DEVELOPMENT OF GEMCITABINE AND CLOFAZIMINE CO-LOADED LIPOSOMAL DELIVERY SYSTEM FOR OSTEOSARCOMA TREATMENT

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DEVELOPMENT OF GEMCITABINE AND CLOFAZIMINE CO-LOADED LIPOSOMAL DELIVERY SYSTEM FOR OSTEOSARCOMA TREATMENT

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ABSTRACT

DEVELOPMENT OF GEMCITABINE AND CLOFAZIMINE CO-LOADED LIPOSOMAL DELIVERY SYSTEM FOR OSTEOSARCOM TREATMENT

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New strategies for more effective treatments need to be developed to cure osteosarcoma patients. Current study was designed to produce a dual drug delivery system with Gemcitabine (GEM) and Clofazimine (CLF) co-loaded liposomes against osteosarcoma diseases. GEM is a second line therapy for osteosarcoma and with combinational use of another regimen it could be much more effective. CLF, an antimycobacterial agent, has been recently recognized as effective on cancer treatment and it was suggested to act on osteosarcoma disease since Wnt signaling pathway is one of the pathway that CLF affects. To prevent the increased toxicities of both agents and control their biodistribution, they were encapsulated into PEGylated liposome. The liposomes were produced in sizes to be administered intravenously (D: 100-150 nm). With the small size of liposomes the tumor cells could be targeted passively by the liposomal system via enhanced permeation and retention effect. Liposomal formulations showed high GEM and CLF loading capacities (90.1±1.16 and 80.1±1.45) and very slow release of GEM and CLF was observed. The cytotoxicity of liposomal formulations were investigated by MTT test and toxicity improvement was

observed for co-loaded liposomes compared with single agent loaded liposomes after 24 h incubation with Saos-2 cells. Free drugs treated groups showed higher cytotoxicities than the liposomal formulations. Flow cytometry results were similar; there were more cells in apoptotic stage in GEM/CLF combinational groups (both free and liposomal formulations) than single agent groups and toxicity of GEM/CLF co-loaded liposomal formulation was numerically lower than the free GEM/CLF group. Cell cycle analysis indicates accumulation of cell population at G0/G1 phase was high when treated with liposomes co-loaded with GEM and CLF. GEM and CLF had apoptotic effects on Saos-2 cells as mitochondrial membrane depolarized cells' ratio increased. Caspase-3 positive cells and early apoptotic cells percentage was increased compared to untreated groups at 24 h. GEM showed a synergistic effect with CLF on Saos-2 cells. With these results, it can be suggested liposomal formulation co-loaded with GEM and CLF would have potential for treating osteosarcoma.

Keywords: Soas-2 cell line, Osteosarcoma, Gemcitabine (GEM), Clofazimine (CLF), Liposome

ÖZ

OSTEOSARKOM TEDAVİSİ İÇİN GEMSİTABİN VE CLOFAZİMİN YÜKLÜ LİPOZOMAL SALIM SİSTEMİ GELİŞTİRİLMESİ

Çalışkan, Yağmur
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Osteosarkom hastalarını tedavi etmek için daha etkili ve yeni tedavi yöntemlerinin geliştirilmesi gerekmektedir. Bu çalışma Osteosarkom hastalığına karşı gemsitabin ve klofazimin yüklenmiş lipozom bazlı ikili salım sisteminin üretimi üzerine tasarlanmıştır. Gemsitabin osteosarkom için ikinci basamak şeklinde bir tedavidir ve farklı bir kemoterapötik ilaçla birlike kullanımıyla osteosarkom üzerinde çok daha etkili olması beklenmektedir. Klofazimin antibakteriyel ajan olmasının yanısıra kanser tedavisinde uygulanmaya başlanmıştır ve Wnt sinyal yolu klofaziminin hedeflediği ve etki ettiği yollardan biri olduğu için osteosarkom üzerinde etkili olması beklenmektedir. Kemoterapötik maddelerin yüksek toksisitesini engellemek ve içeriğin salımını kontrol etmek için, PEG'li lipozomlar yapılmıştır. Bu lipozomlar damar yoluna verilebilecek büyüklüklerde oluşturulmuştur (Ç: 100-150 nm). Lipozomların küçük boyutlu olmasıyla birlikte tümörlü hücreler RES sistemi tarafından pasif şekilde hedef alınabilinmiştir. Lipozomal grupların toksisiteleri MTT ile test edilmiş ve sonuçlar akış sitometre analizi ile pekiştirilmiştir. Saos-2 hücreleri farklı lipozomal gruplar ile 24 saat boyunca inkube edilmiştir.

Gemsitabin ve klofazimin için yüksek yükleme kapasitesi hesaplanmış (90.1±1.16 ve 80.1±1.45) ve her iki ilacın çok yavaş salındığı gözlemlenmiştir. MTT testi ile gemsitabine ve klofazimin yüklü lipozom formülasyon gruplarında sitotoksisitede artış olduğu gözlenmiştir. Serbest ilaçlarla tedavi edilen gruplar, lipozomal formülasyonlara kıyasla daha yüksek sitotoksisite gösterdiler .Akış sitometre analizi ile benzer sonuçlar elde edilmiştir, gemsitabin ve klofazimin yüklü lipozom gruplarında diğer gruplara göre apoptoz hücre oranını anlamlı bir şekilde arttığı gösterilmiştir. Hücre döngüsü analizi sonuçlarına göre, gemcitabin ve klofazimin yüklü lipozomlar Saos-2 hücrelerinin G0/G1 fazında birikimesine neden olmuştur. Gemsitabin ve klofazimin yüklü lipozomlar Saos-2 hücrelerini apoptoza götürmüş ve mitokondri zarının depolarize olmasına sebep olmuştur. Kaspaz-3 pozitif hücreleri ve apoptotik ölümün erken fazlarında bulunan hücrelerin yüzdesi 24 saatte kontrol gruplarına kıyasla artmıştır. Bu sonuçlar, gemsitabin ve klofazimin yüklü lipozomal formülasyon kullanımının osteosarkom tedavisi üzerinde potensiyele sahip olduğunu göstermektedir

Anahtar kelimeler: Soas-2 hücre hattı, Osteosarkom, Gemsitabin, Klofazimin, Lipozom

to my sisters for always being with me and supporting me in all conditions

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVATIONS

ANOVA: Analysis of Variance

CHOL: Cholesterol

DLS: Dynamic Light Scattering

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl Sulphoxide

DNA: Deoxyribonucleic acid

DPPC: 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine

DSPE-mPEG(2000): 1,2 - distearoyl-sn-glycero - 3 - phosphoethanolamine - N - methoxy (polyethylene glycol) - 2000

GEM: Gemcitabine

CLF: Clofazimine

EE: Encapsulation efficiency

ELISA: Enzyme-Linked immunosorbent assay

EPR: Enhanced permeability and retention

FBS: Fetal bovine serum

LUVs: Large unilamellar vesicles

MLVs: Multilamellar vesicles

mPEG: Methoxy polyethylene glycol

MTT: 3-(4,5-dimethylthiazol–2-yl)-2,5-diphenyltetrazolium bromide

MW: Molecular weight

PBS: Phosphate buffered saline

PDI: Polydispersity index

PEG: Polyethylene glycol

RES: Reticuloendothelial system

RT: Room temperature

SUVs: Small unilamellar vesicles

TEM: Transmission electron microscopy

UV: Ultraviolet

Z-average diameter: Average hydrodynamic diameter

CHAPTER 1

1. INTRODUCTION

1.1. Osteosarcoma

Cancer is a disease that is caused by uncontrolled growth of cells. Osteosarcoma is the cancer in the bones which is also called osteogenic sarcoma. It is the most common type of cancer in the bones and after lymphoma and brain tumors it is the third most common cancer type in teens (Kushner et al., 1996). Generally, osteosarcomas occur in children and young people but still it can be seen at any age. Osteosarcoma occurs in bones where the growth rate is fast such as near the ends of the long bones, around knee in the distal femur and in the proximal tibia. Especially in adults, osteosarcoma develops in the bones of the pelvis, shoulder and jaw. Histologically, osteosarcoma is composed of pleomorphic spindle-shaped cells which produce the osteoid matrix which is weaker than the healthy bone matrix (Pan, Chan, & Chia, 2010). The occurrence rate of osteosarcoma is higher in males than females. The genetics is an important reason for growth of the osteosarcoma. In the osteosarcoma patients, the mutation rate is about 3-4 % (Kushner et al., 1996). Other factors that may lead to osteosarcoma are environmental factors such as physical, chemical and biological agents. Fuchs and Pritchard observed that chemicals, radiation and miscellaneous are the causative agents of osteosarcoma (Fuchs & Pritchard, 2002). Viral etiology of osteosarcoma was reported. The Rous sarcoma virus includes a gene which has a potential to be an oncogene of osteosarcoma (Bunker, Magarkar, & Viitala, 2016). The misregulations of growth factors and transcription factors can cause the

osteosarcoma. In addition, radiation plays a critical role in cancer formation and histological findings were showed about radiation effect on osteosarcoma (Fuchs & Pritchard, 2002).

There are some symptoms of osteosarcoma that can be seen weeks to months before the diagnosis such as pain, swelling, decreased joint motion, fractures in bones. The most common one is pain according to a clinical study in 2010, at first pain may not be constant and may be worse at night and increases with the activity. In addition, if the tumor site is in the leg, it may cause limp. Swelling is another common symptom; it may be seen weeks after the pain starts (Pan et al., 2010).

1.1.1. Treatment of Osteosarcoma

Limb salvage surgery is the standard treatment of osteosarcoma. Limb salvage is a surgery to control the osteosarcoma but still in some cases amputation is also valid. Limb salvage surgery is the removal of all tumor part and replacing with an implant which can be made with metal, a bone allograft or combination of bone graft or metal implant Not only removal of the tumor site from the body but also function of the limb is important. Therefore, surgery is considered after medication. (Ferguson & Goorin, 2001). On the other hand, amputation is the removal of all organ since sometimes limb salvage cannot be done. There is still a need for developing functional organ and reconstruction of the tumor site. After the limb salvage surgery, there are some late effects of treatment such as nonunion, limb-length discrepancy, prosthetic loosening and contractures. After the surgery, the ends of replaced bone might not be healing completely and nonunion occurs between bone ends. Generally, tumors are located near the bone plate where bone grows. After the removal of the bone plate following limb salvage surgery, reconstructed bone cannot regrow and limb-length discrepancy occurs. Especially for physically inactive people, after surgery, tightening of joint can occur

and for physically active people, prosthetic loosening can be observed. These are some of the disadvantages of limb salvage surgery; yet amputation is more disadvantageous since it covers the removal of total organ (Tiwari, 2012). For both surgeries, there is a risk of infection especially for repeated limb salvage surgeries which is a disadvantage of all surgeries (Tiwari, 2012).

Neoadjuvant chemotherapy, the surgical resection was followed by an adjuvant chemotherapy is the common treatment in osteosarcoma. The chemotherapy is the valuable part of the treatment (Luetke, Meyers, Lewis, & Juergens, 2014). Until today, the overall survival rate could not be increased so much with the common treatment strategies. Neoadjuvant chemotherapy is an important step since the increase in the response at that step means increase in the survival rates compared to poor responses (Ferguson & Goorin, 2001). In addition, before the surgery, the neoadjuvant chemotherapy decreases the tumor volume. Therefore, chemotherapy plays a standard role in the treatment of osteosarcoma. Recently, cisplatin is commonly used in the treatment of osteosarcoma but it causes systemic toxicities. Wilkins et al. presented that 87% of patients with localized osteosarcoma had better response to the cisplatin treatment than other patients who were treated with methotrexate and doxorubicin. The survival rate increased to "10 year" with the combination of cisplatin with other regimens like doxorubicin or methotrexate.

After the surgical removal of tumor, the recurrence of the disease is 80-90 % which is probably caused by microscopic metastasis of osteosarcoma (Ferguson & Goorin, 2001). This outcome shows the necessity of usage of anticancer agents for osteosarcoma. In literature, there are studies reporting that the adjuvant chemotherapy is effective treatment in osteosarcoma patients, but there are also conflicting results about effectiveness of adjuvant chemotherapy. In a study, after removal

of tumor part by surgery, the chemotherapy was given to a group of patient. According to this study, there was a significant difference between results of only surgery and adjuvant chemotherapy after the surgery (Ferguson & Goorin, 2001). In this study, the time of diagnosis was different in patients. There are other studies to show effectiveness of the surgical resection plus adjuvant chemotherapy. There was an increase in the survival rate of patients that had surgery followed by adjuvant chemotherapy, and the overall survival rate reached to 60 %, while it was around 40 % with only surgery (Ferguson & Goorin, 2001).

Generally, anticancer agents for low grade osteosarcoma patients is not recommended; for all stages of high grade osteosarcoma, chemotherapy is needed. For nonmetastatic osteosarcoma, 2-3 cycles of chemotherapy before the operation and 3-4 cycles after the operation is recommended (Choy, 2015).

First line treatment of osteosarcoma includes different combinations of doxorubicin, cisplatin, methotrexate, ifosfamide, etoposie and epirubicin.In the doxorubicin and cisplatin treatment, 75 mg/m^2 doxorubicin with 100 mg/m^2 cisplatin is applied on day 1, and the cycle is repeated every 21 days. In the combination of methotrexate, cisplatiın, and doxorubicin treatment, 8-12 mg/m^2 methotrexate is given over 4 h on weeks 1, 5, 6, 13, 14, 18, 19, 23, 24, 37 and 38, alternating 60 mg/m^2 cisplatin and doxorubicin 37.5 mg/m^2 on second day and on week 2, 7, 25 and 28. For ifosfamide and etoposide, 9 g/m^2 ifosfamide over 5 day is given with 100 mg/m^2 etoposide daily for five day. Epirubicin 90 mg/m^2 and cisplatin 100 mg/m^2 on plus 2g/m^2 on day 1 and once in every 21 days can be a choice of treatment as first line (Luetke et al., 2014).

The second line therapies are suggested as; Docetaxel and gemcitabine, cyclophosphamide and etoposide; Cyclophosphamide and topotecan; Gemcitabine alone; Ifosfamide and etoposide; Ifosfamide alone; carboplatin, and etoposide, methotrexate, etoposide, and ifosfamide. Docetaxel 100 mg/m^2 on day 8 and every 21 days with gemcitabine 675

mg/m^2 on days 1 and 8 can be given. cyclophosphamide (500 mg/m^2) with 100 mg/m^2 etoposide for 5 days is another treatment. Gemcitabine can be used alone as 1000 mg/m^2 dose weekly for seven weeks. Etoposide 100 mg/m^2 can be used with 3.5 g/m^2 ifosfamide as a second line treatment. Ifosfamide can be used 2.5 mg/m^2 daily with carboplatin 400 mg/m^2 and 100 mg/m^2 etoposide (Luetke et al., 2014). Generally, for localized but unresectable osteosarcomas, chemotherapy could provide long term control of the disease. For patients who give poor pathological response to these anticancer agents, new drug delivery systems need to be developed to overcome drug resistance and result in much more potent anticancer effect.

1.2. Mechanism of Action and Clinical Applications of Agents Used in This Study

1.2.1. Gemcitabine

GEM is a deoxycytidine analog that is highly specific for deoxycytidine and it is one of the widely used chemotherapy agent. The mechanism of GEM is related with DNA incorporation. Briefly, it is activated by deoxycytidine kinase to dFdC-5′-monophosphate (dFdCMP) or deaminated by deoxycytidine deaminase to 2′, 2′-difluorodeox-uridine (dFdU). Then, dFdCMP is further metabolized and dFdC-5’- diphosphate and dFdC-5’-triphosphate were produced, followed by incorporation into DNA, it results in chain termination (Brown, Weymouth-Wilson, & Linclau, 2015). There are some similarities in structure of GEM and other agents like Ara-C, but, GEM has much broader antitumor activity. Depending on the cancer type, the effective dose can change, but commonly, 1000 mg/m^2 IV is used weekly in practice for 3 weeks followed by one week rest (Toschi, Finocchiaro, Bartolini, Gioia, & Cappuzzo, 2005).

GEM has been used in different cancer types as alone or in combination with other chemotherapeutic agents. According to the World Health Organization (WHO), GEM is metabolized well but its toxicity can change with the infusion time; with the standard 30 min infusion only 1.6 % of the cases showed grade 4 anemia, 2.9 % of them had grade 4 neutropenia and Mild and moderate edema was reported for 23.8 % of the cases. While grade 3 thrombocytopenia was seen in 6 % and hemorrhage occurred in 0.6 %, cutaneous toxicity was seen in 27.4 % of the patients, in 3.9 % of patients pulmonary toxicity was reported (Saif & McGee, 2005). There are non-hematological side effects such as nausea, vomiting, diarrhea, asthenia, anorexia.

In literature, there is no report on specific drug interaction for GEM. The most commonly used drug with GEM is cisplatin. These two anticancer agents showed a synergistic interaction by forming a platinum-DNA adduct; incorporation of dFdC by dFdCTP in DNA and inhibition of exonuclease and DNA repair (Moorsel, 1999). Etoposide is another agent that is widely used with GEM and again shows a synergism. Combination of both compounds was suggested to increase the phosphorylation of dFdC that leads to increase in the dFdCTP accumulation and DNA strand break is also favored. DNA strand break probably occurred because of inhibition of DNA repair by dFdC which was increased with combinational usage of both drugs (Moorsel et. al., 1999).

GEM is one of the most effective anticancer agent and it is used as a first line therapy for lung cancer, pancreatic, bladder, breast and ovarian cancer. It has a low toxicity profile compared to the other agents, and combinational usage with other agents decrease the dose amount, thereby lowering the existing side effects. The most common usage of GEM (1000 mg/m^2 per 3 weeks and 1 week rest) resulted in the least toxicity profile. However, for much more

effective results, the prolonged usage is recommended since the catalyzation of triphosphate is done by deoxycytidine kinase and this enzyme is saturated at over 30 min infusion period. Therefore, by increasing the infusion time the activity is increased without increasing the dose amount (Toschi et al., 2005).

1.2.2. Clofazimine

CLF was initially developed for the treatment of tuberculosis but it did not show effective results in an animal model (Barry et al., 1957). It was realized in that study that CLF was concentrated in macrophages which is suggested as a pathway to kill intracellular parasites. Therefore, it was started to be used to treat leprosy. In some studies, it was also shown that CLF was effective on multi-drug resistant strains of *Mycobacterium tuberculosis* and it was a sign that it could also be used in the treatment of cancer. Actually, CLF binds to guanine base of DNA and blocks the template function of DNA during replication and the proliferation can not occur (Xu, Jiang, & Xiao, 2012). It also increases the phospholipase A2 which makes the release of lysophospholipids that are toxic for bacterial proliferation (Ren et al., 2008). There are some immunosuppressive effects of CLF. Generally, immunosuppressive drugs target the pathway of calcium signaling cascade. They induce the complex formation with calcium and blocks the calcineurin which is the protein that makes calcium influx to the plasma. This inhibition leads to prevention of dephosphorylation with the nuclear translocation of nuclear factor of activated T cell (Ren et al., 2008). The pathway causes the inhibition of expression of interleukin gene-2. By inhibiting the calcium pathway, Kv1.3 channels are blocked by CLF. Kv1.3 is highly found in memory T cells of multiple sclerosis patients, psoriasis and type 1 diabetes. This shows that CLF can be used as immunosuppressant agent in addition to acting as

antimicrobial agent. In addition to that, it has the antileprosy and anti-inflammatory activity. It is used against *M. leprae*. It is also effective in chronic skin ulcers because of its antimicrobial effects and partly effective on diseases that are caused by *Mycobacterium avium* (Arbiser & Moschella, 1995).

CLF is a lipophilic dye and it accumulates in fat, bile, macrophages, reticulo-endothelial system and skin tissues. Therefore, the most common adverse events are skin discoloration, lymphedema, and decrease in sweating and tearing (Cariello et. al., 2015).

