MULTICOMPONENT TARGETED PROAPOPTOTIC ANTICANCER DRUG DELIVERY SYSTEM

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And approved by

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ABSTRACT OF THE DISSERTATION

MULTICOMPONENT TARGETED PROAPOPTOTIC ANTICANCER DRUG DELIVERY SYSTEM

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Cancer is one of the leading causes of death in United States. The current approaches for the treatment of solid tumor available are the surgery for the removal of primary tumor followed by chemotherapy and /or radiation.

The efficacy of chemotherapy is limited by the following factors; (1) adverse side-effects on healthy organs, (2) development of cellular resistance and (3) low solubility of many anticancer drugs.

The primary objective of this dissertation is to overcome all the above stated limitations. The methods employed in this dissertation includes designing, synthesizing and evaluating a novel drug delivery system comprising of 1) multiple copies of an anticancer drug, Camptothecin (CPT) ; 2) a polymeric carrier, Polyethylene glycol (PEG); 3) multiple copies of a targeting moiety to cancer cells, Luteinizing hormone releasing hormone (LHRH) peptide; 4) multiple copies of a suppressor of antiapoptotic cellular

defense, BH3 peptide both *in vitro* and *in vivo* on A2780 human ovarian carcinoma cells and malignant ascites and animal models respectively. Conjugates of CPT, LHRH and CPT, LHRH, BH3 were evaluated for cytotoxicity, cancer specificity and antitumor activity.

The multicomponent anticancer delivery system will significantly enhance the efficacy of an anticancer drug in the treatment of all the stages of cancer when compared with free drug, non-targeted delivery systems or the system consisting of one copy of each active component.

DEDICATION

To my wonderful Parents;

Who has always supported me and encouraged me to follow my dreams.

To my advisor, Professor Tamara Minko;

Who has always supported me.

To my Loving Husband Raj;

Who has always being patient with me and encouraged me.

To my lovely daughter, Risha;

I am very fortunate to have you in my life.

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ABBREVIATIONS USED

CPT, Camptothecin; FITC, Fluorescein isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DDS, Drug Delivery System; LHRH, Luteinizing hormone releasing hormone; PEG, Polyethylene glycol; DMSO, Dimethylsulfoxide; DCM, Dichloromethane.

1. INTRODUCTION.

According to the National Center for Health Statistics, cancer is the second leading cause of death in the United States (NCHS, 2004). Despite the advances in cancer treatment and improvements in life style and health care, death rates from cancer have not changed significantly during the last 50 years. In contrast, mortality from heart disease, the leading cause of death, declined almost 2.5-fold for the same period (1). Therefore, the increase in the efficacy of cancer treatment is an essential task for modern medicine.

Although localized tumors can be successfully removed by surgery, the treatment of spreading or metastatic tumors requires high-dose chemotherapy. However, the efficacy of chemotherapy is limited by the rapid development of tumor resistance. Chemotherapeutic agents are known to induce programmed cell death or apoptosis. The activation of cellular antiapoptotic defense that prevents the translation of drug-induced damage into cell death is considered to be the key factor in cellular resistance to a broad spectrum of anticancer drugs (2, 3, 4, 5). The balance between proapoptotic and antiapoptotic factors in the cancer cell is what determines its fate. The inability of cancer cells to initiate apoptosis in response to a variety of stimuli, including cytotoxic agents, significantly limits the efficacy of cancer therapy. Thus, an increase in apoptosis induction during chemotherapy should significantly increase cancer cell death and the efficacy of chemotherapeutic drugs (3, 6, 7).

The efficacy of cancer chemotherapy is limited by severe adverse side effects induced by anticancer drugs. Most of the anticancer drugs used in chemotherapy require modifications to increase solubility, decrease adverse side effects, limit nonspecific

activity, increase circulation time, modify biodistribution, and so on. Various drug delivery systems (DDS) have been developed to provide these modifications.

The BCL-2 protein family plays a central role in the cellular protection against apoptosis (8, 9). It consists of proapoptotic and antiapoptotic members. Antiapoptotic members help to maintain the integrity of mitochondrial membrane preventing the leakage of cytochrome c into the cytoplasm, limit the activation of executors of apoptosis, caspases and therefore protect cells from apoptotic cell death. In contrast, proapoptotic members stimulate apoptosis mainly by suppression of the activity of antiapoptotic BCL-2 family proteins (8, 9, 10).

