., Mule, J., and Baker, J. R., Jr. (2002) Design and Function of a Dendrimer-Based Therapeutic Nanodevice Targeted to Tumor Cells Through the Folate Receptor. *Pharmaceutical Research* 19, 1310-1316.
- (52) Thomas, T. P., Majoros, I. J., Kotlyar, A., Kukowska-Latallo, J. F., Bielinska, A., Myc, A., and Baker, J. R., Jr. (2005) Targeting and inhibition of cell growth by an engineered dendritic nanodevice. *Journal of Medicinal Chemistry* 48, 3729-3735.

- (53) Patri, A. K., Kukowska-Latallo, J. F., and Baker, J. R. (2005) Targeted drug delivery with dendrimers: Comparison of the release kinetics of covalently conjugated drug and non-covalent drug inclusion complex. *Advanced Drug Delivery Reviews* 57, 2203-2214.
- (54) Padilla De Jesus, O. L., Ihre, H. R., Gagne, L., Frechet, J. M. J., and Szoka, F. C., Jr. (2002) Polyester Dendritic Systems for Drug Delivery Applications: In Vitro and In Vivo Evaluation. *Bioconjugate Chemistry* 13, 453-461.
- (55) Majoros, I. J., Myc, A., Thomas, T., Mehta, C. B., and Baker, J. R., Jr. (2006) PAMAM Dendrimer-Based Multifunctional Conjugate for Cancer Therapy: Synthesis, Characterization, and Functionality. *Biomacromolecules* 7, 572-579.
- (56) Zhuo, R. X., Du, B., and Lu, Z. R. (1999) In vitro release of 5-fluorouracil with cyclic core dendritic polymer. *Journal of Controlled Release* 57, 249-257.
- (57) Choe, Y. H., Conover, C. D., Wu, D., Royzen, M., Gervacio, Y., Borowski, V., Mehlig, M., and Greenwald, R. B. (2002) Anticancer drug delivery systems: multi-loaded N4-acyl poly(ethylene glycol) prodrugs of ara-C. II. Efficacy in ascites and solid tumors. *Journal of Controlled Release* 79, 55-70.
- (58) Schiavon, O., Pasut, G., Moro, S., Orsolini, P., Guiotto, A., and Veronese, F. M. (2004) PEG-Ara-C conjugates for controlled release. *European Journal of Medicinal Chemistry* 39, 123-133.

- (59) Malik, N., and Duncan, R. (2002) pp 29 pp , Division of U S Ser No 111,232, (The Dow Chemical Company, UK). Application: US
- (60) Kobayashi, H., Sato, N., Saga, T., Nakamoto, Y., Ishimor, T., Toyama, S., Togashi, K., Konishi, J., and Brechbiel, M. W. (2000) Monoclonal antibody-dendrimer conjugates enable radiolabeling of antibody with markedly high specific activity with minimal loss of immunoreactivity. *European Journal of Nuclear Medicine* 27, 1334-1339.
- (61) Patri, A. K., Myc, A., Beals, J., Thomas, T. P., Bander, N. H., and Baker, J. R., Jr. (2004) Synthesis and in vitro testing of J591 antibody-dendrimer conjugates for targeted prostate cancer therapy. *Bioconjugate Chemistry* 15, 1174-1181.
- (62) Wilbur, D. S., Pathare, P. M., Hamlin, D. K., Buhler, K. R., and Vessella, R. L. (1998) Biotin Reagents for Antibody Pretargeting. 3. Synthesis, Radioiodination, and Evaluation of Biotinylated Starburst Dendrimers. *Bioconjugate Chemistry* 9, 813-825.
- (63) Kobayashi, H., Wu, C., Kim, M.K., Paik, C.H., Carrasquillo J.A., Brechbiel, M.W. (1999) Evaluation of the in vivo biodistribution of indium-111 and yttrium-88 labeled dendrimer-1B4M-DTPA and its conjugation with anti-Tac monoclonal antibody. *Bioconjugate Chemistry* 10, 103-111.
- (64) Wu, G., Barth, R. F., Yang, W., Kawabata, S., Zhang, L., and Green-Church, K. (2006) Targeted delivery of methotrexate to epidermal growth factor receptor-positive brain tumors by means of cetuximab (IMC-C225) dendrimer bioconjugates. *Molecular Cancer Therapeutics* 5, 52-59.