Recently, it is started to be used in cancer therapy. CLF inhibits the Wnt signaling. Wnt signaling plays major role in cell fate, proliferation and migration in the developmental stages and it is not active in adult stages. So, reactivation of it may cause some disorders especially like cancer. The Wnt signaling can be seen in the b-catenin, the planar cell polarity and the calcium related signaling. The over reactivation of Wnt signaling pathway is seen in many cancer types including osteosarcoma. Interaction between Wnt and its receptor increases as a result of increase in the concentration of both receptor and ligand. Increased interaction between Wnt and its receptor was seen in aggressive triple-negative breast cancer, so use of CLF might be an effective treatment for many cancers (Koval et al., 2014).

Lots of studies have demonstrated that Wnt signaling pathway is involved in bone cancers. Particularly, for differentiation and proliferation of cells, b-catenin plays a critical role and the overexpression of Wnt leads to activation of Wnt/b-catenin pathway that is closely associated with many cancer types as well as osteosarcoma (Cai, 2014).

Kv1.3 was overexpressed in chronic lymphocytic leukemia patients (B-CLL) compared to the healthy people. Therefore, CLF can be used to treat B-CLL patients. Kv 1.3 is expressed in the plasma membrane and also in the inner mitochondrial membrane. Mitochondrial channels are the target of Bcl-2 associated X protein (Bax). In apopototic cells Kv 1.3 provides a physical interaction between apoptosis proteins. When the Bax and mitochondrial Kv1.3 come together, the reactive oxygen species (ROS) are produced and cytochrome c is released. CLF inhibits this pathway by blocking the Kv1.3 channel (Leanza et al., 2013). In addition, without existence of Bax protein, CLF has the capability to activate the intrinsic apoptotic path. In the literature, it was shown that in a mouse melanoma model, CLF reduced tumor size by 90 % (Koval et al., 2014). These results indicate that CLF may be a good treatment regimen for cancer.

In literature there are a few publications about combination studies CLF with a drug. One of them was published in 2015 by Koot et al. They used Riminocelles (lipopolymeric micelle) co-encapsulating with synergistic fixed-ratio combination of paclitaxel (PTX) and CLF. Although they showed the synergistic effect of PTX and CLF the delivery system they used was not as successful as they planned. CI values for PTX: CLF ratios with a greater proportion of CLF were found to be synergistic (CI<1) at all simulated fa levels (0.1–0.9) at the intrinsic Pgp-expressing colorectal adenocarcinoma (COLO 320DM, ATCC CCL-220). The trend is for synergy to be greater at lower fa levels. They concluded that lipopolymeric micellular delivery system requires improvement so as to maintain and selectively deliver the fixed-ratio drug combination (Szabo, 2015).

1.3. Liposomes

Liposomes can be a single membrane, a unilamellar (small unilamellar vesicle (SUV), large unilamellar vesicle (LUV)) or multilamellar vesicle (MLV). They were first described by British hematologist Alec D. Bangham (Bangham, 1964). He explained liposome formation based on the amphiphilic nature of the phospholipids and their behavior in aqueous environment. Phospholipids tend to form lipid bilayer consisting of two sheet of lipid molecules with polar heads align through the aqueous part and hydrophobic tails come together through the inside of the bilayer, and the polar heads at the innerface comes together again and produce a hydrophilic core as shown in Figure 1.1 (modified from https://global.britannica.com/science/liposome). These properties of liposomes provide encapsulation of both hydrophilic and hydrophobic molecules to the liposomes. The water soluble molecules can be encapsulated to hydrophilic core or to the hydrophilic surface, water insoluble molecules can be entrapped at the hydrophobic part. With these properties, liposomes could be used in a wide range of different areas such as cosmetics, food industry, and medicine (Bozzuto & Molinari, 2015).

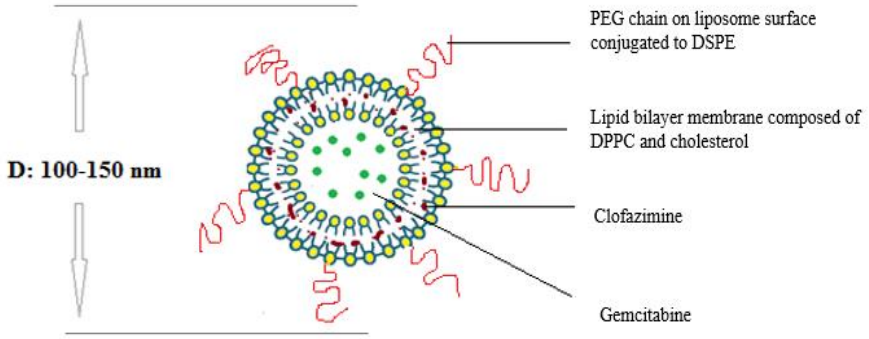


Figure1.1 Liposome bilayer structure (modified from https://global.britannica.com/science/liposome).

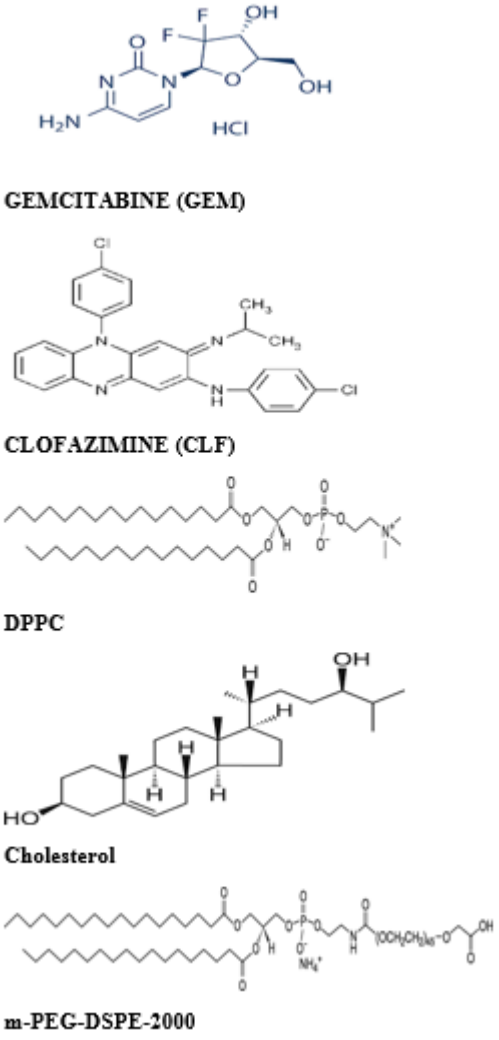


Figure 1.2 Chemical structures of GEM, CLF, DPPC, Cholesterol, and m-PEG-DSPE

1.3.1. Liposomal Drug Delivery Systems

Gregory Gregoriadis was the first scientist who proposed the use of liposomes as drug delivery systems and also showed their potential for targeting strategies. By the end of 70s they were accepted as a delivery system for anticancer agents (Perrie, 2008). By the end of the 80s they started to be used for topical drug delivery, gene therapy and anti-inflammatory agents' delivery. With these developments, the major barrier to overcome was their short bioavailability owing to short

circulation half-life. PEGylation on their surface became a standard application for solving this problem with liposomes and they were called stealth liposomes (Klibanov, 1990). The first approved drug loaded liposome for clinical use was Doxil (doxorubicin loaded PEGylated liposome) (Barenholz, 2012). PEGylation may lead to an increase in uptake of liposomes by cells. According to a study; different chain length of a PEG molecule could result a decrease or increase in tumor cell uptake of liposomes (Sadzuka, 2003).

Then, liposomes were started to be used in a wide range of different treatment applications such as, cardiovascular disease, dermatological disease, ocular disease and tuberculosis (Wang, Huang, 1989). But still, they are used mainly to carry the chemotherapy agents (Wang, Huang, 1989). The main aim of drug research is reaching to the maximum effectiveness of treatment with minimum toxicity. Many conventional drugs are either too difficult to reach the target site at enough concentration or too toxic, without proper dose profile in blood circulation (Bunker et al., 2016). To overcome these problems, nanotechnology offers new drug delivery systems like liposomes. In the literature, there are many liposome studies as a drug delivery system (Zhou, Zhao, 2015). The drug encapsulation is done in different ways according to the properties of the drug molecules. Hydrophobic ones are encapsulated within the lipid bilayer, hydrophilic ones are loaded into the hydrophilic core. The most common method to encapsulate drug molecules is lipid film hydration technique. Hydrophobic drugs could be mixed with lipid mixture in nonpolar solution and hydrophilic drugs could be added in hydration solution of the lipid film. Then through extrusion process, nanosized liposomes could be obtained (Bunker et al., 2016). For intravenous route of administration the size of the liposomes are aimed around 200 nm. (X. Wang et al., 2015).

Liposomes are good drug carrier systems which are biodegradable, biocompatible, non-toxic and non-immunogenic. Their usage area is

wide and they mostly present good drug release profiles. The composition of liposomes could be changed according to the need. Generally, they are PEGylated to increase the circulation time in blood and to prevent the uptake by reticuloendothelial system and macrophages (Gasselhuber, Dreher, Rattay, Wood, & Haemmerich, 2012). The lipid composition is generally arranged to keep the drug encapsulation at the highest possible value and to control the release at the same time. Another important issue to increase the bioavailability is the surface charge of long circulated liposome should be arranged accordingly since slightly negative liposomes have higher half-life in bloodstream than neutral and positively charged liposomes (Gabizon, 1988).

There are many advantages of liposomes other than possibility of encapsulation of both water and lipid soluble drugs. For example they are nonionic, biodegradable and they can improve protein stabilization, and alter the pharmacodynamics and pharmacokinetics of drugs by targeting and/or controlling their release rate (Mansoori, Agrawal, Jawade, & Khan, 2012). The major disadvantage of plain liposome formulation is their short blood circulation time due to Reticuloendothelial system (RES). This can be overcame with the modification with molecules like Polyethylene glycol (PEG) to decrease their opsonization during blood circulation and thereby preventing their recognition by macrophages of the RES.

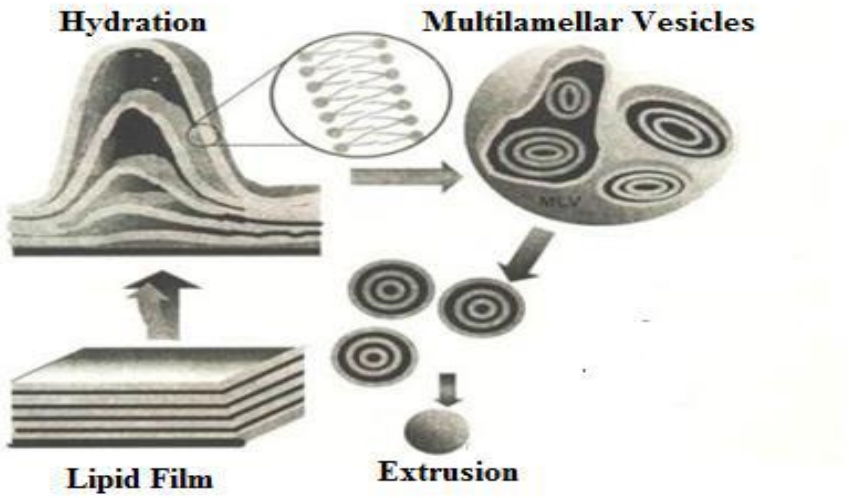


Figure 1.3. Illustration of liposome formation with lipid film hydration method (Mansoori et al., 2012)

1.3.2. In vivo Properties of Liposomes

1.3.2.1. Route of Administration, Stability, and Biodistribution

The liposomes can be administered in various ways according to the intended application. The possible routes of administration are oral, nasal, intravenous, and dermal. Generally, nutrient supplies are given orally and the cosmetic molecules are taken by dermal route owing to good penetrating ability of liposomes in various skin layers. For respiratory system treatments, liposomes are given through nasal route (Mansoori et al., 2012).

Mostly, liposomes are designed to administer intravenously to bypass the gastrointestinal system. The conventional liposomes are easily captured by reticuloendothelial system, therefore the surfaces of the liposomes are modified with polymers like polyethylene glycol (PEG) as mentioned before. PEGylation of liposomes increases the bioavailability and decreases the interaction with the

plasma proteins. It increases the stability of the liposomes while showing good cellular binding (Immordino & Cattel, 2016).

The conventional liposomes can reach their target tissues by passive targeting method via the enhanced permeability and retention effect (EPR) especially for cancer targeting purposes. The small molecules tend to accumulate especially at the tumor site via EPR effect, it is called passive targeting since the vascularization is increased and capillaries are discontinous with high permeability at these sites. Liposomes could easily pass from these vessels and be kept there because of the absence of lymphatic drainage as shown in Figure 1.4 (Gabizon et al., 1997).

Lipid composition, bilayer fluidity, size, surface charge and pH sensitivity are the major factors that affect the biodistribution of the liposomes in body. In vivo structural changes of liposomes affect their stability in blood. Among these, the lipid composition is one of the most important factor for stability and release characteristics. According to the specific treatment and target site properties the lipid choice should be done. For example saturated phospholipids are more stable and can resist to the oxidation and hydrolysis. In the liposome preparation steps, the temperature should be arranged according to the phase transition temperature of lipids since above this temperature spherical structure can be achieved. This is one of the important criteria for phospholipid selection since each phopholipid has unique phase transition temperature. Also, the heat stability of the encapsulated molecule needs consideration as it might be affected from this phase transition temperature during liposome formation. So this might be another criteria for phospholipid selection (Shailesh, Neelam, Sandeep, & Gd, 2009). Thus, overall system design has to be done with considering specific

component characteristics like lipid composition, bilayer fludity, size, surface charge and pH sensitivity.

To modify the membrane fluidity, elasticity and permeability, cholesterol is usually added to the lipid mixture when preparing the liposomes. Cholesterol acts as stabilizer of phospholipids and gives interconnectivity between phopholipids. The ratio of cholesterol in the lipid composition can be arranged according to the need for stability and release kinetics (Anwekar, Patel, & Singhai, 2011).

Size and surface charge are two other parameters affecting the bioavailability and stability of liposomes since uptake by RES is affected mainly by size and surface charge properties. In the literature, it was determined that, when the size of liposomes is between 70 nm and 300 nm, their bioavailability is increased. Charge of liposomes can be arranged according to the intended application by choosing negatively charged or positively charged phospholipids. The highest circulation time in blood could be achieved in the neutral liposomes. Therefore, modification of liposome surface like PEGylation can affect the surface charge of the liposomes. These two parameters can be changed to optimize the effectiveness of the drug loaded liposomes' in terms of biodistribution and release properties (Anwekar et al., 2011).

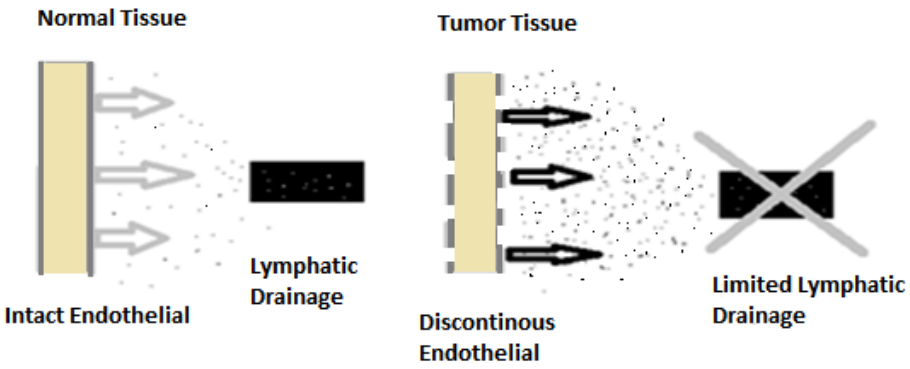


Figure 1.4. Accumulation at the Tumor site by EPR effect modified from: (Gabizon et al., 1997)

1.4. Aim of the Study

Osteosarcoma is the cancer type in the bones. The advances in medications towards combinational treatments increased the survival rate in recent years. Still, the survival rate needs to be improved without systemic toxicities to the patients. Therefore, this study was designed to investigate the cytotoxic and apoptotic effect of Gemcitabine and Clofazimine against Saos-2 cell line.

The liposomal formulation is expected to have the ability to accumulate at tumor site via passive targeting and therefore, improve the anti-cancer effect via GEM's anti-cancer activity and by CLF's act on the Wnt signaling pathway that is overexpressed in many cancer types including osteosarcoma. It is the first time that CLF is evaluated for its cytotoxicity and GEM was used in liposomal formulation together with CLF for dual loading, release and anticancer activity investigations on Saos-2 cells. Commonly in cancer tissues, Wnt molecule is overexpressed and CLF could kill the saos-2 cells by targeting the Wnt signaling pathway in osteosarcoma.

This liposomal delivery system is proposed to bring a novel approach for treatment of osteosarcoma with increased effectiveness and decreased side effects.

CHAPTER 2

2. MATERIALS AND METHODS

2.1. Materials

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), Cholesterol, 1,2-Distearoyl-sn-glycero-3-Phosphoethanolamine-N-[Methoxy (Carboxy Polyethylene Glycol)2000 (18:00 mPEG(2000)-DSPE-COOH), L-α-phosphatidylethanolamine-N-(lissamine Rhodamine B sulfonyl) (Ammonium Salt) (Egg-transphosphatidylated, Chicken), Mini-extruder set, filter supports, Nucleopore Track-Etch Membranes (100, 400, 800 nm) were purchased from Avanti Polar Lipids, Inc. (USA). Gemcitabine Hydrochloride, dialysis sacks, benzoylated dialysis tubing, chloroform (HPLC grade), methanol (HPLC grade), and ethanol (HPLC grade) were the products of Sigma-Aldrich Chem. Co. (USA). Clozafimine was purchased from Sigma Aldrich (USA). Dimethyl Sulfoxide (DMSO) was purchased from Sigma-Aldrich (USA). Sephadex G-75, PD-10 Disposable Columns were purchased from GE Healthcare (UK). Saos-2 cell line was obtained from ATCC (USA). RPMI 1640 medium with phenol red obtained from GIBCO-BRL Biochrom AG (Germany). FBS (10%) was purchased from Biological Industries (Israel), and L-glutamine and 1× penicillin-streptomycin were obtained from Invitrogen (USA). MTT reagent (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) was obtained from Invitrogen (USA). Cell culture plastic-wares were the products of Orange Scientific (Germany).

2.2.Methods

2.2.1. Preparation of Liposome

2.2.1.1. Preparation of Conventional Liposome

Lipid film hydration method was used to form MLVs in aqueous salt solution. DPPC: CHOL lipids were used at molar ratio of 6:3 to produce conventional liposomes. Lipids were incubated for 15 min at room temperature to equilibrate before use. Lipid amounts were calculated according to molar ratio and put into round bottom eppendorf tubes and dissolved in 100 µl chloroform. Nitrogen gas was applied for 2 h to evaporate chloroform. Then, obtained lipid film was incubated overnight in vacuum oven (Nüve EV 018, Turkey) to remove the remnants of the organic solvent. Hydration solution was prepared by ammonium sulfate solution (120 mM) in 1mL dH_2O. In 1 hour, hydration was carried out by continues two min cycles of vortexing (CAT VM3, France) and heating in water bath at 65ºC. After hydration, the obtained solution was subjected to extrusion process with mini-extruder set (Avanti Polar Lipids, USA) to decrease the size of liposomes. During this process, hydrated liposome emulsion was passed from polycarbonate filters having membranes with pore sizes; 800, 400, 200 and 100 nm. The hydrated liposome emulsion was passed through each membrane 11 times at 65 ºC.

2.2.1.2.Preparation of PEGylated Liposome

PEGylated liposomes were prepared with the same method of conventional liposomes. Lipid composition of PEGylated liposome was DPPC: CHOL: DSPE-PEG2000-COOH (6:3:1). DPPC: CHOL: DSPE-m-PEG (2000) lipids were used at molar ratio of 6:3:1 to produce PEGylated liposomes. Lipids were incubated for 15 min at room temperature to equilibrate before use. Lipid amounts were calculated according to molar ratio and put into round bottom eppendorf tubes and dissolved in 100 µl chloroform. Nitrogen gas was applied for 2 h to evaporate chloroform. Then, obtained lipid film

was incubated overnight in vacuum oven (Nüve EV 018, Turkey) to remove the remnants of the organic solvent. Hydration solution was prepared by ammonium sulfate solution (120 mM) in 1mL dH_2O. In 1 hour, hydration was carried out by continues two min cycles of vortexing (CAT VM3, France) and heating in water bath at 65ºC. After hydration, the obtained solution was subjected to extrusion process with mini-extruder set (Avanti Polar Lipids, USA) to decrease the size of liposomes. During this process, hydrated liposome emulsion was passed from polycarbonate filters having membranes with pore sizes; 800, 400, 200 and 100 nm. The hydrated liposome emulsion was passed through each membrane 11 times at 65 ºC.

2.2.1.3. Preparation of Gemcitabine Loaded Liposome

Ammonium sulfate gradient method was used to load GEM into LUV form liposomes which were prepared in ammonium sulfate solution like doxorubicin loading to liposomes by same method in another study (Dalgic, 2016). Briefly, LUVs were formed in 120 mM solution of ammonium sulfate that forms both intra and extra-liposomal aqueous phases. Dialysis method was used after hydration with ammonium sulfate solution and extrusion steps to change extra liposomal phase with NaCl (0.9 %) for 20 h. After that, both liposome and drug solution were heated to 65 ºC, mixed and incubated for 10 min for drug encapsulation. Loading of GEM was done in dark due to its light sensitivity. Then the solution mixture was put into the ice bath suddenly. Column chromatography set up was used to remove unencapsulated drug. Sephadex G-75 column (GE Healthcare, USA) was prepared in 0.9 % NaCl solution. The vacuum (Nüve EV 018, Turkey) was applied to the solution for 6 h to remove air bubbles. Column chromatography was performed in the dark and all liposome amount were introduced to set up. Then, 1 mL fractions of liposomes were collected. Turbidity reading of each tube was performed at 410 nm by using UV-Visible spectrophotometer (Hitachi U-2800A, Japan). According to turbidity reading, liposome fractions were pooled. Liposomes were freshly prepared

before characterization and cell culture studies. Collected liposomes were used for other characterization and cell culture studies. Both conventional and stealth liposomes were loaded with GEM with the same method.

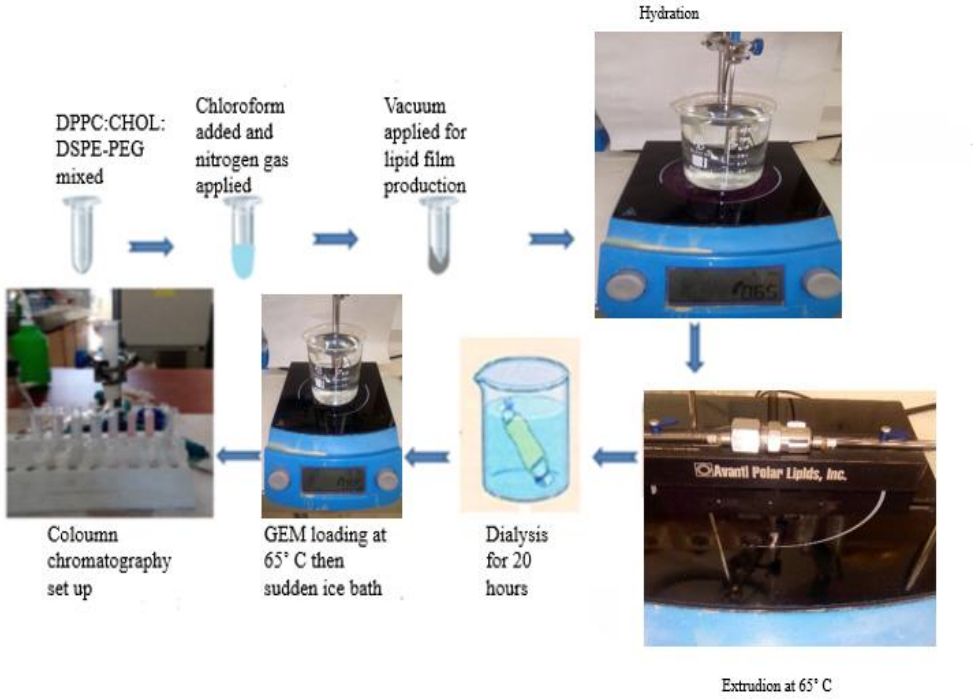


Figure 2.1 GEM loaded PEGlyated liposome (DPPC: CHOL: DSPE-m-PEG (2000) (6:3:1)) production

2.2.1.4. Preparation of Clofazimine Loaded Liposome

CLF (600 µg) added to lipid solution before thin lipid film formation. Then, both CLF loaded conventional and stealth LUV form liposomes were prepared with the same method as used for preparing liposome production in section 2.2.1.3.

2.2.1.5. Preparation of GEM and CLF co-loaded Liposome

According to the methods described in the previous parts for separate loading of GEM and CLF, they were co-loaded into same liposome for preparation of dual drug loaded liposomes. Briefly, CLF (600 µg) was added to the lipid solution before thin lipid film formation. Dialysis method

was used after hydration with ammonium sulfate solution and extrusion steps to change extra liposomal phase with NaCl (0.9 %) for 20 h. After that, both liposome and drug solution were heated to 65 ºC, mixed and incubated for 10 min for drug encapsulation. The other all procedures are same as in Section 2.2.1.3.

2.2.2. Quantification of Gemcitabine

Quantification of GEM was done with UV-Visible spectrophotometer (Hitachi U-2800A, Japan) at 268.8 nm wavelength. Calibration curve was obtained in methanol for loading calculations and in PBS for release experiments at the concentration range; 0. 01 – 5000 ng/mL (Appendix A). To quantify GEM amount loaded into liposomes 1 ml of GEM loaded liposome was disrupted in methanol and the optical densities were obtained. These values were then converted to loaded drug amounts by using calibration curve, non-interference of lipids in methanol was checked at the same wavelength

2.2.3. Quantification of Clofazimine

Quantification of CLF was done with UV-Visible spectrophotometer (Hitachi U-2800A, Japan) at 452 nm wavelength. Calibration curve was prepared in methanol at the concentration range between 0.01- 2000 μM (Appendix A). To measure loaded CLF amount, as in the GEM loaded liposomes, liposomes were disrupted in methanol, and optical densities were obtained. The values were then calculated by using calibration curve. Interference from the lipids was subtracted using plain liposome measurements at the same wavelength.

2.2.4. Quantification of Phospholipid Amounts (DPPC)

Stewart Assay was used to quantify phospholipid amounts of liposomes (Dalgic,2016). Briefly, Stewart solution was prepared by mixing 2.7 g ferric (III) chloride and 3 g ammonium thiocyanate in 100 mL distilled water. Liposome samples (20 μl) were dissolved in 2 mL chloroform by mixing gently and then, 2 mL ferrothiocyanate solution was added and vortex mixed for 20 seconds. The solutions were centrifuged at 2000 rpm for 5 min (Hettich EBA 20, UK). Also, a blank solution was prepared with chloroform- ferrothiocyanate solution mixture for use as baseline in spectrophotometer analysis. After that, chloroform phase was taken with a syringe and measured at 488 nm with UV-Visible spectrophotometer (Hitachi U-2800A, Japan). The amount of phospholipids was calculated using the standard curve of phospholipids prepared in chloroform (Appendix A).

2.2.5. Characterization of Liposomes

2.2.5.1. Particle Size and Zeta Potential Measurement

Particle size measurement was done with dynamic light scattering method. Liposomes were diluted with 0.9 % NaCl solution at 1:10 (v/v) ratio. The average diameter and size distribution with zeta potential of liposomes were measured with particle size analyzer (Malvern Mastersizer 2000) in Central Laboratory, Middle East Technical University, METU. All liposomal formulations were analyzed with the same method to obtain the particle size and zeta potential.

2.2.5.2. Morphological Characterization

Morphological characterization of liposomes was done with Transmission Electron Microscopy (TEM). Liposome sample (100 μL) was placed on copper grid (Formvar-Carbon Film on 300 square mesh Copper Grids) after 1:50 dilution with PB solution (0.1 M, pH 7.4), and left overnight to dry.

Then, the liposomes were dyed with 2% uranyl acetate (Sigma-Aldrich Co., USA) solution. The characterization of stained sample was done at 80 kV in bright field imaging mode with High Contrast Transmission Electron microscopy (TEM) (FEI Technai G2 Spirit BioTwin CTEM, USA) in Central Laboratory (Middle East Technical University)).

2.2.5.3. Loading and Encapsulation Efficiency

2.2.5.3.1. Gemcitabine Loading and Encapsulation Efficiency

Encapsulation efficiency and loading percentages of liposomes were calculated for GEM using GEM amounts in disrupted liposomes. Encapsulation efficiency was calculated as the ratio of the total GEM amount loaded into liposomes to the GEM amount initially used in preparation of liposomes. Loading efficiency was calculated by the ratio of encapsulated GEM to lipid amount of dissolved liposome. These values were calculated for all liposomal formulations of GEM. Equations are as follow:

$$\text{EE \%} = \frac{mg\ GEM\ in\ LUVs}{mg\ GEM\ initially\ added} \text{x } 100$$

$$\text{Loading \%} = \frac{mg\ GEM\ in\ LUVs}{mg\ DPPC\ in\ LUVs} \text{x}100$$

2.2.5.3.2. Clofazimine Encapsulation Efficiency and Loading Capacity

Encapsulation efficiency and loading percentages of liposomes were calculated for CLF using CLF amounts in disrupted liposomes. Encapsulation efficiency was calculated as the ratio of the total CLF amount loaded into liposomes to the CLF amount initially used in preparation of liposomes. Loading efficiency was calculated by the ratio of encapsulated CLF to lipid amount of dissolved liposome.

These values were calculated for all liposomal formulations of CLF. Equations are as follow:

$$\text{EE \%} = \frac{mg\ CLF\ in\ LUVs}{mg\ CLF\ initially\ added} \text{x } 100$$

$$\text{Loading \%} = \frac{mg\ CLF\ in\ LUVs}{mg\ DPPC\ in\ LUVs} \text{x}100$$

2.2.5.4. In vitro Release Experiments

2.2.5.4.1. Gemcitabine Release

GEM loaded liposomes were examined for evaluating in vitro release profile of GEM with respect to time. Briefly, 1 ml of liposome was put into dialysis bag (12000 Da MWCO, Sigma-Aldrich, USA) and then placed in polypropylene tubes containing 10 mL of phosphate buffered saline (PBS, 0.1 M, pH 7.4). Tubes were transferred into the shaking water bath (NÜVE ST 402, Turkey) and incubated at 37°C. Aliquots (1 mL) from release media around dialysis tubes were taken at 2, 4, 6, 12, 24, and 48 h. After 24 hour of release experiment, total release media was changed with fresh PBS. Then, released GEM amounts were determined measuring by GEM calibration curve (Appendix A) and then converted to cumulative amounts. All GEM release experiments were carried out in triplicates.

2.2.5.4.2. Clofazimine Release

CLF release profiles of CLF loaded liposomes were obtained using similar release experiment set up with GEM release experiments. The only difference was in analysis of CLF amounts in release media. Release samples that had been taken from release medium were left to dry and solved again in methanol The samples were measured at 452 nm by UV-Visible spectrophotometer (Hitachi U-2800A, Japan) and CLF amounts were calculated by using calibration curve (Appendix A).

2.2.6. Stability of Liposomes

Stability of liposome formulations were examined by following the change in particle size after incubation at two different temperatures; 4°C and 25°C. Particle size measurements were examined after first and second week incubations for GEM and CLF loaded and also with co-loaded liposome formulation by using Dynamic Light Scattering (Malvern Nano ZS90, UK) in Central Laboratory, Middle East Technical University) and compared with fresh liposome formulations.

2.2.7. Cell Culture Studies

2.2.7.1.Cell Culture Conditions

Saos-2 cells were cultured in RPMI 1640 medium, supplemented with 10% FBS and 2 mM L-glutamine and penicillin-streptomycin. Cells were incubated at 37°C under humidified atmosphere of 5% CO_2 and 95% air in incubator (SHEL LAB, USA). In every two or three days, medium was changed with the fresh one. Cells were passaged when they reached about 70-80 % confluence.

2.2.7.2.In Vitro Cytotoxicity Assay

Cytotoxicity experiments was done on Saos-2 cells with free GEM, CLF solutions at different concentrations as well as with GEM, CLF and their co-loaded liposomal formulations. Initially $1x10^4$ cells were seeded to each well of a 96 well-plate and incubated overnight to adhere. For cytotoxicity tests, MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay was used. Briefly, needed amount of MTT and DMEM were mixed at a ratio of 5:1. Then, prepared MTT solution was diluted with PBS (0.01 M, pH 7.4) at 1:10 ratio. All procedures were done under sterile conditions. After incubation of all test groups with cells for 24 h, all media was removed from the wells and washed with PBS (0, 01 M, pH 7.4) twice. MTT solutions (100 μl) were added to each well and plates were incubated

for 5 h at 37^0C in dark. The MTT solution was removed and 100 µl DMSO was added to each well for 15 min by continuous shaking. Then, the optical densities were measured by spectrophotometer with a microplate reader (GMI Biotech 3550, USA) at 570 nm. Empty liposomes, plain and PEGylated liposomes were the control groups for investigating lipid toxicity. Each group had 3 replicates. All in vitro experiments were considered for 24 and 48 h incubation periods except co-loaded liposomes which were incubated for 24 h.

2.2.7.3.Combination Index

Effects of drugs combination used in this study were evaluated using the combination index equation which was based on the multiple drug-effect equation of Chou-Talalay52. CI<1 indicates synergism, CI=1 indicates additive effect, and CI>1 indicates antagonism. The analysis was done with CompuSyn software (ComboSyn Inc., Paramus, USA).

This theory provides algorithms for computer simulation of synergism and/or antagonism. This can be at any effect and concentration/dose level, as shown in the CI plot and isobologram, respectively. (Appendix B).

2.2.7.4. Flow Cytometry Analysis

For apoptosis studies PE annexin v/7 AAD, Caspase-3, JC-1 apopototic markers were quantified and cell cycle analysis were done. Cells were seeded at a density of 1 x 10^6 cells per well in a 6-well plate and allowed to attach for 6 h. Then, they were treated with drug solutions or liposome groups (GEM, CLF, GEM/CLF, GEM-Lip, CLF-Lip and GEM/CLF co-loaded Lip) and incubated for 24 h. After that, cells were harvested by trysinization, washed with PBS and centrifuged at 20 000 g for 5 min.

2.2.7.4.1. PE Annexin v/7AAD assay

Cell apoptosis analysis was performed with PE Annexin V/7AAD assay (BD Biosciences). Saos-2 cells (1×10^5 cells/well) in all apoptosis assays were cultured at 6-well plate and incubated for 24 h with different groups. Then, cells were washed with cold PBS and resuspended in 100 µl 1X binding buffer, followed by addition of 5 µl PE Annexin V-PE and 5 µl 7-AAD. The cells were incubated for 15 min at room temperature in the dark. Finally, 400 µl 1X binding buffer were added to the cells, which were analyzed by flow cytometry. A total of at least 10.000 events were collected and analyzed by Accuri C6 Flow Cytometer (Accuri Cytometer, Ann Arbor, MI, USA) at MERLAB in Middle East Technical University.

2.2.7.4.2. Detection of Active Caspase-3

Detection of active caspase-3 by flow cytometry was used to assess apoptosis induced by different formulations treatment after 24 h. The cells were washed twice with PBS and resuspended in Cytofix/Cytoperm solution (Becton Dickinson) for 20 min on ice. After two washes with Perm/Wash Buffer (Becton Dickinson) at room temperature, the pellets were resuspended in FITC-conjugated monoclonal active caspase-3 antibody (Becton Dickinson) containing Perm/Wash buffer and incubated for 30 min at room temperature. Each sample was then washed with Perm/Wash buffer and then analyzed by Accuri C6 flow cytometer (Accuri Cytometer, Ann Arbor, MI, USA) at MERLAB in Middle East Technical University.

2.2.7.4.3. Detection of Membrane Potential

Saos-2 cells were incubated with different formulations for 24 h. Then, cells are washed with PBS and 5µl of the JC-1 staining solution (Becton Dickinson) incubated in 5% CO_2 incubator at 37°C for 20 min protected from light. After washing two times with buffer solution, cells were analyzed by flow cytometer. After that, samples were analyzed by Accuri

C6 flow cytometer (Accuri Cytometer, Ann Arbor, MI, USA) at MERLAB in Middle East Technical University.

2.2.7.4.4. Cell Cycle Analysis

Cells were treated with different liposomal formulations for 24 h and harvested. After cold PBS wash, samples were fixed with 70 % ethanol and kept on ice for 2 h. Following centrifugation at 800 g for 5 min, cells were washed with PBS and stained with 25 μg/mL propidium iodide with 30 min incubation at 37˚C. Staining solution added then samples were analyzed by Accuri C6 flow cytometer (Accuri Cytometer, Ann Arbor, MI, USA) at MERLAB in Middle East Technical University.

2.2.8. Statistical Analysis

For statistical analysis one-way Analysis of Variance (ANOVA) test was used to compare groups for single parameter. Tukey's Multiple Comparison Test for the post-hoc pairwise comparisons (SPSS-22 Software Programme, SPPS Inc., USA) was used; $p < 0.05$ was considered as statistically significant result.

CHAPTER 3

3. RESULTS AND DISCUSSION

3.1. Characterization of Liposome Formulations

3.1.1. Encapsulation Efficiency, Loading and Lipid Recovery of Liposome Formulations

3.1.1.1. GEM Encapsulation Efficiency, Loading and Lipid Recovery of Liposome Formulations

Encapsulation efficiency and loading of GEM were calculated for both conventional and PEGylated liposomes, besides their lipid recovery efficiencies. Ammonium sulfate gradient method was used in loading GEM to liposomes as it was reported to increase encapsulation efficiency (Dar Li & Huang, 2009). As expected from this method, both liposome formulations encapsulated 95 % of GEM (Table 3.1). High encapsulation efficiency was obtained by the ammonium sulfate gradient loading method in which high ion gradient force creates GEM aggregates inside the liposome. The ammonia concentration difference makes ammonia ions rush outside and protonated hydrogen ions create an acidic environment inside of liposome which makes GEM accumulate inside. GEM enters the liposome and get loaded into the liposome hydrophilic core beyond its solubility by conjugating with SO4-. In literature, 10 % sucrose, 5 % glucose and 0.9 % NaCl were also suggested as exchange salt solution. In order to not disturb osmotic forces between inside and outside of liposomes, hydration solution and extra-liposomal solution should be at high molarity. For example, if ammonium sulfate solution at high molarity (250-300 mM) is selected as hydration solution, the extra-liposomal solution should also be at high

molarity (10 % sucrose, 5 % glucose (Tu & Mcginnis, 2010). When the osmotic balance is not arranged, the liposome structure and tonicity are affected, leading to a decrease in the loading efficiency (Dar Li & Huang, 2009). Here, NaCl solution at low concentration was used to change extra-liposomal solution by dialysis method. In literature, several studies used ammonium sulfate at high concentrations (250 mM) and 0.9 % NaCl solution were carried out and encapsulation efficiency was found to be around 25 % for loading of hydromorphone hydrochloride (Tu & Mcginnis, 2010). However, in this study a lower concentration of ammonium sulfate (120 mM) was used and higher encapsulation efficiency (about 95 %) was achieved. In accordance with encapsulation efficiencies high GEM loadings were obtained for both formulations of liposomes; conventional ones and PEGylated ones were similar (Table 3.1).