- (65) Hong, S., Bielinska A.U., Keszler B., Beals, J.L., Shi, X., Balogh, L., Orr, B.G., Baker, J.R.Jr. (2004) Interaction of polyamidoamine dendrimers with supported lipid bilayers and cells: hole formation and the relation to transport *Bioconjugate Chemistry* 15, 774-782.
- (66) Kihara, F., Arima, H., Tsutsumi, T., Hirayama, F., and Uekama, K. (2003) In Vitro and In Vivo Gene Transfer by an Optimized α -Cyclodextrin Conjugate with Polyamidoamine Dendrimer. *Bioconjugate Chemistry* 14, 342-350.
- (67) Wiwattanapatapee, R., Lomlim, L., and Saramunee, K. (2003) Dendrimers conjugates for colonic delivery of 5-aminosalicylic acid. *Journal of Controlled Release* 88, 1-9.
- (68) Khandare, J., Kolhe, P., Pillai, O., Kannan, S., Lieh-Lai, M., and Kannan, R. M. (2005) Synthesis, cellular transport, and activity of polyamidoamine dendrimer-methylprednisolone conjugates. *Bioconjugate Chemistry* 16, 330-337.
- (69) Kolhe, P., Khandare, J., Pillai, O., Kannan, S., Lieh-Lai, M., and Kannan, R. M. (2005) Preparation, cellular transport, and activity of polyamidoamine-based dendritic nanodevices with a high drug payload. *Biomaterials* 27, 660-669.
- (70) Landers, J. J., Cao, Z., Lee, I., Piehler, L. T., Myc, P. P., Myc, A., Hamouda, T., Galecki, A. T., and Baker, J. R., Jr. (2002) Prevention of influenza pneumonitis by sialic acid-conjugated dendritic polymers. *Journal of Infectious Diseases* 186, 1222-1230.

- (71) Liu, M., Kono, K., and Frechet, J. M. J. (1999) Water-soluble dendrimer-poly(ethylene glycol) starlike conjugates as potential drug carriers. *Journal of Polymer Science, Part A: Polymer Chemistry* 37, 3492-3503.
- (72) Sato, N., Kobayashi, H., Saga, T., Nakamoto, Y., Ishimori, T., Togashi, K., Fujibayashi, Y., Konishi, J., and Brechbiel, M. W. (2001) Tumor targeting and imaging of intraperitoneal tumors by use of antisense oligo-DNA complexed with dendrimers and/or avidin in mice. *Clinical Cancer Research* 7, 3606-3612.
- (73) Backer, M. V., Gaynutdinov, T. I., Patel, V., Bandyopadhyaya, A. K., Thirumamagal, B. T. S., Tjarks, W., Barth, R. F., Claffey, K., and Backer, J. M. (2005) Vascular endothelial growth factor selectively targets boronated dendrimers to tumor vasculature. *Molecular Cancer Therapeutics* 4, 1423-1429.
- (74) Thomas, T. P., Myaing, M. T., Ye, J. Y., Candido, K., Kotlyar, A., Beals, J., Cao, P., Keszler, B., Patri, A. K., Norris, T. B., and Baker, J. R., Jr. (2004) Detection and analysis of tumor fluorescence using a two-photon optical fiber probe. *Biophysical Journal* 86, 3959-3965.
- (75) Yordanov, A. T., Kobayashi, H., English, S. J., Reijnders, K., Milenic, D., Krishna, M. C., Mitchell, J. B., and Brechbiel, M. W. (2003) Gadolinium-labeled dendrimers as biometric nanoprobe to detect vascular permeability. *Journal of Materials Chemistry* 13, 1523-1525.
- (76) Malik, N., Wiwattanapatapee, R., Klopsch, R., Lorenz, K., Frey, H., Weener, J.W., Meijer E.W., Paulus, W., Duncan, R. (2000) Dendrimers:

Relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of ¹²⁵I-labeled polyamidoamine dendrimers in vivo. *Journal of Controlled Release* 65, 133-148.

- (77) Nigavekar, S. S., Sung, L.Y., Llanes, M., Lawrence, T.S., Beceker, C.H., Balogh, L., Khan, M.K. (2004) 3H dendrimer nanoparticle organ.tumor distribution. *Pharmaceutical Research* 21, 476-483.
- (78) Kobayashi, H., Saga, T., Kawamoto, S., Sato, N., Hiraga, A., Ishimori, T., Konishi, J., Togashi, K., and Brechbiel, M. W. (2001) Dynamic micro-magnetic resonance imaging of liver micrometastasis in mice with a novel liver macromolecular magnetic resonance contrast agent DAB-Am64-(1B4M-Gd)64. *Cancer Research* 61, 4966-4970.
- (79) Roberts, C. J., Bhalgat, M.K., Zera R.T. (1996) Preliminary biological evaluation of polyamidoamine (PAMAM) Starbust dendrimers. *Journal of Biomedical Materials Research, Part A* 30, 53-65.
- (80) Kobayashi, H., Jo, S.-K., Kawamoto, S., Yasuda, H., Hu, X., Knopp Michael, V., Brechbiel Martin, W., Choyke Peter, L., and Star Robert, A. (2004) Polyamine dendrimer-based MRI contrast agents for functional kidney imaging to diagnose acute renal failure. *Journal of magnetic resonance imaging : JMRI* 20, 512-8.
- (81) Kobayashi, H., Kawamoto, S., Saga, T., Sato, N., Hiraga, A., Ishimori, T., Akita, Y., Mamede, M. H., Konishi, J., Togashi, K., and Brechbiel, M. W. (2001) Novel liver macromolecular MR contrast agent with a polypropylenimine diaminobutyl dendrimer core: Comparison to the

vascular MR contrast agent with the polyamidoamine dendrimer core. *Magnetic Resonance in Medicine* 46, 795-802.