Lipid recovery was calculated for conventional and PEGylated liposomes. Lipid recovery was also slightly (but not significantly) lower in PEGylated liposomes than conventional ones (Table 3.1).

Table 3.1. GEM encapsulation efficiency, loading and percent lipid recovery results of conventional liposome (DPPC: CHOL 6:3) and PEGylated liposome (DPPC: CHOL: PEG 6:3:1) formulations.

Liposome Type	GEM EE (%)	GEM Loading (%)	Lipid recovery (%)
Conventional	96.13±1.36	47.56±1.42	69.57±2.12
PEGylated	95.31±1.24	46.47±1.36	68.35±2.17

3.1.1.2.Clofazimine Encapsulation Efficiency, Loading and Lipid Recovery of Liposome Formulations

CLF was added to the lipid solution during lipid film preparation step. CLF is a hydrophobic drug and it could be mixed with lipid mixture to entrap within bilayer of the liposomes. CLF encapsulation efficiency, loading capacity and lipid recovery percentage were calculated as in the GEM loaded liposomes. The results were similar with the GEM loaded liposomes for encapsulation efficiency, loading and lipid recovery. Table 3.2 gives the CLF encapsulation efficiency, loading capacity and lipid recovery percentages. In the literature, hydrophobic molecules entrapment efficiency was lower than hydrophilic molecules because of the rigidity of acyl chain of PC-based lipids (S. Hong, S.H. Kim, 2015). In this study, rigidity of the lipid membrane was suitable to allow CLF loading at a high rate. As in the GEM loaded stealth liposomes, CLF loaded stealth liposomes showed slightly decreased encapsulation efficiency, loading and lipid recovery because of the addition of PEG molecule.

Table 3.2. CLF encapsulation efficiency, loading and percent lipid recovery of conventional liposome (DPPC: CHOL 6:3) and stealth liposome (DPPC: CHOL: PEG 6:3:1) formulations

Liposome Type	CLF EE (%)	CLF Loading (%)	Lipid recovery (%)
Conventional	93.23±1.36	44.5±1.17	70.5±1.16
PEGylated	91.43±1.12	41.1±1.14	69.4±1.23

3.1.1.3. Encapsulation Efficiency, Loading and Lipid Recovery of GEM and CLF co-Loaded Liposomes

GEM and CLF co-loaded liposomes' encapsulation efficiency, loading and lipid recovery results (Table 3.3) were not significantly different than their single loading results. Combinational loading of both drugs together

resulted in only small decrease in encapsulation efficiency, loading and lipid recovery with addition of hydrophobic drug to the liposome structure. In literature; there are other studies showing similar results for hydrophobic and hydrophilic drugs co-loaded liposomes. Especially for Tamoxifen (TMX), GEM loading to the liposomes cause destabilization of lipid bilayer structure which leads to the lowering in TMX encapsulation percent. (Cosco, 2011). There was again a small difference between conventional and PEGylated liposomes which is expected according to the previous results and literature. Addition of PEG molecule could decrease the internal volume of liposomes and this could resulted in lower encapsulations of GEM and CLF. The CLF is a hydrophobic drug and it was encapsulated by mixing in the lipid solution. CLF aggregated quickly in the lipid film preparation step and the encapsulation of CLF could be lower than GEM because of solubility reasons.

Table 3.3. Encapsulation efficiency, loadingy and percent lipid recovery results of GEM/CLF co-loaded conventional (DPPC: CHOL 6:3) and PEGylated liposome (DPPC: CHOL: PEG 6:3:1) formulations

Combinational Drug Loaded Liposome Type	GEM EE (%)	GEM Loading (%)	CLF EE (%)	CLF Loading (%)	Lipid Recovery (%)
Conventional	91.1±1.2	40.3±2	83.1±1.3	35.8±1.3	67.2±1.2
PEGylated	90.1±1.2	39.1±1.6	80.1±1.5	31.5±1.3	65.3±1.2

3.1.2. Drug Release Profile

3.1.2.1.GEM Release Profile

GEM loaded conventional (DPPC: CHOL 6:3) and PEGylated (DPPC: CHOL: DSPE-PEG 2000 6:3:1) liposomes were evaluated for in vitro release profiles. The cumulative percent release curves of the conventional and PEGylated liposomes are shown in Figure 3.1. The burst release and

cumulative release profiles of liposomal formulations were similar. The burst release at 2 h was 10.3 % for conventional and 8.6 % for PEGylated liposomes. For higher bioavailability, a longer blood circulation is needed. So, liposomes are usually modified by PEGylation to obtain "stealth liposomes" which are more stable and circulate in bloodstream for longer time than the conventional liposomes (Immordino & Cattel, 2006). In addition, PEGylated liposomes usually show slower drug release than the conventional ones. In parallel to these studies slightly slower GEM release was observed with PEGylated formulations as shown in Figure 3.1. It was thought that the PEG chains on the liposome surface affect the drug release probably by retaining drug molecules within the entangled PEG chains. In many studies, PEGylated liposomes showed slower drug release profile than conventional liposomes (Immordino & Cattel, 2016). At this stage it is important to get slow release as this event will occur during blood circulation before the liposomes reach to their target (cancerous) tissue. Therefore, the less GEM is released the more cytotoxic agents liposomes would carry to cancer cells. Besides this would also take advantage of decreasing the systemic amounts of free GEM, and thus, its cytotoxic effects on other tissues.

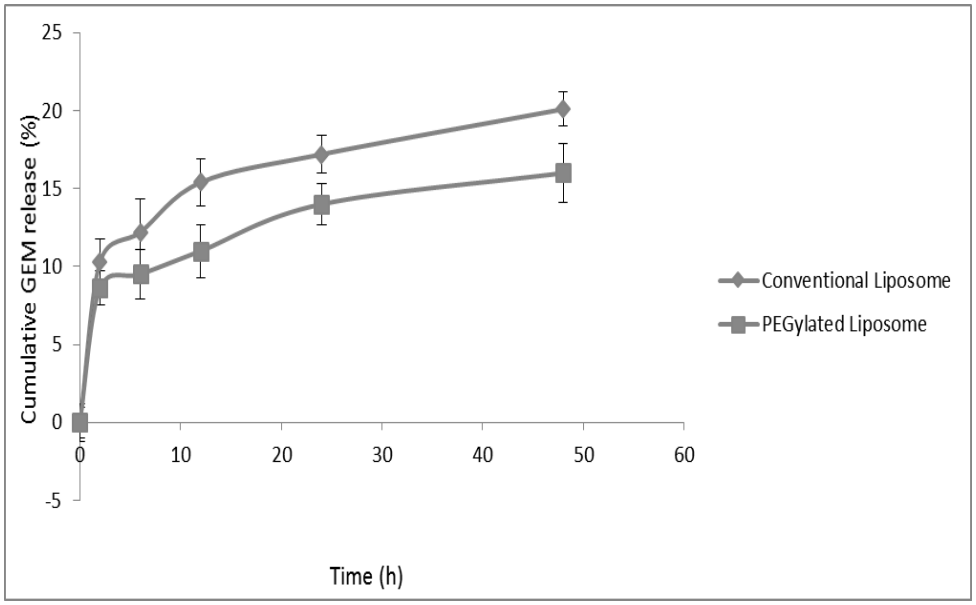


Figure 3.1. GEM release profiles of conventional liposome (DPPC: CHOL 6:3) and PEGylated liposome (DPPC: CHOL: DSPE-PEG 6:3:1) in PBS (pH 7.4) at 37˚C (n=3).

3.1.2.2. Clofazimine (CLF) Release Profile

CLF loaded liposomes were also evaluated for in vitro release profiles using conventional (DPPC: CHOL 6:3) and PEGylated (DPPC: CHOL: DSPE-PEG 2000 6:3:1) formulations. The cumulative percent release of the conventional and PEGylated liposomes are shown in Figure 3.2. The burst release was not observed in CLF loaded liposomes as reported in other studies on hydrophobic drugs (Sun, Zhou, Zhang, Li, & Liu, 2015). The CLF is a hydrophobic drug and it is encapsulated inside of lipid bilayer which is the hydrophobic region of liposome.. That is why that the burst release is not generally expected in hydrophobic drug loaded drug delivery systems. As in the GEM loaded liposomes, PEGylation caused the liposome to release CLF slower than the conventional liposome. This outcome will provide carrying much more amount of cytotoxic agent while creating fewer side effects because of a controlled manner release as in other

studies (Vijayakumar, 2016). Thus, PEGylation of liposomes make the release sustained without any burst release of drug molecule and provide the advantage that by using lower amount of liposomes, the effective rate of treatment could be achieved. This might also lead to the fewer side effects.

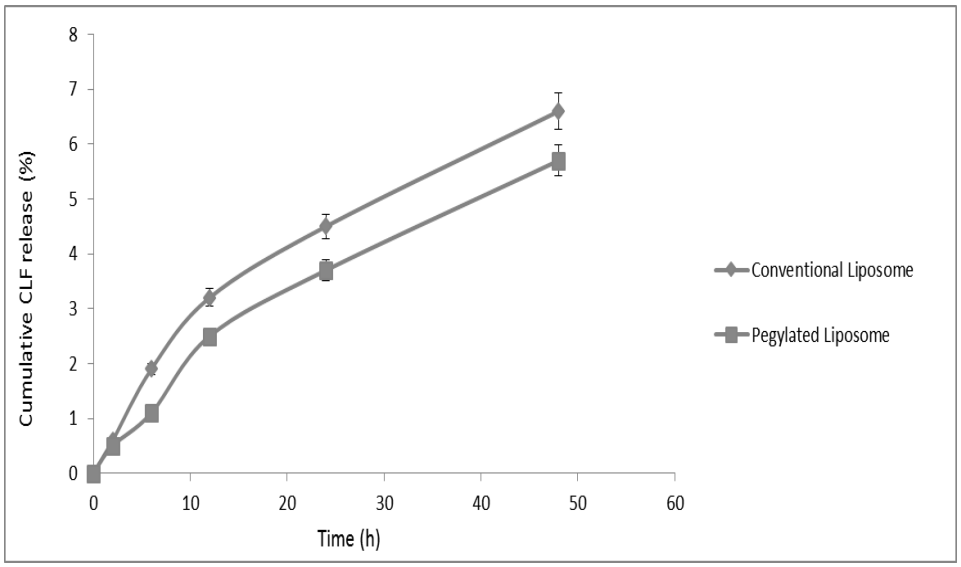


Figure 3.2. CLF release profiles of conventional liposome (DPPC: CHOL 6:3) and PEGylated liposome (DPPC: CHOL: DSPE-PEG 6:3:1) in PBS (0.1 M, pH 7.4 at 37˚C (n=3)

3.1.2.3.GEM and CLF Release Profile in the Co-Loaded Liposomes

GEM and CLF release profiles were also studied from their co-loaded PEGylated liposomes (Figure 3.3). Accordingly, there is a slight difference between GEM release profile of only GEM-loaded liposome and co-loaded liposomes. Release of GEM was slightly lower in co-loaded liposomes probably due to presence of hydrophobic drug (CLF) in lipid bilayer. In another research, GEM release was reported to be slower when it was co-loaded with tamoxifen (a hydrophobic drug). It was stated that his could be due to addition of tamoxifen, a hydrophobic molecule, to the lipid bilayer. The release was lowered around 5 % at the end of the 24 h as in our study;

GEM release was nearly 3 % lower in co-loaded liposomes than only GEM loaded liposomes (Cosco, 2011). CLF release was almost same in both liposomal formulations as expected considering its entrapment site in liposome structure.

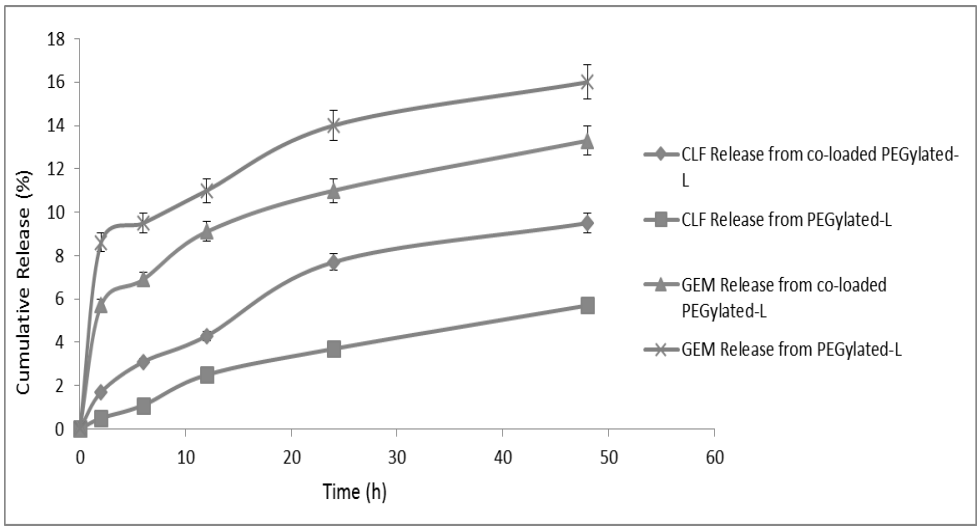


Figure 3.3. GEM and CLF release profiles of PEGylated liposome (DPPC: CHOL: DSPE-PEG 6:3:1) in PBS (0.1 M, pH 7.4) at 37˚C (n=3).

GEM release from single loaded and co-loaded liposomes was faster than CLF due to its higher water solubility than CLF.

3.1.3. Particle Size Distribution, Surface Charge, and Morphology

In literature, size of liposomes was suggested as one of the most important parameter to affect the bioavailability and clearance from blood circulation (Immordino & Cattel, 2016). The liposomes which are smaller than 200 nm can escape from the reticuloendothelial system (RES). The larger size ones are opsonized and recognized by the macrophages in the RES (Dar Li & Huang, 2009).

In the current study, considering this requirement, conventional and PEGylated liposomes were prepared to be below 200 nm in diameter and

the number of passes through extruder kit was arranged to have size distribution of liposomes between 100 and 200 nm. After eleven passages through extruder kit at predetermined extrusion temperature liposomes were easily obtained within this size range. The conventional liposomes are known to accumulate at tumor site by passive targeting due to their small size. The enhanced permeability and retention effect enables small molecules to accumulate at the tumor site with prolonged circulation time in the blood. Because of the high vascularization at the tumor site there is high blood supply to these tissues and there is lack of lymphatic drainage. These properties lead to accumulation of large molecules with more fluid transport to the tumor site. This property has been shown to increase the drug concentration at tumor site with many in vivo studies on liposomal drug delivery systems.

Dynamic light scattering method was used to determine the particle size of liposomes which were summarized in Table 3.4. The liposome groups had similar particle size values. There was a slight increase in average diameter when two agents were loaded together.

Table 3.4 Zeta potential and Particle size of GEM-CLF loaded liposomes

Liposome	Zeta-potential (mV)	Z-average (nm)	Width (nm)	90^{th} percentile	PDI
GEM-Conventional-L	-7.7	120.0	30.2	129.8	0.029
GEM-PEGylated-L	-7.2	128.0	30.8	134.7	0.032
CLF-PEGylated-L	-11.2	131.0	30.3	135.8	0.034
Co-loaded-PEGylated-L	-9.3	135.0	31.3	140.2	0.037

Figure 3.4 shows the size distribution of the GEM loaded conventional and PEGylated liposomes obtained by DLS analysis. PEGylated liposomes had slightly larger size than the conventional liposomes since PEG is a hydrophilic molecule and the higher hydrodynamic diameter is expected (Yinghuan et al., 2011). Still the size distribution of PEGylated liposome was below 200 nm with PDI $\leq$ 0.1. Liposome groups had a uniform size and the zeta potential values were slightly under zero. The PEG molecule makes liposome surface hydrophilic and open to the cellular binding which was shown in many studies (Immordino & Cattel, 2016). Zeta potential for the PEGylated liposomes is slightly lower than the conventional ones indicating the stability of stealth liposomes being higher since the PEG molecule does not only give hydrophilicity to the liposomes but also cause some neutrality to increase cellular binding (Sun et al., 2015).

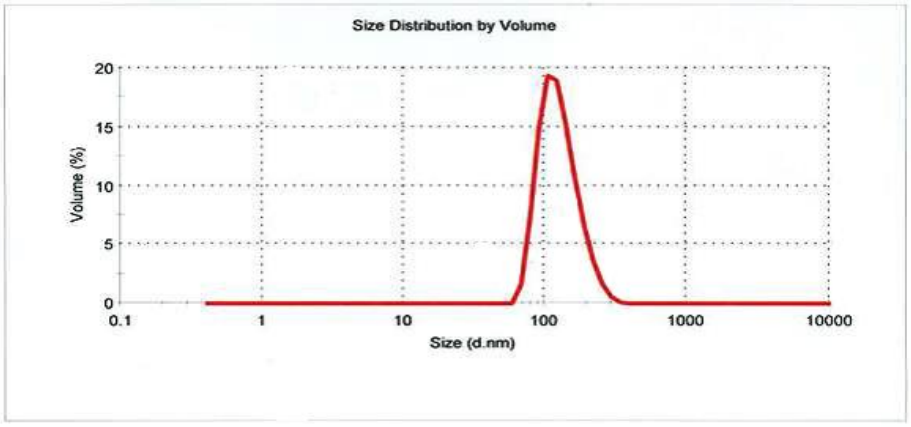
Therefore, zeta potential value is important to determine the systemic

stability of liposomes.

Figure 3.5 shows the size distribution curve of CLF and combinational drug loaded stealth liposomes obtained by DLS analysis. Both liposome groups had negative zeta potential and a uniform size below 200 nm as in the GEM loaded liposomal groups. With the increase in the zeta potential of liposomes, the bioavailability is increased. Therefore, slightly negative zeta potential value is promising for liposomes (Paolino, 2010). Results of CLF loaded PEGylated ones were similar to GEM loaded ones. Zeta potential values of CLF and combinational drug loaded PEGylated liposomes were again slightly lower than the conventional liposomal groups owing to the existence of the PEG molecule on the surface. The slight decrease in the zeta potential of combinational drug loaded stealth liposomes can be explained by the addition of hydrophobic drug to the liposome structure. There was also a slight increase in the average size, probably due to same effect of CLF. This is supported with other studies in the literature as addition of hydrophobic molecule like tamoxifen lowered the zeta potential value of liposomes (Cosco, 2011). All liposomal groups were within expected ranges according to the size distribution, surface charge and morphology.

Liposome structures were evaluated with TEM analysis. Figure 3.6 shows TEM image of GEM loaded liposome. Unilamellar morphology with mean diameter between 100 and 200 nm was observed and in the TEM images there was no aggregation for both formulations. These results were in agreement with the size and surface charge analysis results of liposomes.

a)



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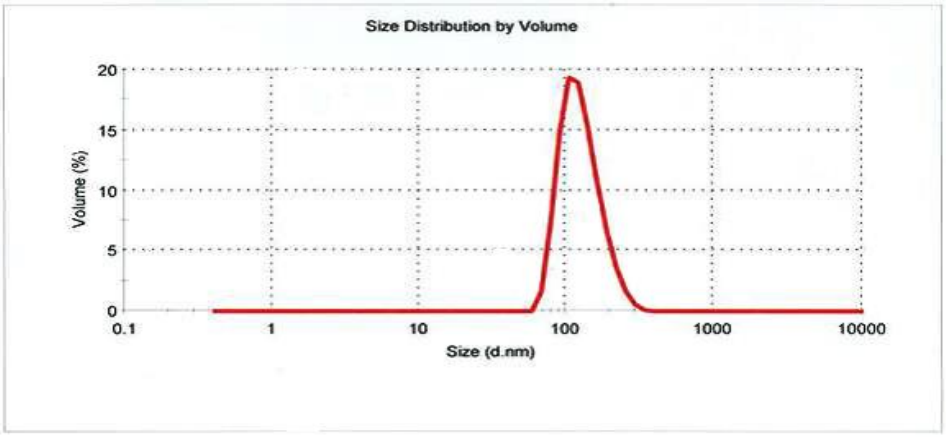
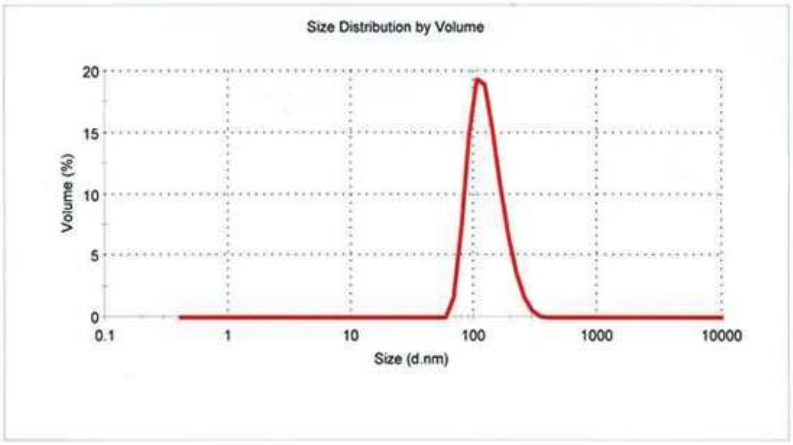


Figure 3.4. Size distribution of GEM loaded liposomes a) Conventional (DPPC: CHOL). b) PEGylated (DPPC: CHOL: DSPE-PEG2000)

a)



b)

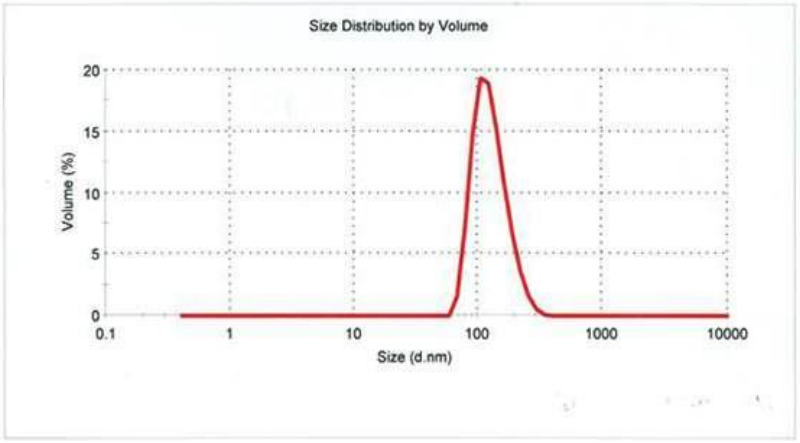


Figure 3.5. Size distribution of liposomal groups a) CLF loaded liposomes b) Co-loaded liposomes

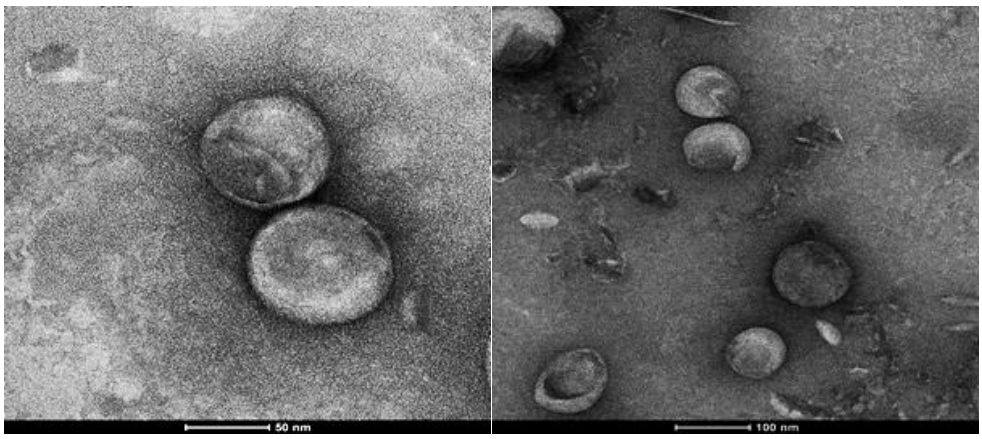


Figure 3.6 TEM image of GEM loaded liposomes

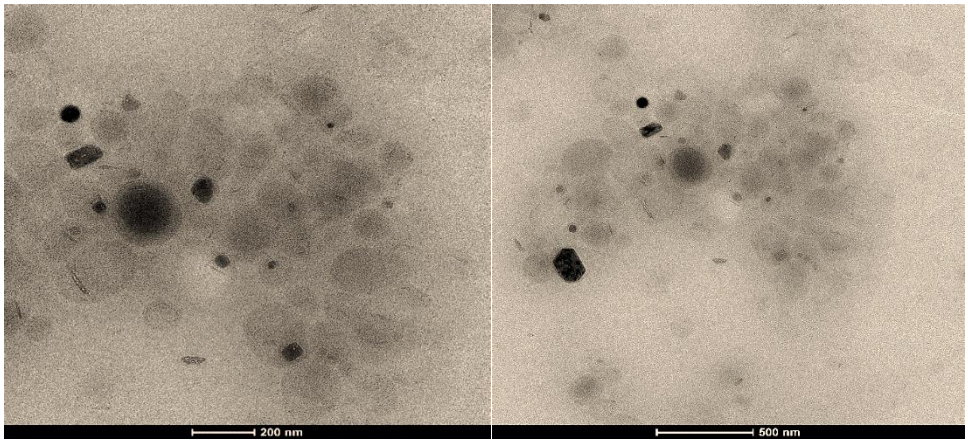


Figure 3.7 TEM image of CLF/GEM co-loaded liposomes

3.2.Cell Culture Studies

The cytotoxicity of free GEM and CLF solutions and their liposomal formulations were evaluated with the cell culture studies using Saos-2 cell line. Saos-2 cells are cancer cell lines from human primary osteogenic sarcoma.

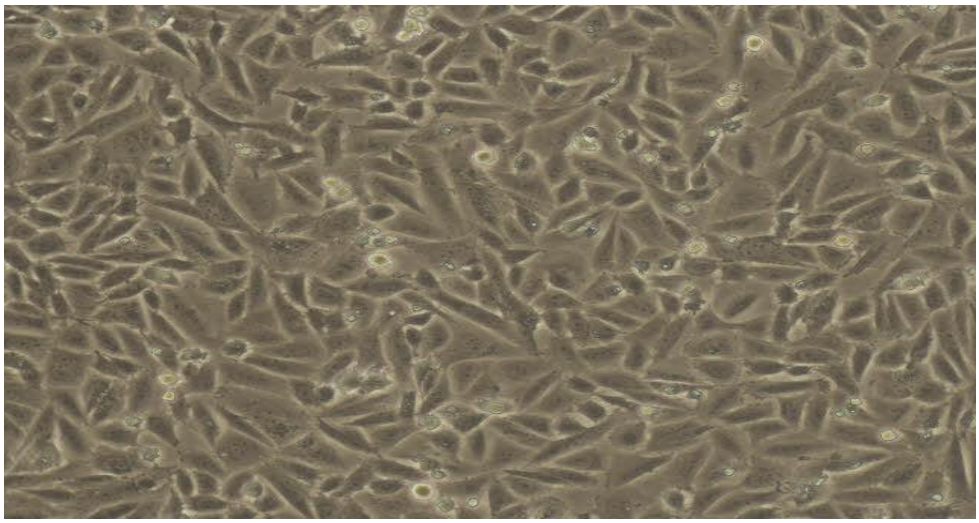


Figure 3.8 Light microscopy image of Saos-2 cells (11^{th} passage) 20X magnification

3.2.1. Cellular Toxicity of Gemcitabine

In order to determine the effective dose and IC_{50} value of GEM on Saos-2 cells different concentrations (5 ng/ mL, 10 ng/ mL, 20 ng/ mL, 50 ng/ mL) of GEM were used . Figure 3.9 shows the cytotoxicity of GEM treated Saos-2 cells after 24 and 48 hour incubations. As shown in the figure, cell viability decreased with the increase in the concentration of GEM and the incubation time. Accordingly, the IC_{50} value was determined as 46 ng/mL after 24 hour incubation. This value was used for other cytotoxicity studies with liposomal formulations of GEM. In another study, GEM IC_{50} value on Saos-2 cell line was given as 7.8 ng/mL (Leu et al., 2004). This value is around 5 times lower than our experimental result which is 46 ng/ mL. This difference might have resulted from the resistance of Saos-2 cells to the GEM with increase in the passage number. In addition, the seeded cell number was not defined in the article, the cell number could be different and it could be the reason of the different IC_{50} values. For osteosarcoma treatment GEM is not first line therapy; it is used alone and in combination with the docetaxel as second line therapy. GEM is used 1000 mg/m^2 weekly for 7 weeks when it is used alone or in combination with docetaxel, is used 675mg/m^2 on day 1 and day 8 then docetaxel 100 mg/m^2 weekly (Palmerini et al., 2016). Treatment of osteosarcoma was developed with the usage of multi-agent chemotherapy. The combinational therapy of GEM with docetaxel was much more effective than GEM alone in osteosarcoma patients. The median survival rate was 17.9 months for GEM/docetaxel group and 11.5 months for only GEM group (Pautier, 2016) which showed that second agent of chemotherapy produced a superior result in osteosarcoma patients since in the GEM/docetaxel arm, the dose of agents was decreased to reduce the grade and number of side effects. With the low doses of agents, the combinational treatment gave better results than the GEM treatment alone (L. Wang, Liu, Huang, & Xu, 2007).

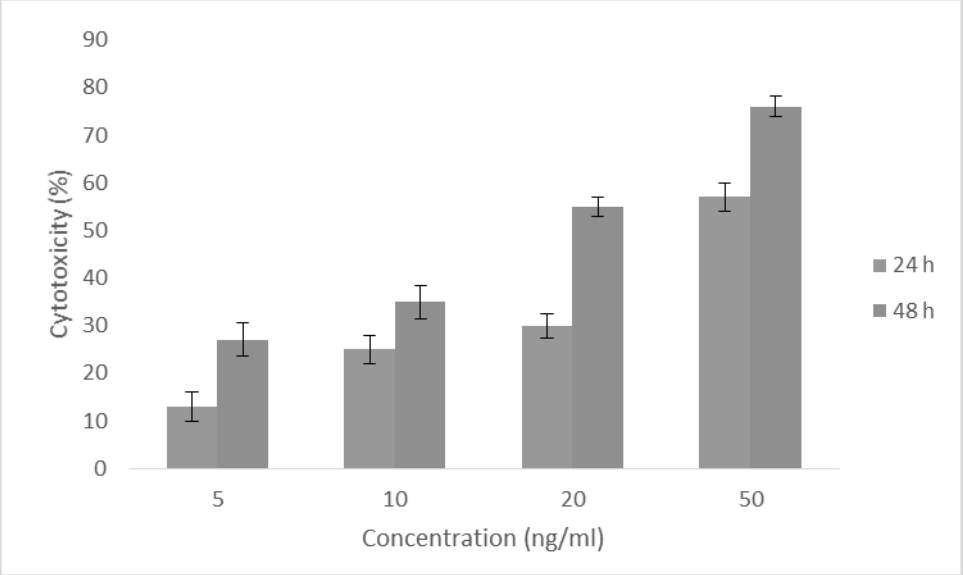


Figure 3.9 Relative cytotoxicity results of Saos-2 cells treated with different concentrations of GEM after 24 and 48 h incubations. (n=3).

3.2.2. Cellular Toxicity of Gemcitabine Loaded Liposomal Formulations

Saos-2 cells were incubated for 24 and 48 h to evaluate the cytotoxicity of empty and GEM loaded conventional and PEGylated liposomes. In each experimental group GEM amount in liposome formulation was at IC_{50} dose. The empty liposomes were diluted in order to obtain same concentrations with GEM loaded liposomes. Table 3.5 represents the experimental groups. Figure 3.11 shows the cytotoxicity of liposomal groups after 24 and 48 h incubations as percentage of untreated control cells.

There was a numerical increase in the cytotoxicity after 48 h of incubations for all liposomal formulations. However, percent cytotoxicities of liposomal groups were quite low for all liposomal groups after 24 and 48 h incubation. This shows that cytotoxic effect of the drug was lowered when they were encapsulated inside the liposomes. So, in the conventional liposomal

formulations, cytotoxicity percentage was not around 50 % since GEM was entrapped by liposomes and released slowly with time. This is not a common result as shown in other studies (Sun et al., 2015). In this study, after 48 h incubation the liposome released 70 % of its content but in our study, the liposome released around 16 % of its contents at the end of 48 h incubation period. The release amount was lower than the other reported liposomal studies (Cosco, 2011), this could be the reason for lower cytotoxicities of liposomal groups. At 48 h, cytotoxicity percentages were a little higher than the 24 h incubations because of the increase in the released amount in GEM and longer time incubation with liposomes.

Sometimes cells are prone to much more amount of free drug than its liposomal formulation. Hence, while some cells receive high amounts of drug some might have received very small due to less liposome intake. This effect is thought to be eliminated when liposome formulation is applied in vivo as they will accumulate mostly at the tumor site.

All GEM loaded liposomal formulations showed significantly higher cytotoxicities than empty liposomal formulations. Therefore, GEM loaded liposomal formulations had time dependent cytotoxic effect on Saos-2 cells ($p<0.05$).

Table 3.5 Experimental groups of conventional liposomes and percentage cytotoxicity at 24 and 48 h

Groups	24 h Cytotoxicity (%)	48 h Cytotoxicity (%)
Empty -C-L	13±0.21	16±0.19
2x Empty -C-L	21±0.32	25±0.24
IC_{50} dose-free GEM	47±0.78	54±0.71
2 x IC_{50} dose-free GEM	91±0.98	97±0.12
IC_{50} GEM-C-L	30±0.28	36±0.32
2 x IC_{50} GEM-C-L	70±0.77	76±0.41

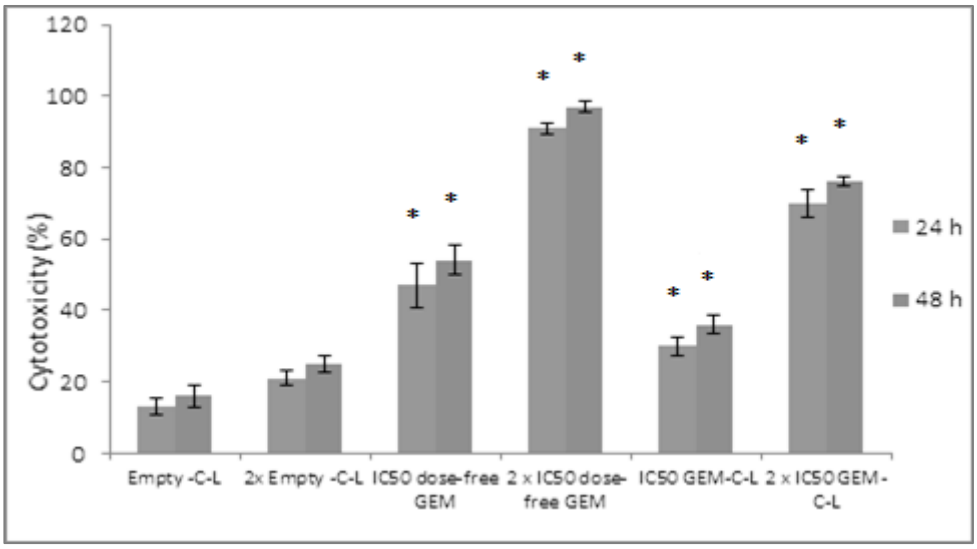


Figure 3.10 Relative cytotoxicity of GEM loaded conventional liposomes and control groups. Asterisks * denote statistical significance at $p<0.05$. Empty-C-L is the control group of IC_{50} dose free GEM and IC_{50} GEM-C-L. 2 x Empty-C-L is the control groups of 2 x IC_{50} dose free GEM and 2 x IC_{50} GEM-C-L. (n=3).

Table 3.6 Experimental groups of GEM loaded PEGylated liposomes and percentage cytotoxicity at 24 and 48 h

Groups	Cytotoxicity at 24 h (%)	Cytotoxicity at 48 h (%)
Empty -PEGylated-L	15±0.13	18±0.17
2x Empty -PEGylated -L	23±0.22	26±0.21
IC_{50} dose-free GEM	48±0.32	56±0.41
2 x IC_{50} dose-free GEM	93±0.56	96±0.65
IC_{50} GEM- PEGylated -L	31±0.38	34±0.39
2 x IC_{50} GEM-PEGylated -L	67±0.33	71±0.59

Table 3.6 shows the cytotoxicity results for PEGylated liposome groups. In previous results, PEGylated liposomes showed slower release than the conventional liposomes because PEGylation of liposomes results in a much more stable and complex structure. As in the case of conventional liposomes, PEGylated ones also had lower cytotoxicity than free GEM solutions. As seen in Figure 3.12 cytotoxicity percentage results for PEGylated liposomes were slightly lower than the conventional liposomes as observed in other studies (Immordino & Cattel, 2016). But the difference between conventional and stealth liposomes was not significant.

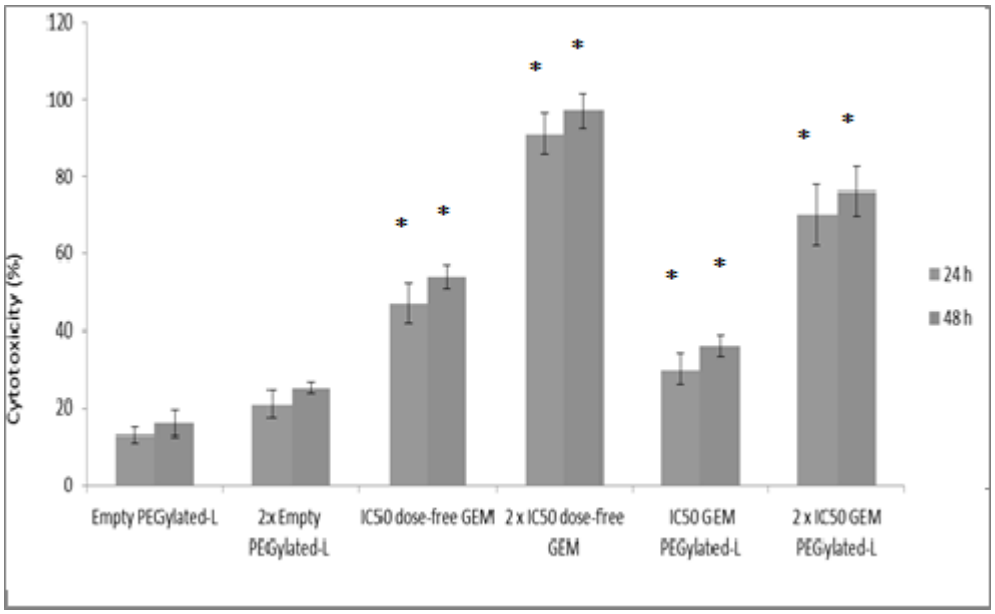


Figure 3.11 Relative cytotoxicity of GEM loaded stealth liposomes and control groups. Asterisks * denote statistical significance at $p<0.05$ Empty-PEGylated-L is the control group of IC_{50} dose free GEM and IC_{50} GEM-PEGylated-L. 2 x Empty-PEGylated-L is the control groups of 2 x IC_{50} dose free GEM and 2 x IC_{50} GEM-PEGylated-L. (n=3).

In literature, the effective dose of liposomal formulation of GEM was not determined on Saos-2 cell line, but the common in vitro effective cytotoxic dose changes between 0.01 μM and 0.1 μM that is compatible with in vivo administration for treatment of cancer diseases (Paolino, 2010). In our

study, the IC_{50} dose was calculated as 46 ng/mL for free GEM and it is around 0.15 μM that is close to the value in the literature.

All GEM loaded liposomal formulations showed significantly higher cytotoxicities than empty liposomal formulations. Therefore, GEM loaded liposomal formulations had time dependent cytotoxic effect on Saos-2 cells ($p<0.05$).