- (82) Shukla, S., Wu, G., Chatterjee, M., Yang, W., Sekido, M., Diop Lamine, A., Muller, R., Sudimack Jennifer, J., Lee Robert, J., Barth Rolf, F., and Tjarks, W. (2003) Synthesis and biological evaluation of folate receptor-targeted boronated PAMAM dendrimers as potential agents for neutron capture therapy. *Bioconjugate chemistry* 14, 158-67.
- (83) Fischer, D., Li Y., Ahlemeyer, B., Krieglstein, J., Kissel, T. (2003) In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* 24, 1121-1131.
- (84) Jevprasesphant, R., Penny, J., Attwood, D., D'Emanuele, A. (2003) Engineering of dendrimer surfaces to enhance transepithelial transport and reduce cytotoxicity. *Pharmaceutical Research* 20, 1543-1550.
- (85) Chen, H., Neerman M.F., Parrish, A.R., Simanek, E.E. (2004) Cytotoxicity, hemolysis, and acute in vivo toxicity of dendrimers based on melamine, candidate vehicles ofr drug delivery. *Journal of American Chemical Society* 126, 10044-10048.
- (86) Jevprasesphant, R., Penny, J., Jalal, R.,McKeown, N.B., D'Emanuele, A. (2003) The influence of surface modification on the cytotoxicity of PAMAM dendrimers. *International Journal of Pharmaceutics* 252, 263-266.
- (87) Haensler, J., and Szoka, F. C., Jr. (1993) Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chemistry* 4, 372-9.

- (88) El-Sayed, M., Ginski, M., Rhodes, C., Ghandehari, H. (2002) Transepithelial transport of polyamidoamine dendrimers across CAco-2 cell monolayers. *Journal of Controlled Release* 81, 355-365.
- (89) Bowman, B. J., and Ofner, C. M., III. (2000) Characterization and in vitro methotrexate release from methotrexate/gelatin conjugates of opposite conjugate bond polarity. *Pharmaceutical Research* 17, 1309-1315.
- (90) Kosasih, A., Bowman, B. J., Wigent, R. J., and Ofner, C. M., III. (2000) Characterization and in vitro release of methotrexate from gelatin/methotrexate conjugates formed using different preparation variables. *International Journal of Pharmaceutics* 204, 81-89.
- (91) Rosowsky, A., Forsch, R., Uren, J., and Wick, M. (1981) Methotrexate analogs. 14. Synthesis of new g-substituted derivatives as dihydrofolate reductase inhibitors and potential anticancer agents. *Journal of Medicinal Chemistry* 24, 1450-5.
- (92) Rosowsky, A. (1973) Methotrexate analogs. 2. Facile method of preparation of lipophilic derivatives of methotrexate and 3',5'-dichloromethotrexate by direct esterification. *Journal of Medicinal Chemistry* 16, 1190-3.
- (93) Pignatello, R., Guccione, S., Forte, S., Di Giacomo, C., Sorrenti, V., Vicari, L., Uccello Barretta, G., Balzano, F., and Puglisi, G. (2004) Lipophilic conjugates of methotrexate with short-chain alkylamino acids as DHFR inhibitors. Synthesis, biological evaluation, and molecular modeling. *Bioorganic & Medicinal Chemistry* 12, 2951-2964.

- (94) Pignatello, R., Puleo, A., Puglisi, G., Vicari, L., and Messina, A. (2003) Effect of Liposomal Delivery on In Vitro Antitumor Activity of Lipophilic Conjugates of Methotrexate with Lipoamino Acids. *Drug Delivery* 10, 95-100.
- (95) Pignatello, R., Spampinato, G., Sorrenti, V., Di Giacomo, C., Vicari, L., McGuire, J. J., Russell, C. A., Puglisi, G., and Toth, I. (2000) Lipophilic methotrexate conjugates with antitumor activity. *European Journal of Pharmaceutical Sciences* 10, 237-245.
- (96) Pignatello, R., Spampinato, G., Sorrenti, V., Vicari, L., Di Giacomo, C., Vanella, A., and Puglisi, G. (1999) Aliphatic α,ω -bis(amides) of methotrexate. Influence of chain length on in-vitro activity against sensitive and resistant tumor cells. *Pharmacy and Pharmacology Communications* 5, 299-305.
- (97) Riebeseel, K., Biedermann, E., Loeser, R., Breiter, N., Hanselmann, R., Muelhaupt, R., Unger, C., and Kratz, F. (2002) Polyethylene Glycol Conjugates of Methotrexate Varying in Their Molecular Weight from MW 750 to MW 40000: Synthesis, Characterization, and Structure-Activity Relationships in Vitro and in Vivo. *Bioconjugate Chemistry* 13, 773-785.
- (98) Selinger, Z., and Lapidot, Y. (1966) Synthesis of fatty acid anhydrides byreaction with dicyclocarbodiimide. *Journal of Lipid Research* 7, 174-175.
- (99) Hu, H., Fan, X.-d., and Cao, Z.-l. (2005) Thermo- and pH-sensitive dendrimer derivatives with a shell of poly(N,N-dimethylaminoethyl

methacrylate) and study of their controlled drug release behavior. *Polymer* 46, 9514-9522.