3.2.3. Cellular Toxicity of CLF

Different concentrations of CLF (25 μM, 50 μM, 75 μM, and 100 μM) were used to determine suitable dose for loading to the liposomes and IC50 value on Saos-2 cells upon incubation for 24 and 48 h. In Figure 3.13 cytotoxicity levels were higher after 48 h incubation than 24 h incubation. With the increasing concentrations of CLF, the cell viabilities decreased with time and IC_{50} value was determined as

77 μM after 24 h. This value was used for other cytotoxicity studies with liposomal formulations of CLF. For our knowledge, there is no other research in the literature to show CLF IC_{50} concentration on Saos-2 cells. This IC_{50} value may be a high dose alone for treatment. There is no other research to show dose information of CLF on Saos-2 cell line, but there is a study on breast cancer cells (HTB19 cell line). Many cancers including the breast cancer depends on the overreactivation of Wnt signaling pathway by increasing the concentration of Wnt ligands with these receptors and CLF plays role on this pathway and prevent reactivation of Wnt signaling. In this study, IC_{50} was estimated between 3-6 μM depending on the treatment period (Koval et al., 2014). In the literature, there are several studies about pharmacokinetic results of CLF and CLF distribution was very broad and tend to accumulate in tissues depending on the tissue type. The concentration of CLF accumulation was between 10 μM and 500 μM in different tissues (Connor, Sullivan, & Richard, 1996). Therefore, it is advantageous to use CLF with another regimen like GEM and also in co-

loaded liposomal formulation to reach the most effective dose without causing their side effects. In addition, with the Wnt signaling reactivation, β-catenin protein nuclear localization has occurred which is found in cancer tissues but not in normal tissues (Polakis, 2000). This mechanism could also make accumulation of CLF in the target tissues that could decrease the side effects.

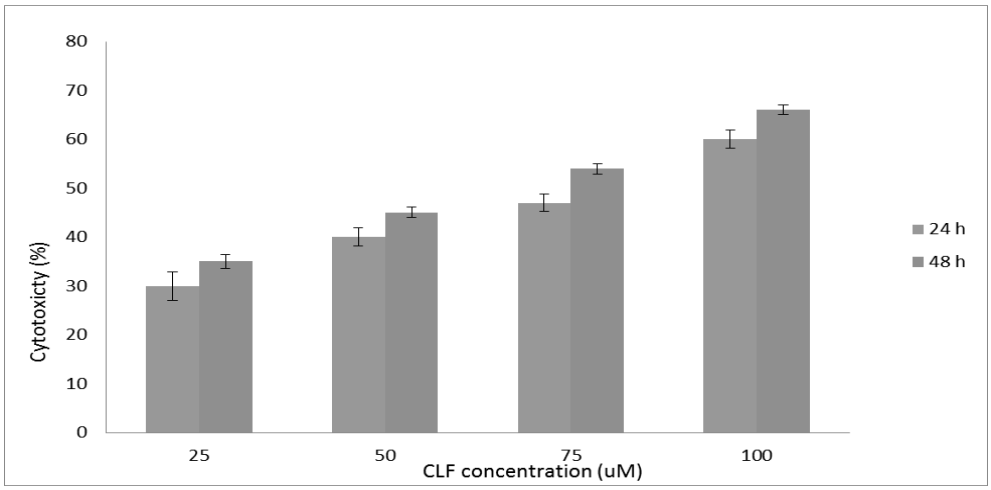


Figure 3.12 Relative cytotoxicity results of Saos-2 cells treated with different concentrations of CLF in for 24 and 48 h incubations. (n=3).

3.2.4. Cellular Toxicity of CLF Loaded Liposomes

Saos-2 cells were incubated for 24 and 48 h to evaluate the cytotoxicity of empty and CLF loaded PEGylated liposomes. In each experimental group CLF amount in liposome formulation was at IC_{50} dose. The empty liposomes were diluted in order to obtain same lipid concentrations with CLF loaded liposomes. Table 3.7 shows all experimental groups and Figure 3.14 represents the cytotoxicity percentages of liposomal formulations of CLF.

There was a numerical increase in the cytotoxicity from 24 to 48 h of incubations for all liposomal formulations. As in the previous liposomal

formulations, CLF loaded liposomes showed lower cytotoxicity than free CLF solutions since the release of CLF occurs in prolonged period.

Table 3.7 Experimental groups of CLF and relative cytoxicities at 24 and 48 h

Groups	24 h Cytotoxicity (%)	48 h Cytotoxicity (%)
Empty -PEGylated-L	14±0.1	17±0.12
2x Empty -PEGylated-L	21±0.14	24±0.15
IC_{50} dose-free CLF	47±0.18	54±0.21
2 x IC_{50} dose-free CLF	91±0.37	98±0.44
IC_{50} CLF-PEGylated-L	27±0.11	33±0.17
2 x IC_{50} CLF-PEGylated-L	63±0.22	68±0.28

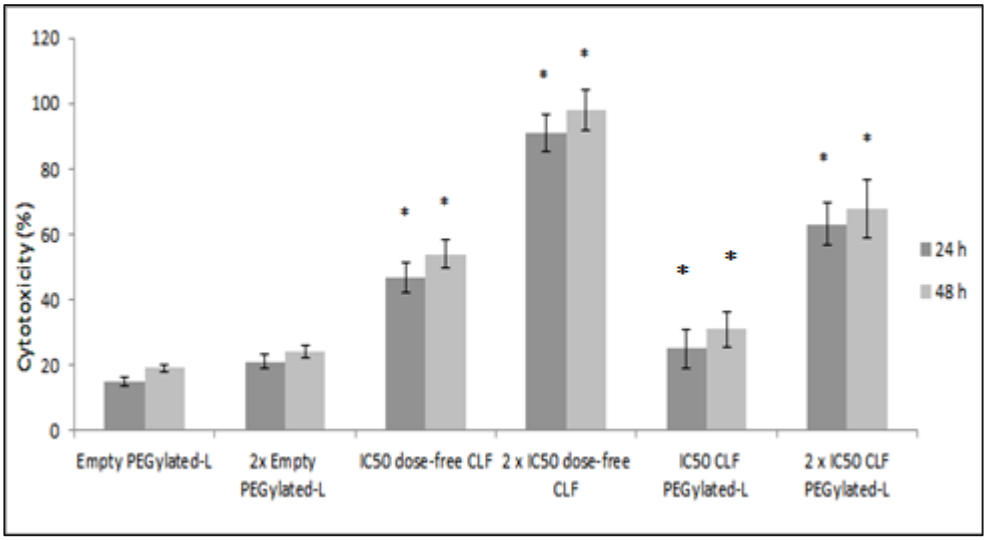


Figure 3.13 Relative cytotoxicity of CLF loaded PEGylated liposomes and control groups. Asterisks * denote statistical significance at $p<0.05$. Empty-PEGylated-L is the control group of IC_{50} dose free CLF and IC_{50} CLF-PEGylated-L. 2 x Empty-PEGylated-L is the control groups of 2 x IC_{50} dose free CLF and 2 x IC_{50} CLF-PEGylated-L. (n=3)

All CLF loaded liposomal formulations showed significantly higher cytotoxicities than empty liposomal formulations. Therefore, CLF loaded liposomal formulations had time dependent cytotoxic effect on Saos-2 cells ($p<0.05$).

3.2.5. Cellular Toxicity of Combination of GEM and CLF

Different concentrations of CLF (25 µM, 50 µM, 75 µM, and 100 µM) were used with GEM at IC_{50} concentration to show the change in cytotoxicity level of GEM with CLF. With the increase in the CLF concentration, the cytotoxicity percentage increased. In the previous experiments, CLF IC_{50} concentration was calculated as 77 µM and as in the Figure 3.15 with the increase in the CLF concentration, the cytotoxicities were increased. Addition of CLF to the GEM treated groups, enhanced the cytotoxic effect since when GEM and CLF were used together, the percentage cytotoxicities was higher than the only GEM treated group at the same concentration.

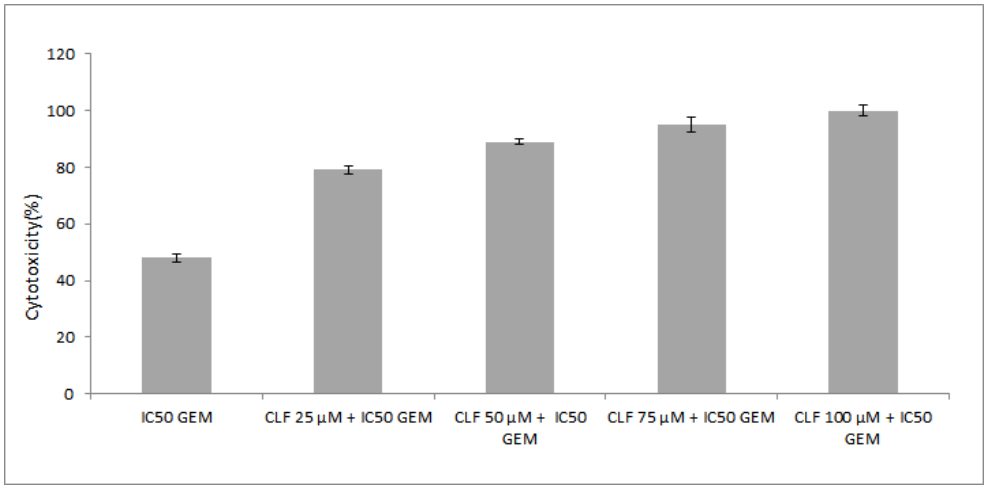


Figure 3.14 Relative cytotoxicity of combination of free GEM and CLF on Saos-2 cells. (n=3)

3.2.6. Cellular Toxicity of GEM and CLF Co-Loaded Liposomes

Table 3.8 shows the cytotoxicity percentages of different experimental groups for GEM and CLF co-loaded liposomes at 24 h. Saos-2 cells were incubated with liposomes for 24 h to evaluate the cytotoxicity of empty and GEM/CLF co-loaded stealth liposomes. In experimental group GEM amount in liposome formulation was at IC_{50} dose. The empty liposomes were diluted in order to obtain same lipid concentrations with GEM loaded liposomes. In each well, the amount of GEM was 9.2 μg (IC_{50} dose per well), co-loaded CLF was calculated as 11 μM per well. This value for CLF was almost 7 times lower than IC_{50} dose of CLF per well so there was no expectation about getting 50 % cytotoxic percentage with CLF. In addition, because of the slow release of drug molecules from liposomal formulation, the cytotoxicity percentages were expected to be lower than free drug treatments. Sometimes PEG brush could act as a barrier for uptake of liposomes by cells, since in the case of endocytosis PEG brush could preclude the escape of liposomes from endosomes and this could resulted in lower cytotoxicities for liposomal formulations than free forms (Deshpande, 2014).

The liposomal formulations of co-loaded liposomes showed higher cytotoxicity than GEM alone after 24 h incubation period (Figures 3.12 and 3.16).

When the liposomal formulation of GEM and GEM/CLF co-loaded liposomes were compared, there was an increase in the cytotoxicity on Saos-2 cells after 24 h incubation period. When the liposomes were diluted to obtain GEM at its IC_{50} concentration, CLF concentration was also lowered than its IC_{50} value. Even at this lower drug concentration, the co-loaded liposomes were much more effective than only GEM loaded liposomes.

Table 3.8 Experimental groups of co-loaded stealth liposomes and percentage cytotoxicity at 24 h

Liposome Types	24 h Cytotoxicity (%)
Empty IC_{50}GEM/CLF-L	14.01±0.18
Empty 2x IC_{50}GEM/CLF-L	20.52±0.11
IC_{50}dose-free GEM/CLF	69.51±0.28
2 x IC_{50}dose-free GEM/CLF	99.12±0.14
IC_{50}GEM/CLF-L	40.22±0.23
2 x IC_{50}GEM/CLF-L	77.06±0.45

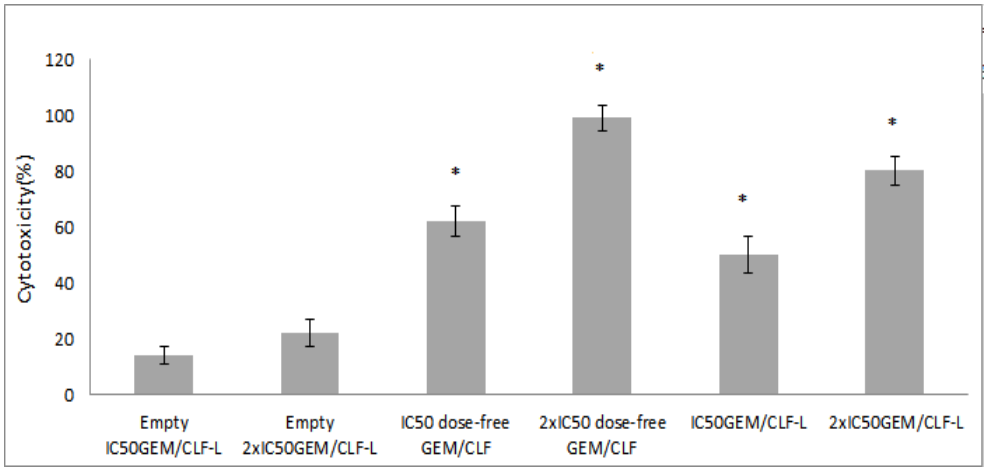


Figure 3.15 Relative cytotoxicity of GEM/CLF co-loaded PEGylated liposomes on Saos-2 cells Asterisks * denote statistical significance at $p<0.05$. Empty IC_{50} GEM/CLF-L is the control group of IC_{50} dose free GEM/CLF and IC_{50} GEM/CLF-L. 2 x Empty IC_{50} GEM/CLF-L is the control group of 2 x IC_{50} dose free GEM/CLF and 2 x IC_{50} GEM/CLF-L. (n=3)

As in the previous results, the liposomal formulations showed lower cytotoxicites than the free forms. All GEM/CLF loaded liposomal formulations showed significantly higher cytotoxicities than empty liposomal formulations. Therefore, GEM/CLF loaded liposomal formulations had cytotoxic effect on Saos-2 cells ($p<0.05$).

In literature, GEM/Docetaxel combinational therapy was tested on high – grade osteosarcoma and spindle cell sarcoma patients. These two drugs could be used in a combination since they have synergistic effect on osteosarcoma. While GEM results in DNA chain elongation and inhibition of cell growth because of the active triphosphate incorporation of DNA, by promoting microtubule assembly docetaxel inhibits the microtubule de-polymerization (Leu et al., 2004). There is a clinical study on patients who are receiving GEM and Docetaxel combinational therapy. Patients were compared to the other patients having only GEM treatment, and the survival rate and complete or partial responses were higher in the GEM/Docetaxel treatment than only GEM treatment (Palmerini et al., 2016).

There is another study about GEM/Tamoxifen co-loaded liposomal treatment on breast cancer cells. As a conclusion of this study, two drugs have different pathways to kill cancer cells and could prevent the resistance of cancer tissue to the anticancer agents by decreasing the effective dose. In the GEM mode of action, cell nucleoside transporter is bypassed by colloidal device that leads to the accumulation of drug in tissues. The liposomal formulation of GEM/Tamoxifen was the most effective treatment on breast cancer cells since the liposomal formulation could lead to decrease effective dose of drugs with the slower release of drugs compared to the free forms. The combinational loading of drugs were much more effective than single loading of both drugs and free forms of them (Cosco, 2011). In addition to the slower release of drugs, the endocytosis of negatively charged liposomes could be harder than the positive or neutral charged liposomes. The repulsive forces between liposomal surface and cell surface could make the liposome endocytosis slower and this could make a decreased in cytotoxicity of liposomal formulations than free forms of drugs (Miller, 1998).

3.2.7. Synergistic Effect of GEM with CLF

Both GEM and CLF alone are able to inhibit proliferation of Saos-2 cells. To investigate whether CLF could enhance the anti-tumor effect of GEM, combination effect was examined. Four different concentrations of CLF (25, 50, 75, 100 μM) and a single dose of GEM (0.17 μM) were evaluated by MTT assay after 24 h treatment. All Combination Index (CI) values were smaller than 1. (Appendix C). Therefore, using GEM and CLF together was found to have synergistic effect in all tested combinations.

3.2.8. Flow Cytometry Analysis

3.2.8.1.Cell Membrane Asymmetry

Annexins, bind to phosphatidylserine (PS) to identify apoptosis, a family of calcium-dependent phospholipid binding proteins. During the initiation of apoptosis, PS distribution is changed in the bilayer since PS is translocated to the outer leaflet of the extracellular membrane. This is detectable with fluorescently labeled Annexin V. In early apopototic phase, cells show negative staining with viability dyes since the cell membranes excludes viability dyes like propidium iodide (PI) and 7-AAD. In late apoptotic pheses, cell membrane structure is distrupted and Annexin V binding to PS occurs with the uptake of dyes (PI and 7AAD), therefore this quantification is widely used to identify apoptotic stages of cells (Elmore, 2007).

The effect of the GEM and CLF formulations were evaluated using Annexin V PE/7-Aminoactinomycin D (7-AAD) staining through flow cytometry. Annexin V selectively binds to phosphatidylserine (PS) in the cellular membrane that occurs in the early stage of apoptosis. Annexin V binding to cells is the indicator of apoptosis. 7-AAD binds to DNA and it is a red fluorescent and only apoptotic cells are permeable to it (Van Engeland, 1995).