- (100) Kang, H., Kim, J.-D., Han, S.-H., and Chang, I.-S. (2002) Self-aggregates of poly(2-hydroxyethyl aspartamide) copolymers loaded with methotrexate by physical and chemical entrapments. *Journal of Controlled Release* 81, 135-144.
- (101) Wu, G., Barth Rolf, F., Yang, W., Kawabata, S., Zhang, L., and Green-Church, K. (2006) Targeted delivery of methotrexate to epidermal growth factor receptor-positive brain tumors by means of cetuximab (IMC-C225) dendrimer bioconjugates. *Molecular cancer therapeutics* 5, 52-9.
- (102) Wosikowski, K., Biedermann, E., Rattel, B., Breiter, N., Jank, P., Loeser, R., Jansen, G., and Peters, G. J. (2003) In Vitro and in Vivo Antitumor Activity of Methotrexate Conjugated to Human Serum Albumin in Human Cancer Cells. *Clinical Cancer Research* 9, 1917-1926.
- (103) Peterson, J., Allikmaa, V., Subbi, J., Pehk, T., and Lopp, M. (2002) Structural deviations in poly(amidoamine) dendrimers: a MALDI-TOF MS analysis. *European Polymer Journal* 39, 33-42.
- (104) Subbi, J., Aguraiuja, R., Tanner, R., Allikmaa, V., and Lopp, M. (2005) Fragmentation of poly(amidoamine) dendrimers in matrix-assisted laser desorption. *European Polymer Journal* 41, 2552-2558.
- (105) Morgan, J. R., and Cloninger, M. J. (2005) Synthesis of carbohydrate-linked poly(polyoxometalate) poly(amido)amine dendrimers. *Journal of Polymer Science, Part A: Polymer Chemistry* 43, 3059-3066.

- (106) Woller, E. K., and Cloninger, M. J. (2001) Mannose Functionalization of a Sixth Generation Dendrimer. *Biomacromolecules* 2, 1052-1054.
- (107) Malik, N., Evagorou, E. G., and Duncan, R. (1999) Dendrimer-platinate: a novel approach to cancer chemotherapy. *Anti-Cancer Drugs* 10, 767-776.
- (108) Gianasi, E., Wasil, M., Evagorou, E. G., Kedde, A., Wilson, G., and Duncan, R. (1999) HPMA copolymer platinates as novel antitumor agents: in vitro properties, pharmacokinetics and antitumor activity in vivo. *European Journal of Cancer* 35, 994-1002.
- (109) Greene T.W., W. P. G. M. (1999) *Protective Groups in Organic Synthesis*, Third edition ed., John Wiley&Sons, Inc, New York.
- (110) Luo, J., Smith, M. D., Lantrip, D. A., Wang, S., and Fuchs, P. L. (1997) Efficient Syntheses of Pyrofolic Acid and Pteroyl Azide, Reagents for the Production of Carboxyl-Differentiated Derivatives of Folic Acid. *Journal of the American Chemical Society* 119, 10004-10013.
- (111) Chen, Q., Sowa, D. A., Cai, J., and Gabathuler, R. (2003) Efficient One-Pot Synthesis of Doxorubicin Conjugates Through Its Amino Group to Melanotransferrin P97. *Synthetic Communications* 33, 2401-2421.
- (112) Chen, Q., Sowa, D. A., and Gabathuler, R. (2003) Synthesis of Doxorubicin Conjugates Through 14-Hydroxy Group to Melanotransferrin P97. *Synthetic Communications* 33, 2391-2400.
- (113) Jurgens, H., Gobel, U. (1986) Pharmacology, toxicology, and drug interactions of methotrexate. *Rheumatology* 9, 6-19.

- (114) Cannon, W. R., Garrison, B. J., and Benkovic, S. J. (1997) Consideration of the pH-dependent inhibition of dihydrofolate reductase by methotrexate. *Journal of Molecular Biology* 271, 656-668.
- (115) Verkhivker, G. M., Rejto, P. A., Bouzida, D., Arthurs, S., Colson, A. B., Freer, S. T., Gehlhaar, D. K., Larson, V., Luty, B. A., Marrone, T., and Rose, P. W. (1999) Towards understanding the mechanisms of molecular recognition by computer simulations of ligand-protein interactions. *Journal of Molecular Recognition* 12, 371-389.
- (116) Matherly, L. H. (2001) Molecular and cellular biology of the human reduced folate carrier. *Progress in Nucleic Acid Research and Molecular Biology* 67, 131-162.
- (117) Zhao, R., and Goldman, I. D. (2003) Resistance to antifolates. *Oncogene* 22, 7431-7457.
- (118) Rosowsky, A., Lazarus, H., Yuan, G. C., Beltz, W. R., Mangini, L., Abelson, H. T., Modest, E. J., and Frei, E., III. (1980) Effects of methotrexate esters and other lipophilic antifolates on methotrexate-resistant human leukemic lymphoblasts. *Biochemical Pharmacology* 29, 648-52.
- (119) Assaraf Y. G., Babani S., and Goldman I.D. (1998) Increased activity of a novel low pH folate transporter associated with lipophilic antifolate resistance in Chinese hamster ovary cells. *Journal of Biological Chemistry* 273, 8106-8111.