Apoptosis of Saos-2 cells were examined with Annexin V FITC and 7AAD (Figure 3.16). The stained cells were divided in three groups: live (negatively stained), early apoptosis (Annexin V stained) and late apoptosis (Annexin V and 7AAD stained). As seen in figure 3.17, apoptotic cells were observed after 24 h exposure with different formulations of GEM and CLF groups. GEM/CLF and GEM/CLF-L groups apoptotic cells ratio were the highest for both early and late apoptotic cells ratio. They are significantly different than the control groups. There were two control groups. Since CLF is a hydrophobic drug and is dissolved in DMSO that is also toxic for cells, the group control for CLF includes 2% DMSO per well CLF. The comparison of combinational formulations were done with control CLF group. Early apoptotic cells fraction was higher in the GEM/CLF-L formulation than the free GEM/CLF group.This might be related with slow release of content from liposomes at 24 h; around 10 %. In addition, the late apoptotic cells fraction was higher in the GEM/CLF group than the GEM/CLF-L formulation.Similarly this could be due to the slow release from the liposomal formulation after the 24 h. Another reason for lower apoptotic cell ratio is due to less amount of drug accumulation in the cells due to longer internalization process of liposomes compared to free forms in solution. Late apoptotic cell ratios were higher with free GEM/CLF treated groups. Slower internalization of liposomes may also lead to higher values of early apoptotic cell ratios than late apoptotic cell ratios. This can also explain the higher value of early apoptotic cell ratio for liposomes

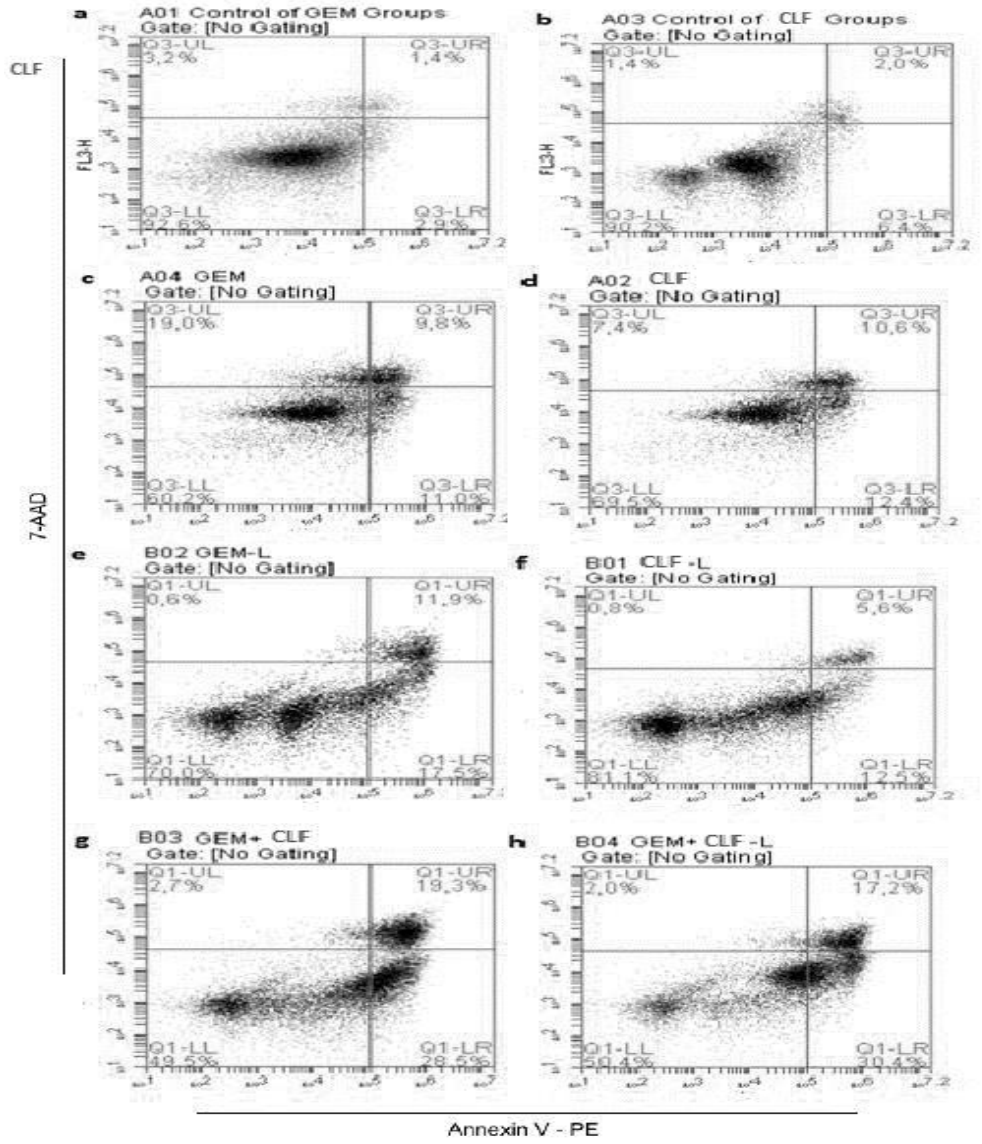


Figure 3.16 Cell membrane asymmetry of Saos-2 cells treated with different formulations of GEM and CLF detected and quantified at 24 h with Annexin V-PE/7-AAD stains. Flow cytometric analysis resultsof Annexin V-PE vs 7-AAD signal gated as live, early apoptotic and late apoptotic quadrants as shown in a) Control for GEM treated cells b) Control for CLF treated cels c) GEM d) CLF e) GEM loaded liposome f) CLF loaded liposome g) free GEM/CLF combination and h) GEM/CLF co-loaded liposome

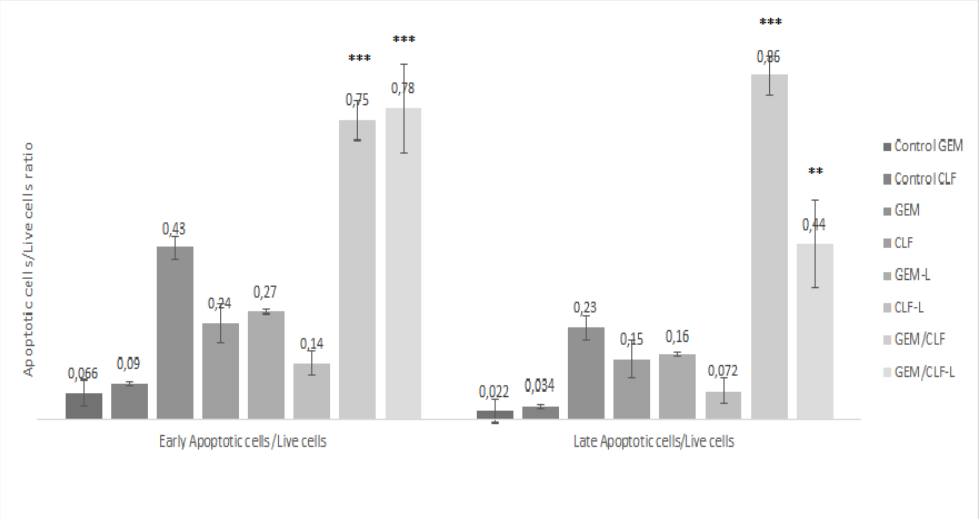


Figure 3.17 Cell population column chart of apoptotic cells/Live cells ratio (n=3). Asterisks ** and *** denote statistical significance between control and treatment populations at $p<0.01$and $p< 0.001$, respectively.

3.2.8.2.Caspase-3 Activity

Caspase-3 is the main protease that is activated in the early stage of apoptosis. It is an inactive proenzyme and activated in the apoptotic cells by activation of other caspases. Active caspases activate other caspases and produce a caspase cascade which cleaves proteins that are important for cell survival. In the final stage of this cascade the controlled cell death occurs. There are two types of cascades; initiators and effectors. Caspase-3 is an effector type and active form of it has two large and two small subunits that cleaves the substrate at the carboxyl terminus of aspartate residues. In the early apoptotic pathway, upon the activation of caspase-3 by other caspases the active sites of subunits come together and contribute to the substrate binding and catalysis therefore caspase-3 is used as a marker of early apoptosis (Elmore, 2007).

Caspase-3 was detected in the treatment groups by flow cytometer analysis. As seen in Figure 3.19, the combination of GEM/CLF group and its liposomal formulation showed the highest caspase-3 activity and the differences from the control group were statistically significant. The

liposomal formulation of GEM/CLF showed less caspase-3 activity than the free GEM/CLF group as in the other flow cytometer results.This may be because of above mentioned reasons related with release and internalization differences.

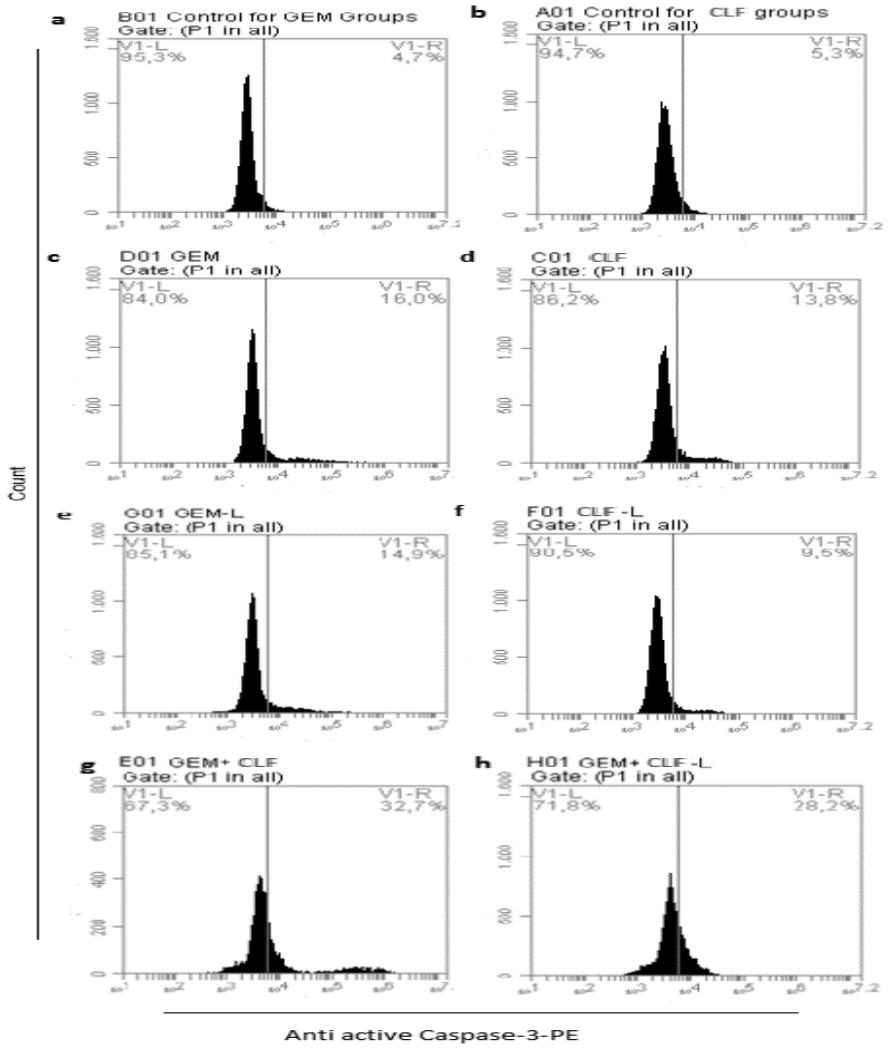


Figure 3.18 Effects of different formulations of GEM and CLF on caspase activation of Saos-2 cells at 24 h. Flow cytometry fluorescence intensity histograms of cells stained with anti-active caspase-3 PE are shown: a) Control for GEM treated cells b) Control for CLF treated cels c) GEM d) CLF e) GEM loaded liposome f) CLF loaded liposome g) free GEM/CLF and h) GEM/CLF co-loaded liposome

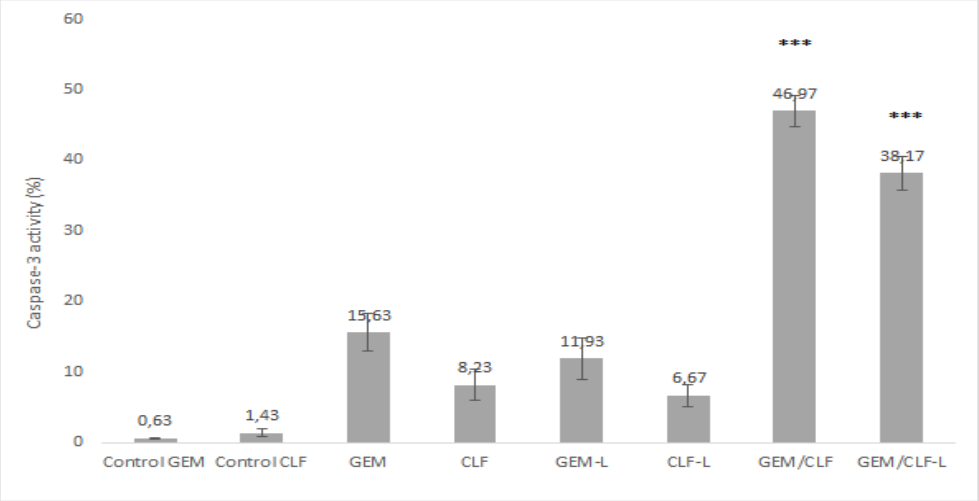


Figure 3.19 Column chart of corresponding histograms are plotted. Asterisks *** denote statistical significance at $p<0.001$, respectively. (n=3).

3.2.8.3.Depolarization of Mitochondrial Membrane

JC-1, a membrane permeable dye, is used to determine mitochondrial membrane potential. JC-1 aggregates when the membrane polarization occurs and the emitted light shifts from 530 nm to 590 nm that is caused by selectively entering of dye to the mitochondria. At 530 nm, the emission of light occurs from JC-1 monomeric form and at 590 nm, from J-aggregate. The shift provide a quantitative measurement in flow cytometer and used to indicate the initiation of apoptosis (Petit & Mignotte, 1995).

Depolarization of the mitochondria is one of the first events in apoptotic pathway. During the mitochondrial respiratory chain, redox reactions produce a negative gradient and JC-1 as a cationic dye can selectively enter mitochondria. The color changes from green to red (Perelman, 2012).

As seen in Figure 3.21, the depolarized cells' fraction was the highest in the groups that GEM and CLF were used together and the differences are significant when comparison was done with the control group. This results

supports the previous outcomes that when GEM and CLF are used in combination they are much more effective on cancer cells.

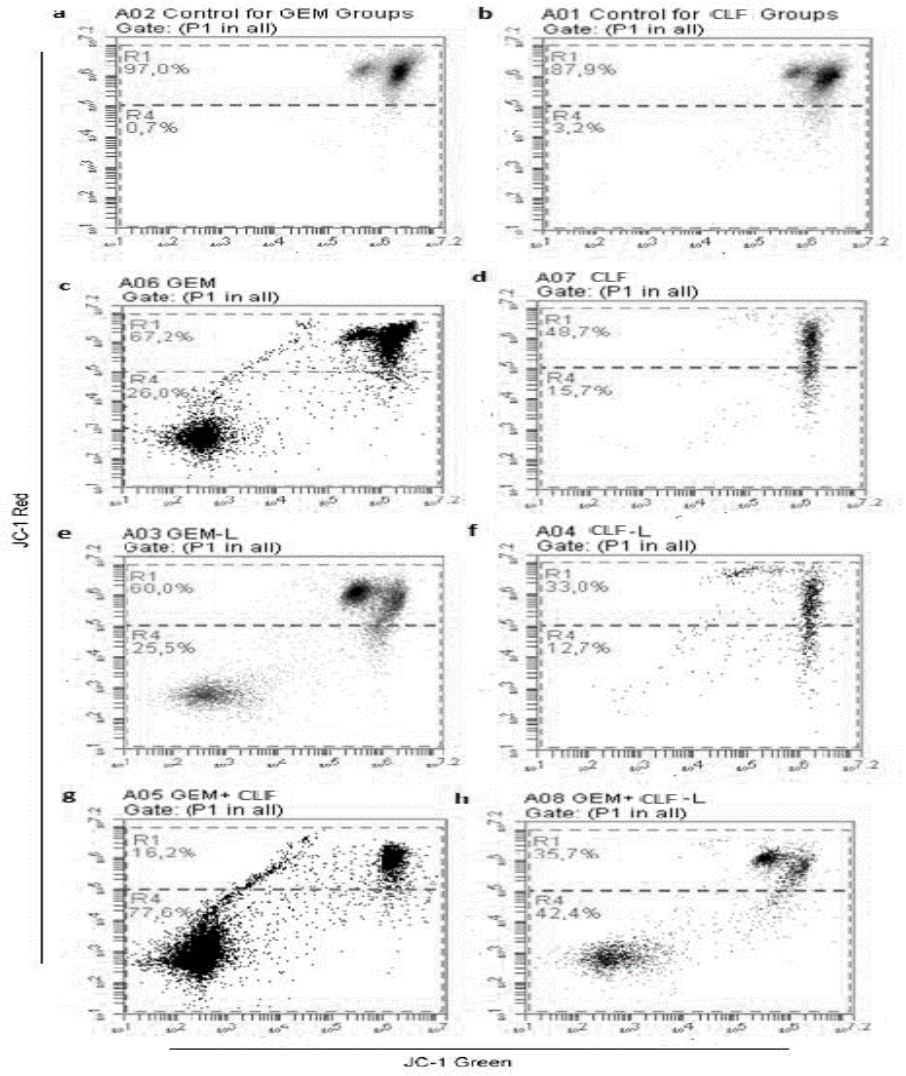


Figure 3.20 Effect of different treatments with GEM and CLF on mitochondrial membrane potential at 24 h. Cells were stained with JC-1 dye. Gated fluorescence intensity values for polarized and depolarized states were labeled. a) Control for GEM treated cells b) Control for CLF treated cels c) GEM d) CLF e) GEM loaded liposome f) CLF loaded liposome g) free GEM/CLF and h) GEM/CLF co-loaded liposome

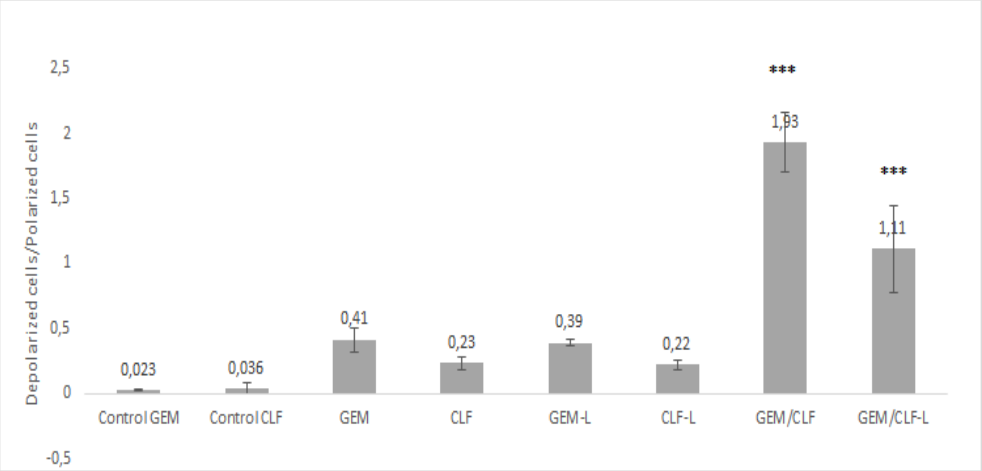


Figure 3.21 Column chart of depolarized/polarized cell ratio. Asterisks *** denote statistical significance at $p<0.001$, respectively. (n=3)

3.2.8.4. Cell Cycle Analysis

Cell cycle analysis was done to determine the percentage of apoptotic and alive cells after treatments. Normal cells have a uniform DNA content in G1 phase and in G2/M phase the DNA content is twice that in G1. In normal conditions, when the DNA is stained by dyes, the G1 and G2/M cells should be uniform. In practical experiments, the G1 and G2 cells are shown by peaks with various width on frequency histograms. When diploid cells stained with a dye that stochiometrically binds to DNA were analyzed by flow cytometry, a "narrow" distribution of fluorescent intensities was obtained. This is displayed as a histogram of fluorescence intensity (X-axis) vs. number of cells with each observed intensity. Since all G1 cells have the same DNA content, exactly the same fluorescence should in theory be detected from every G1 cell, and only a single channel in our histogram would be filled (i.e. there would be a very sharp spike in the histogram at the G1 fluorescence intensity). The greater the observational variation, the broader the resulting Gaussian peak. The term "Coefficient of Variation" (CV) is used to describe the width of the peak. CV is a normalized standard deviation defined as CV = 100 * Standard Deviation / Mean of peak. Generally, there is a high coefficient of variation of G1 cell populations in

improper staining conditions. Generally, apoptotic cells have fractional DNA and fragmented DNA extracted during the staining. The fractions of DNA are represented by the sub-G1 peak in frequency histograms, the quantification of sub-G1 cells should be done with flow cytometry analysis (Schönthal, 2004).

Flow cytometer analysis was done to analyze cell cycle distribution and the results are represented in Figure 3.22. In the literature, G0/G1 phase increased in the GEM treated cells compared to the control group. It was reported that GEM treated cells were arrested at G0/G1 phase in human urinary bladder TCC cell line 5637 (S. Wang, 2010). There is no study about cell cycle analysis of GEM treated Saos-2 cells but the MG63 osteosarcoma cells were shown to be arrested at G2/M phase when they were treated with GEM (Sun et al., 2015). In the present study results, it was shown that the individual GEM treatment led to an increase in G0/G1 phase. The fraction of G0/G1 phase is the highest for combination groups of GEM and CLF. G0/G1 phase increased 2 fold in GEM/CLF group and 1. 5 fold in GEM/CLF-L formulation when they were compared with the control group. There is also no study about cell cycle analysis of Saos-2 cells that are treated with CLF but in these results it was shown that it is also arresting Saos-2 cells in G0/G1 phase. As in the other flow cytometry assay results, in cell cycle analysis the concentration of arrested cells are the highest in GEM/CLF treated cells and the liposomal formulation of combination of GEM/CLF treatment seem to arrest cells less than the free GEM/CLF. The difference between free form and liposomal formulation of GEM/CLF was not significant.