- (120) Gruner, B. A., and Weitman, S. D. (1999) The folate receptor as a potential therapeutic anticancer target. *Investigational New Drugs* 16, 205-219.
- (121) Antony, A. C. (1996) Folate receptors. *Annual Review of Nutrition* 16, 501-521.
- (122) Sabharanjak, S., and Mayor, S. (2004) Folate receptor endocytosis and trafficking. *Advanced Drug Delivery Reviews* 56, 1099-1109.
- (123) Zhao, R., Seither, R., Brigle, K. E., Sharina, I. G., Wang, P. J., and Goldman, I. D. (1997) Impact of overexpression of the reduced folate carrier (RFC1), an anion exchanger, on concentrative transport in murine L1210 leukemia cells. *Journal of Biological Chemistry* 272, 21207-21212.
- (124) Jansen, G., Westerhof, G. R., Jarmuszewski, M. J. A., Kathmann, I., Rijksen, G., and Schornagel, J. H. (1990) Methotrexate transport in variant human CCRF-CEM leukemia cells with elevated levels of the reduced folate carrier. Selective effect on carrier-mediated transport of physiological concentrations of reduced folates. *Journal of Biological Chemistry* 265, 18272-7.
- (125) Sierra, E. E., Brigle, K. E., Spinella, M. J., and Goldman, I. D. (1995) Comparison of transport properties of the reduced folate carrier and folate receptor in murine L210 leukemia cells. *Biochemical Pharmacology* 50, 1287-94.

- (126) Genestier, L., Paillot, R., Quemeneur, L., Izeradjene, K., and Revillard, J. P. (2000) Mechanisms of action of methotrexate. *Immunopharmacology* 47, 247-257.
- (127) Goldman, I. D., and Matherly, L. H. (1985) The cellular pharmacology of methotrexate. *Pharmacology & Therapeutics* 28, 77-102.
- (128) Matherly, L. H., Angeles, S. M., and McGuire, J. J. (1993) Determinants of the disparate antitumor activities of (6R)-5,10-dideaza-5,6,7,8-tetrahydrofolate and methotrexate toward human lymphoblastic leukemia cells, characterized by severely impaired antifolate membrane transport. *Biochemical Pharmacology* 46, 2185-95.
- (129) Wong, S. C., Zhang, L., Witt, T. L., Proefke, S. A., Bhushan, A., and Matherly, L. H. (1999) Impaired membrane transport in methotrexate-resistant CCRF-CEM cells involves early translation termination and increased turnover of a mutant reduced folate carrier. *Journal of Biological Chemistry* 274, 10388-10394.
- (130) Volk Erin, L., Farley Kate, M., Wu, Y., Li, F., Robey Robert, W., and Schneider, E. (2002) Overexpression of wild-type breast cancer resistance protein mediates methotrexate resistance. *Cancer research* 62, 5035-40.
- (131) Volk Erin, L., and Schneider, E. (2003) Wild-type breast cancer resistance protein (BCRP/ABCG2) is a methotrexate polyglutamate transporter. *Cancer research* 63, 5538-43.

- (132) Chu, B. C. F., and Howell, S. B. (1981) Differential toxicity of carrier-bound methotrexate toward human lymphocytes, marrow, and tumor cells. *Biochemical Pharmacology* 30, 2545-52.
- (133) Moutardier, V., Tosini, F., Vlieghe, P., Cara, L., Delpero, J. R., and Clerc, T. (2003) Colloidal anticancer drugs bioavailabilities in oral administration models. *International Journal of Pharmaceutics* 260, 23-38.
- (134) Li, Y., and Kwon, G. S. (1999) Micelle-like structures of poly(ethylene oxide)-block-poly(2-hydroxyethyl aspartamide)-methotrexate conjugates. *Colloids and Surfaces, B: Biointerfaces* 16, 217-226.
- (135) Boratynski, J., Opolski, A., Wietrzyk, J., Gorski, A., and Radzikowski, C. (2000) Cytotoxic and antitumor effect of fibrinogen-methotrexate conjugate. *Cancer letters* 148, 189-95.
- (136) Ou, X.-H., Kuang, A.-R., Liang, Z.-L., Peng, X., and Zhong, Y.-G. (2004) Receptor binding characteristics and cytotoxicity of insulin-methotrexate. *World journal of gastroenterology : WJG* 10, 2430-3.
- (137) Weigand, M., Hartung, G., Roboz, J., Sieger, S., Wolf, M., Sinn, H., Schrenk, H. H., Wiessler, M., and Frei, E. (2001) Mode of action of methotrexate-albumin in a human T-cell leukemia line and activity against an MTX-resistant clone. *Anti-cancer drug design* 16, 227-37.
- (138) Burger, A. M., Hartung, G., Stehle, G., Sinn, H., and Fiebig, H. H. (2001) Pre-clinical evaluation of a methotrexate-albumin conjugate (MTX-HSA) in human tumor xenografts in vivo. *International Journal of Cancer* 92, 718-724.

- (139) Stehle, G., Sinn, H., Wunder, A., Schrenk, H. H., Schutt, S., Maier-Borst, W., and Heene, D. L. (1997) The loading rate determines tumor targeting properties of methotrexate-albumin conjugates in rats. *Anti-Cancer Drugs* 8, 677-685.
- (140) Torres, J. M., Darracq, N., and Uriel, J. (1992) Membrane proteins from lymphoblastoid cells showing cross-affinity for α -fetoprotein and albumin. Isolation and characterization. *Biochimica et Biophysica Acta, Protein Structure and Molecular Enzymology* 1159, 60-6.
- (141) Hartung, G., Stehle, G., Sinn, H., Wunder, A., Schrenk, H. H., Heeger, S., Kranzle, M., Edler, L., Frei, E., Fiebig, H. H., Heene, D. L., Maier-Borst, W., and Queisser, W. (1999) Phase I trial of methotrexate-albumin in a weekly intravenous bolus regimen in cancer patients. *Clinical Cancer Research* 5, 753-759.
- (142) Vis, A. N., van der Gaast, A., van Rhijn, B. W. G., Catsburg, T. K., Schmidt, C., and Mickisch, G. H. J. (2002) A phase II trial of methotrexate-human serum albumin (MTX-HSA) in patients with metastatic renal cell carcinoma who progressed under immunotherapy. *Cancer Chemotherapy and Pharmacology* 49, 342-345.
- (143) Kim C.H., H. S. J., Lee M.G. (1993) The organ targetability of small and large albumin microspheres containing free and HSA conjugates methotrexate *International Journal of Pharmaceutics* 89, 91-102.
- (144) Chau, Y., Tan, F. E., and Langer, R. (2004) Synthesis and Characterization of Dextran-Peptide-Methotrexate Conjugates for Tumor

Targeting via Mediation by Matrix Metalloproteinase II and Matrix Metalloproteinase IX. *Bioconjugate Chemistry* 15, 931-941.

- (145) Hudecz, F., Clegg, J. A., Kajtar, J., Embleton, M. J., Pimm, M. V., Szekerke, M., and Baldwin, R. W. (1993) Influence of carrier on biodistribution and in vitro cytotoxicity of methotrexate-branched polypeptide conjugates. *Bioconjugate Chemistry* 4, 25-33.
- (146) Yuen, N., Hagiwara, A., Kin, S., Fukuda, K.-i., Ito, T., Takagi, T., Fujiyama, J., Sakakura, C., Otsuji, E., and Yamagishi, H. (2004) Intratumoral administration of methotrexate bound to activated carbon particles: Antitumor effectiveness against human colon carcinoma xenografts and acute toxicity in mice. *Journal of Pharmacology and Experimental Therapeutics* 311, 382-387.
- (147) Wong, S. C., Proefke, S. A., Bhushan, A., and Matherly, L. H. (1995) Isolation of human cDNAs that restore methotrexate sensitivity and reduced folate carrier activity of methotrexate transport-defective Chinese hamster ovary cells. *Journal of Biological Chemistry* 270, 17468-75.
- (148) Warlick, C. A., Sweeney, C. L., and McIvor, R. S. (1999) Maintenance of differential methotrexate toxicity between cells expressing drug-resistant and wild-type dihydrofolate reductase activities in the presence of nucleosides through nucleoside transport inhibition. *Biochemical Pharmacology* 59, 141-151.
- (149) Kimura, E., Nishimura, K., Sakata, K., Oga, S., Kashiwagi, K., and Igarashi, K. (2004) Methotrexate differentially affects growth of

suspension and adherent cells. *The international journal of biochemistry & cell biology* 36, 814-25.

- (150) Pinedo, H. M., Zaharko, D. S., Bull, J. M., and Chabner, B. A. (1976) The reversal of methotrexate cytotoxicity to mouse bone marrow cells by leucovorin and nucleosides. *Cancer Research* 36, 4418-24.
- (151) Rosenblatt, D. S., Whitehead, V. M., Matiaszuk, N. V., Pottier, A., Vuchich, M. J., and Beaulieu, D. (1982) Differential effects of folinic acid and glycine, adenosine, and thymidine as rescue agents in methotrexate-treated human cells in relation to the accumulation of methotrexate polyglutamates. *Molecular Pharmacology* 21, 718-22.
- (152) Widemann, B. C., Balis, F. M., and Adamson, P. C. (1999) Dihydrofolate reductase enzyme inhibition assay for plasma methotrexate determination using a 96-well microplate reader. *Clinical chemistry* 45, 223-8.
- (153) Liang, M., and Kovacs, J.A. (2000) Expression and characterization of recombinant human-derived *Pneumocystis carinii* dihydrofolate reductase. *Antimicrobial Agents and Chemotherapy* 44, 3092-3096.
- (154) Christie, R. J., and Grainger, D. W. (2003) Design strategies to improve soluble macromolecular delivery constructs. *Advanced Drug Delivery Reviews* 55, 421-437.
- (155) Yang, H., and Lopina, S. T. (2005) Extended release of a novel antidepressant, venlafaxine, based on anionic polyamidoamine dendrimers and poly(ethylene glycol)-containing semi-interpenetrating networks. *Journal of Biomedical Materials Research, Part A* 72A, 107-114.