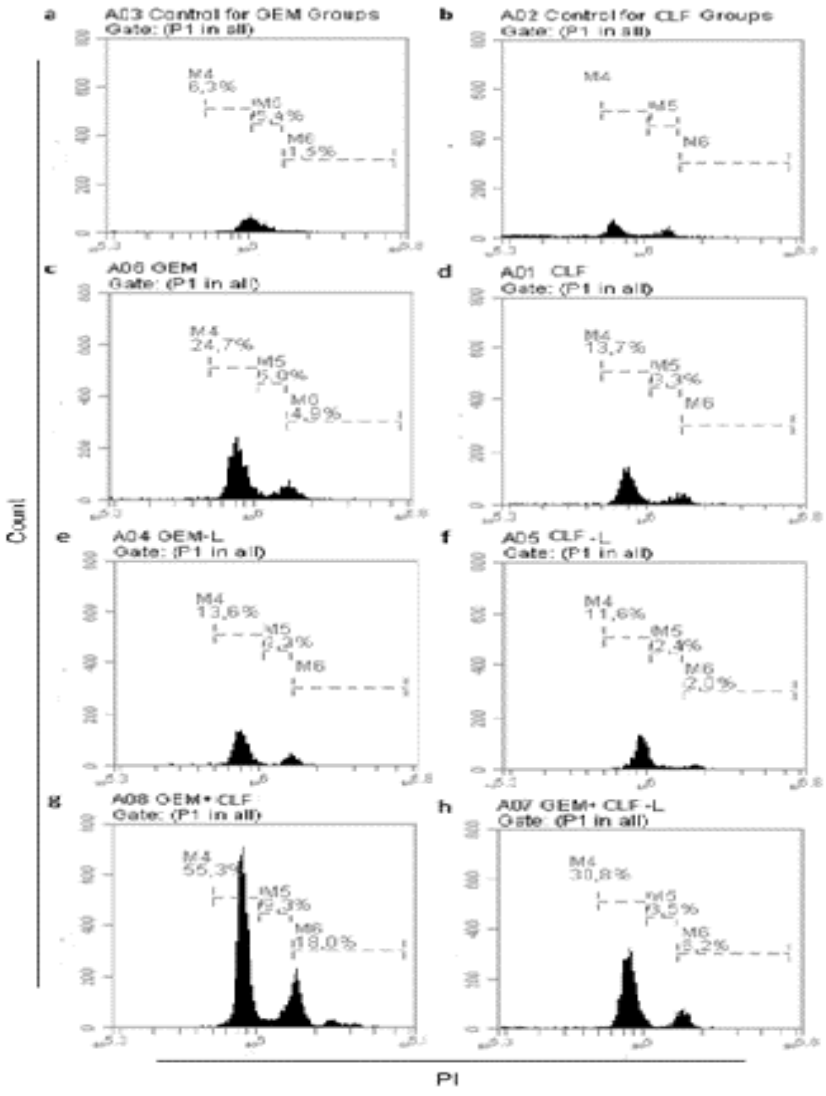


Figure 3.22 Cell-cycle analysis of Saos-2 cells treated with different formulations of GEM and CLF at 24 h. Flow cytometry analysis results showing fluorescence intensity histograms of cells stained with propidium iodide. Intensity ranges for corresponding cell-cycle phases (G0/G1, S and G2/M) were labeled. a) Control for GEM treated cells b) Control for CLF treated cels c) GEM d) CLF e) GEM loaded liposome f) CLF loaded liposome g) free GEM/CLF and h) GEM/CLF co-loaded liposome

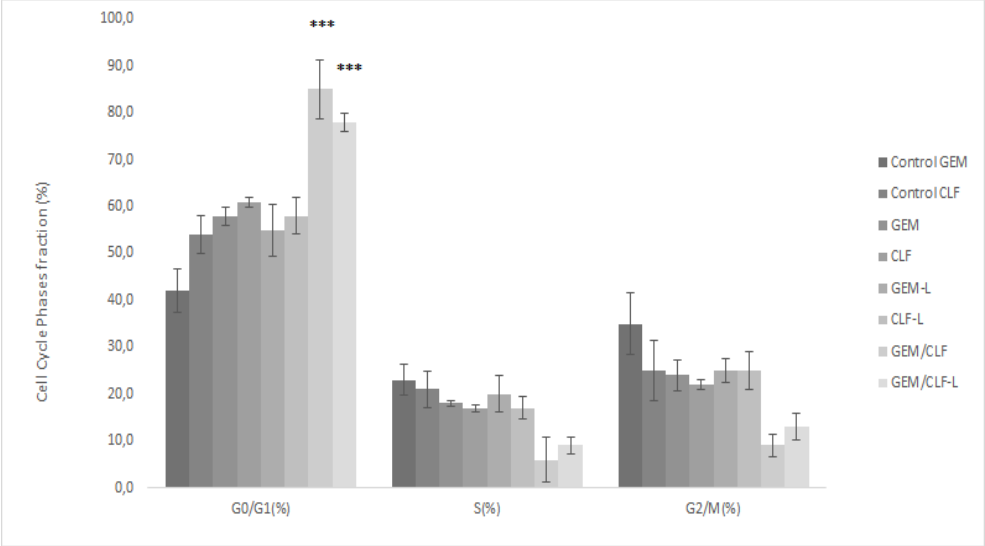


Figure 3.23 Column chart of normalized count values of cell cycle phases' fraction. Asteriks *** denote statistical significance at $p<0.001$ (n=3).

3.3. Stability of Liposome Formulations

Stability of liposome formulations is considered according to the physical, chemical and biological conditions. Generally, chemical and physical properties determine the shelf-life of liposomes. The biological stability determines the circulation time of liposome in blood. To increase this time and prevent the opsonization by immune system, liposomes were PEGylated and to overcome RES effect liposomes' size were optimized around 100 nm (Yadav, Murthy, Shete, & Sakhare, 2011).

Generally, the storage stability of liposomes are examined as its shelf-life as mentioned before. The deformation process of liposomes occurs when the hydrolysis of ester bonds occur and oxidation of lipids decreases the liposome structure integrity. The size distribution, pH and ionic strength should be optimized to obtain stable liposomes (Yadav et al., 2011).

In this study, the stability studies were done by particle size measurements after first and second week of incubations at 4°C and 25°C. Table 3.9 shows

the particle size distribution parameters of liposomes at two different temperatures. The increase in the size and PDI values show the deformation of liposome since oxidation of lipids increase the aggregation rate and liposomes aggregate into larger vesicles with the deformation process (Senior, J. H., 1987).

Table 3.9 Change in particle size distribution of GEM/CLF co-loaded PEGylated liposome

Week	At 4°C		At 25°C	
	Z-average (nm)	PDI	Z-average (nm)	PDI
0	135.0	0.037	135.0	0.035
1	137.2	0.038	143.4	0.047
2	140.1	0.04	155.7	0.07

Accordingly, liposomes were more stable at 4°C than at 25°C. Although they tend to aggregate more at room temperature there wasn't a considerable increase at the average size after incubations at both conditions. It should also be noted that even after 2 weeks of incubation their sizes were within the suitable range for i.v. applications. As the storage condition it could be recommended to keep liposomes at 4°C and use within two weeks.

CHAPTER 4

4. CONCLUSION

In conclusion, in this thesis, different formulations of GEM and CLF encapsulated liposomal systems were developed against osteosarcoma. Increased cytotoxicity cancer cell line, Saos-2 cells, was achieved when GEM and CLF were used in combination. The MTT results of free GEM and CLF combination solution had the highest cytotoxicity as they provide higher and earlier accumulation of both agents than their encapsulated liposomal forms which release the agents in a controlled manner (around 10 % at 24 h). This outcome seems to be a disadvantage for in vitro studies but for in vivo environment it is suggested to be eliminated. With the flow cytometer analysis these results were also similar. The cytotoxicity was enhanced with the combinational usage of GEM with CLF and by the liposomal system. By this aspect, a novel system, liposome co-loaded with GEM and CLF was successfully presented for the first time in this study. Hydrophobic CLF molecule was successfully encapsulated to lipid layer of liposome without altering GEMs' high encapsulation. CLF was encapsulated into liposomes with GEM for the first time and the co-loading of CLF made GEM release slower. The novel co-loaded liposomal GEM/CLF treatment caused higher cytotoxicity than their individual treatment. Apoptotic cells and membrane depolarized cells percent were increased with the co-loaded liposomal GEM/CLF treatment. GEM in combination with CLF had potent synergistic effect. This novel GEM/CLF co-loaded liposomal treatment seems to be a possible therapeutic approach for osteosarcoma.

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APPENDIX A

CALIBRATION CURVES

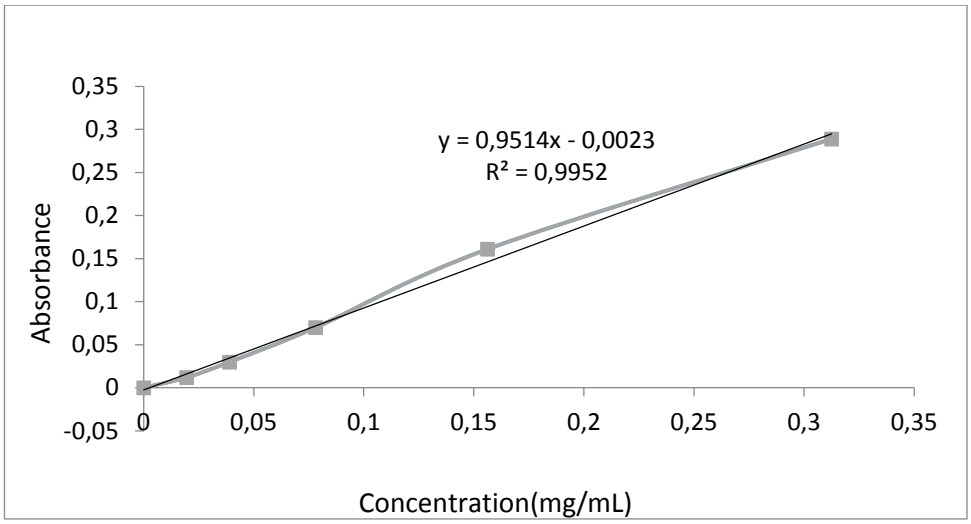


Figure A1: GEM Calibration Curve (in MetOH)

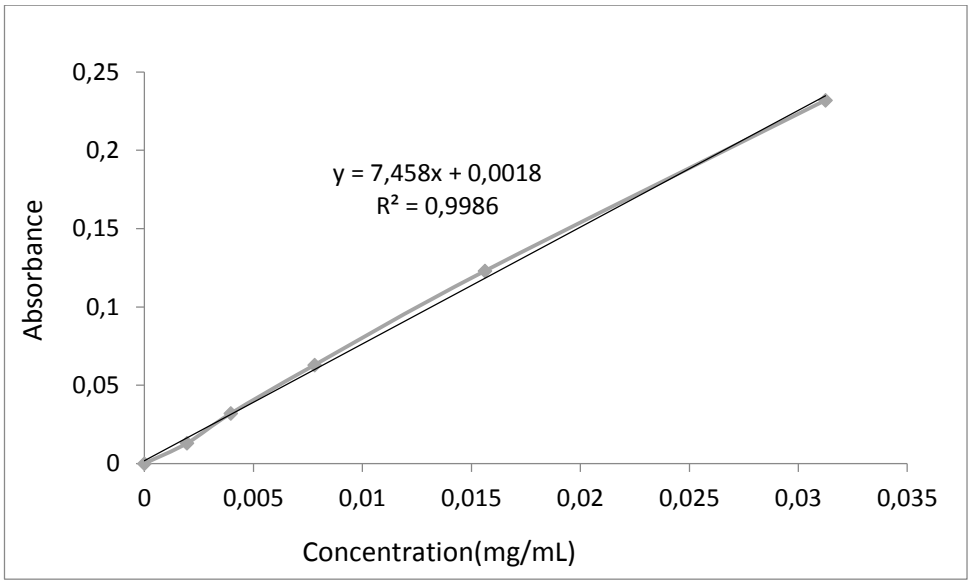


Figure A2: GEM Calibration Curve (in PBS)

APPENDIX B

CALIBRATION CURVES

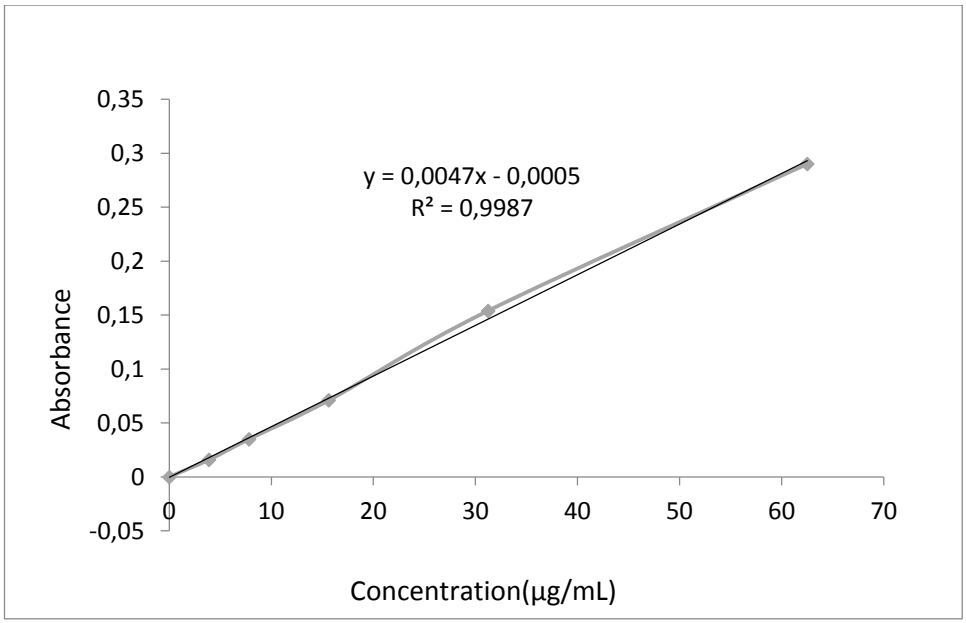


Figure A3: DPPC Calibration Curve

APPENDIX C

COMPUSYN REPORT

Drug: Gemcitabine (GEM) [uM]

Drug: Clofazimine (CLF) [uM]

Drug Combo: Gemcitabine and Clofazimine co-loaded Liposome (GEM/CLF)

Data for Drug: GEM [uM]

Dose	Effect
0.8	0.88
1.6	0.75
3.2	0.65
6.4	0.42

4 data points entered.

Xint:

0.69795

Yint:

0.74767 +/0.04404

m: 1.0712

+/0.09008

Dm: 4.98831

r: 0.9930

Data for Drug: CLF [uM]

Dose Effect

25.0 0.7

50.0 0.67

75.0 0.57

100.0 0.45

4 data points entered.

Xint:

1.99062

Yint:

1.42846 +/0.39798

m: 0.7176

+/0.22644

Dm: 97.8644

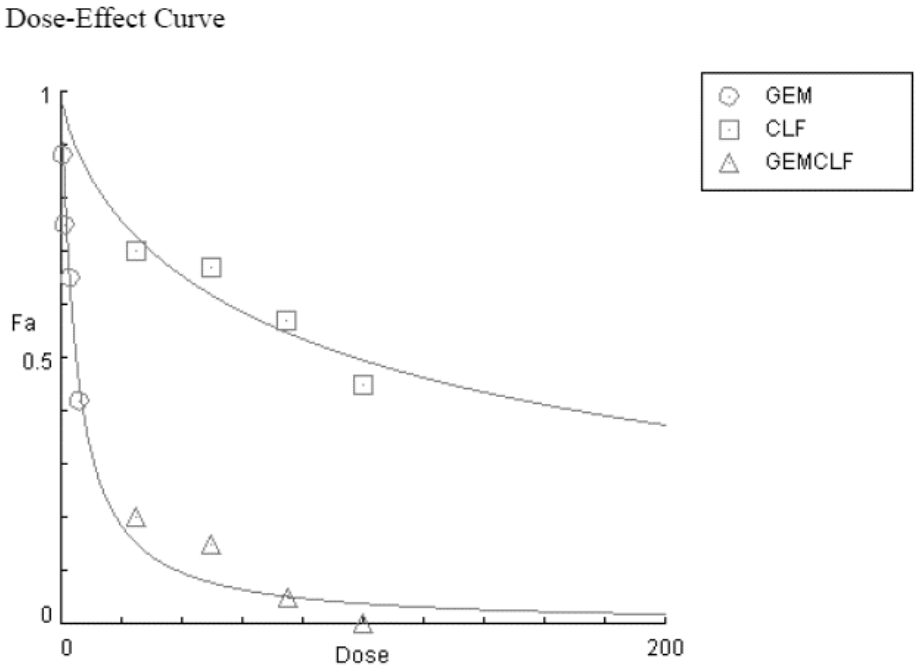
r: 0.9132

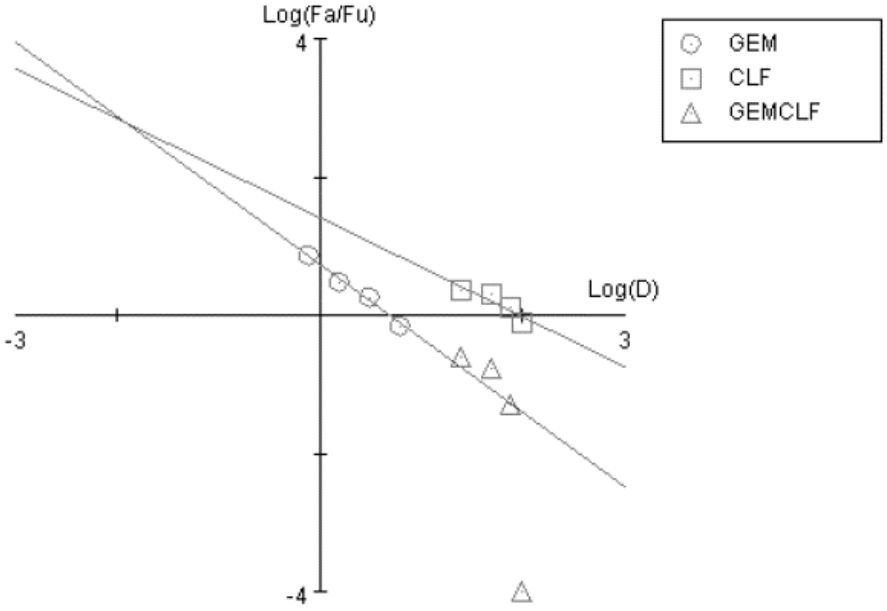
Data for NonConstant

Combo: GEMCLF (GEM+CLF)

Dose GEM	Dose CLF	Effect
0.17	25.0	0.2
0.17	50.0	0.15
0.17	75.0	0.05
0.17	100.0	1.0E-4

4 data points entered.





CI Data for NonConstant

Combo: GEMCLF (GEM+CLF)

Dose GEM	Dose CLF	Effect	CI
0.17	**25.0**	**0.2**	**0.04635**
0.17	**50.0**	**0.15**	**0.05231**

0.17	**75.0**	**0.05**	**0.01484**
0.17	**100.0**	**1.0E-4**	**9.01-E6**

DRI Data for NonConstant

Combo: GEMCLF (GEM+CLF)

Fa	Dose GEM	Dose CLF	DRI GEM	DRI CLF
0.2	**18.1962**	**675.500**	**107.037**	**27.0200**
0.15	**25.1879**	**1097.55**	**148.164**	**21.9510**
0.05	**77.9257**	**5924.23**	**458.387**	**78.9898**
1.0E4	**27036.3**	**3.671E7**	**159037.**	**367084.**

Summary Table

Drug/Combo	Dm	m	r
GEM	**4.98831**	**1.0712**	**0.9930**
CLF	**97.8644**	**0.7176**	**0.9132**