- (156) Choi, W.-M., Kopeckova, P., Minko, T., and Kopecek, J. (1999) Synthesis of HPMA copolymer containing adriamycin bound via an acid-labile spacer and its activity toward human ovarian carcinoma cells. *Journal of Bioactive and Compatible Polymers* 14, 447-456.
- (157) Linebaugh, B. E., Sameni, M., Day, N. A., Sloane, B. F., and Keppler, D. (1999) Exocytosis of active cathepsin B enzyme activity at pH 7.0, inhibition and molecular mass. *European Journal of Biochemistry* 264, 100-109.
- (158) Stachowiak, K., Rodziewicz-Motowidlo, S., Sosnowska, R., Kasprzykowski, F., Lankiewicz, L., Grubb, A., and Grzonka, Z. (2004) Effect of antisense peptide binding on the dimerization of human cystatin C - gel electrophoresis and molecular modeling studies. *Acta Biochimica Polonica* 51, 153-160.
- (159) Duncan, R., Gac-Breton, S., Keane, R., Musila, R., Sat, Y. N., Satchi, R., and Searle, F. (2001) Polymer-drug conjugates, polymer-directed enzyme prodrug therapy (PDEPT) and (polymer-enzyme liposome therapy) PELT: basic principles for design and transfer from the laboratory to clinic. *Journal of Controlled Release* 74, 135-146.
- (160) Dubowchik, G. M., Mosure, K., Knipe, J. O., and Firestone, R. A. (1998) Cathepsin B-sensitive dipeptide prodrugs. 2. Models of anticancer drugs paclitaxel (taxol), mitomycin C and doxorubicin. *Bioorganic & Medicinal Chemistry Letters* 8, 3347-3352.

- (161) Etrych, T., Chytil, P., Jelinkova, M., Rihova, B., and Ulbrich, K. (2002) Synthesis of HPMA copolymers containing doxorubicin bound via a hydrazone linkage. Effect of spacer on drug release and in vitro cytotoxicity. *Macromolecular Bioscience* 2, 43-52.
- (162) Paranjpe, P. V., Chen, Y., Kholodovych, V., Welsh, W., Stein, S., and Sinko, P. J. (2004) Tumor-targeted bioconjugate based delivery of camptothecin: design, synthesis and in vitro evaluation. *Journal of Controlled Release* 100, 275-292.
- (163) Paranjpe, P. V., Stein, S., and Sinko, P. J. (2005) Tumor-targeted and activated bioconjugates for improved camptothecin delivery. *Anti-Cancer Drugs* 16, 763-775.
- (164) Trouet A., M. M., Baurain R., Campeneere D.D.D. (1982) A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier conjugate: in vitro and in vivo studies. *Proc. Natl. Acad. of Sci. USA* 79, 626-629.
- (165) Nichifor, M., Schacht, E. H., and Seymour, L. W. (1996) Macromolecular prodrugs of 5-fluorouracil. 2: Enzymic degradation. *Journal of Controlled Release* 39, 79-92.
- (166) Dubowchik, G. M., Firestone, R.A. (1998) Cathepsin B-sensitive dipeptide prodrugs.1.A model study of structural requirements for efficient release of doxorubicin. *Bioorganic & Medicinal Chemistry Letters* 8, 3341-3346.

- (167) Vasey, P. A., Kaye, S. B., Morrison, R., Twelves, C., Wilson, P., Duncan, R., Thomson, A. H., Murray, L. S., Hilditch, T. E., Murray, T., Burtles, S., Fraier, D., Frigerio, E., and Cassidy, J. (1999) Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: First member of a new class of chemotherapeutic agents-drug-polymer conjugates. *Clinical Cancer Research* 5, 83-94.
- (168) Rubino F.M. (2001) Separation methods for methotrexate, its structural analogues and metabolites. *Journal of Chromatography B* 764, 217-254.
- (169) Lemieux, B., Percival, M. D., and Falgueyret, J.-P. (2004) Quantitation of the lysosomotropic character of cationic amphiphilic drugs using the fluorescent basic amine Red DND-99. *Analytical Biochemistry* 327, 247-251.
- (170) Ohkuma, S., and Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proceedings of the National Academy of Sciences of the United States of America* 75, 3327-31.
- (171) Panyam, J., Zhou, W.-Z., Prabha, S., Sahoo, S. K., and Labhasetwar, V. (2002) Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. *FASEB Journal* 16, 1217-1226.
- (172) Dervieux, T., Lein, D.O., Marcelletti, J., Pischel, K., Smith, K., Walsh, M., Richerson, R. (2003) HPLC determination of erythrocyte methotrexate

polyglutamates after low-dose methotrexate therapy in patients with rheumatoid arthritis. *Clinical Chemistry* 49, 1632-1641.

- (173) Fan J., P. L. E., Vitols K.S., Huennekens F.M. (1990) Visualization of folate transport proteins by covalent labeling with fluorescein methotrexate. *Advances in Enzyme Regulation* 30, 3-8.
- (174) Gaudray P., T. J., Wahl G.M. (1986) Fluorescent methotrexate labeling and flow cytometric analysis of cells containing low levels of dihydrofolate reductase. *The Journal of Biological Chemistry* 261, 6285-6292.
- (175) Fan J., P. L. E., Vitols K.S., Huennekens F.M. (1991) Affinity labeling of folate proteins with the N-Hydroxysuccinimide ester of the gamma-isomer of fluorescein-methotrexate. *Biochemistry* 30, 4573-4580.
- (176) Assaraf Y.G., S. L. C., Schimke R.T. (1989) Characterization by flow cytometry of fluorescein-methotrexate transport in chinese hamster ovary cells. *Cytometry* 10, 50-55.
- (177) Lee, J. H., Lim, Y., Choi, J.S., Choi, M., Yang, C., Park, J. (2003) Quarternized polyamidoamine dendrimers as novel gene delivery system: relationship between degree of quarternization and their influences. *Bull. Korean Chem Soc.* 24, 1637-1640.
- (178) Lee, J. H., Lim, Y., Choi, J.S., Lee, Y., Kim, T., Kim, H., Yoon, J.K., Kim, K., Park, J. (2003) Polyplexes assembled with internally quarternized PAMAM-OH dendrimer and plasmid DNA have a neutral surface and gene delivery potency. *Bioconjugate Chemistry* 14, 1214-1221.

ABSTRACT**SYNTHESIS OF POLYAMIDOAMINE DENDRIMER-METHOTREXATE
CONJUGATES AND *IN VITRO* CHARACTERIZATION**

by

SEZEN GURDAG

August 2006

Advisor: Dr. Rangaramanujam M. Kannan

Major: Chemical Engineering

Degree: Doctor of Philosophy

Dendritic polymers have a wide array of biological applications spanning from drug and gene delivery to imaging mainly due to highly versatile densely packed end functional groups. We investigated the importance of surface charge imparted by the end groups on the *in vitro* activity of polyamidoamine (PAMAM)-Methotrexate (MTX) conjugates in isogenic cell lines of human acute lymphoblastoid leukemia CCRF-CEM and Chinese hamster ovary CHO cells, with drug-sensitive and -resistant sublines. Amide-bonded conjugates were prepared in a dicyclohexylcarbodiimide (DCC)-mediated coupling scheme utilizing either $-NH_2$ or $-COOH$ group on MTX as the linking site to afford anionic conjugate A (PAMAM-G2.5-COOH) and cationic conjugate B (PAMAM-G3-NH₂), respectively. The *in vitro* activity tests demonstrated substantially higher activity of anionic conjugate A not only compared to cationic conjugate B but also to the free drug. This activity enhancement of the conjugated drug was more

pronounced in MTX-resistant cell lines with 24-fold reduction in the IC₅₀ value in RII cells compared to the free drug regardless of the loss of the RFC function. Exploitation of the salvage pathways by co-incubating either with the combination of adenosine and thymidine or leucovorin ensured that conjugated-drug still relies on the inhibition of dihydrofolate reductase (DHFR) activity as the sole mode of antimetabolite action. Contrarily, conjugate B did not show appreciable cytotoxicity on any of the cell lines. The significant differences in the cytotoxicities may be suggestive of the involvement of lysosomes retaining the conjugates for different periods of time determined by the surface charge of the native dendrimer to release the drug to various levels. Our preliminary drug release data suggested that an analogous anionic conjugate liberated approximately 7% of the conjugated-MTX in the presence of the enzyme, cathepsin B. Due to the precipitation of the cationic conjugate, similar experimentation could not be performed. Furthermore, to ease the accessibility of the linking site to the enzyme, we synthesized spacer-linked PAMAM-MTX conjugates with ester bonds, from which we expect to have higher drug release and eventually higher *in vitro* activity.

AUTOBIOGRAPHICAL STATEMENT

Name: Sezen Gurdag

Date of Birth: May 12, 1977

Education:

- Ph.D., Chemical Engineering, Wayne State University, Detroit, MI, 2006
- M.S., Chemical Engineering, Bogazici University, Istanbul, Turkey, 2001
- B.S., Chemical Engineering, Istanbul Technical University, Istanbul, Turkey, 1998

Experience:

- Graduate Research Assistant, Wayne State University, Detroit, MI, 2002-2006
- Graduate Teaching Assistant, Wayne State University, Detroit, MI, 2001-2002
- Graduate Research Assistant, Bogazici University, Istanbul, Turkey, 2000-2001
- Student Research Assistant, Bogazici University, Istanbul, Turkey, 1999-2000