The BCL-2 family is characterized by specific regions of homology termed BCL-2 homology (BH1, BH2, BH3, BH4) domains (**Fig.1.1**). These domains are critical to the functions of these proteins, including their impact on cell survival and their ability to interact with other family members and regulatory proteins (8, 9). BCL-2 homology 3 (BH3) domain of proapoptotic proteins from the BCL-2 family is responsible for the induction of apoptosis (8, 9, 10). Furthermore, expression of small truncated derivatives of BAK protein containing the BH3 domain was sufficient for its cell lethal activity. Moreover, it was found that short synthetic peptides, corresponding to the minimal sequence of BH3 domain when bound to the antiapoptotic BCL-2 family proteins, suppress the cellular antiapoptotic defense (10, 11). Therefore, synthetic peptides homologous to BH3 domain can potentially improve traditional cancer therapy by decreasing the resistance of cancer cells due to the reduction of the normal protective antiapoptotic activity of the BCL-2 family proteins that suppress apoptosis. Therefore, BH3 peptide can provide molecular targeting of antiapoptotic members of BCL2 protein

family and potentially improve traditional therapy of ovarian cancer by decreasing the antiapoptotic cellular defense against anticancer drugs. However, the practical use of the BH3 peptide is limited by its low capacity to permeate cells thus requiring a carrier that is capable of delivering it into cancer cells.

Targeting drug therapy specifically to cancer cells has numerous benefits including the maintenance of low plasma to cell concentrations ratio reducing side effects and increasing anti-cancer effectiveness. A targeted anticancer drug must achieve high cell permeability and retention by the specific cell population. Target cell specificity might be achieved by attaching molecular targeting moieties to the drug that interact precisely with cell surface specific receptors, minimizing adverse side effects on healthy tissues. Luteinizing hormone releasing hormone (LHRH) receptor is expressed in several types of cancer cells (ovarian, breast, prostate) and is not normally expressed in healthy human visceral organs (12). Normally LHRH is released from the hypothalamic region of the brain into the pituitary portal circulation. From there, it is transported to the adenohypophysis, where it triggers the release of the luteinizing hormone into the systemic circulation. Therefore, the LHRH decapeptide can be used to target cell surface receptors to deliver anticancer drug and BH3 peptide specifically to cancer cells expressing these receptors and facilitate cellular uptake of BH3 peptide (**Fig.1.2**).

The first set of studies in this dissertation employed the design and evaluation of a water-soluble multicomponent drug delivery system or polymeric prodrug which is able to simultaneously deliver several copies of anticancer drugs specifically to tumor. The drug delivery system consists of up to three copies of an apoptotic agent or an anticancer drug, Camptothecin, widely used drug in the treatment of ovarian cancer, up to three

copies of LHRH as a targeting agent or penetration enhancer and Polyethylene glycol (PEG) ~ 3000 as a carrier. This approach is based on the use of multivalent spacer which allows the conjugation of multiple copies of drug and targeting agent. The spacer used in the present investigation is citric acid. The number of molecules of conjugated substances per one molecule of PEG is limited mainly by their solubility, not by the design, synthesis of the whole system and steric hindrance of components.

Polyethylene glycol (PEG) polymer is widely used as a carrier for DDS. PEG is a water-soluble nonionic polymer approved by the Food and Drug Administration for pharmaceutical applications. PEG is essentially non-toxic; PEG conjugated proteins have already been approved for human use; PEG can be obtained with low polydispersity, is easily activated for conjugation, and PEG is relatively inexpensive for large scale processes (9). The foremost advantage of using PEG as a carrier specifically with respect to anticancer drug is that it improves the solubility of poorly soluble drugs. It is highly significant in terms of delivery and formulation of these drugs. Secondly, it provides a protective shell to the drug, impeding it from the interactions from outside resulting in longer circulation half-life (9). The final advantage of using PEG as a carrier is its ability to override multidrug resistance. The copolymer utilizes the receptor-mediated endocytosis for entry into the cancer cells, thus avoiding the elimination by P-glycoprotein (P-gp) or multidrug resistance pump. The cellular entry via this route also accommodates the release of drug from the polymer by lysosomal enzymes by integration of spacer between the components. The most frequently used anticancer drugs have a dual effect on cancer cells. On one hand, they provoke cell death mainly by activation of the central apoptotic signal. On the other hand, they trigger antiapoptotic cellular defense

primarily by inducing the overexpression of antiapoptotic members of the BCL2 protein family (8).

The second set of studies are based on the inclusion of a synthetic analogue of BCL2 homology 3 (BH3) peptide or antisense oligonucleotides targeted to BCL2 mRNA in the same drug delivery system as with LHRH will provide for concurrent tumor targeting and suppression of antiapoptotic cellular defense (**Fig.1.3**). Our previous experimental data showed that LHRH peptide enhanced the uptake of DDS by cancer cells and limited the accumulation of an anticancer drug in healthy tissues but did not prevent the overexpression of antiapoptotic members of the BCL2 protein family (5, 6, 7). BH3 peptide substantially enhanced the cytotoxicity of traditional chemotherapeutic drugs but was unable to prevent their accumulation in healthy organs, thus increasing the risk of adverse side effects. The system also uses a novel multivalent branched spacer (citric acid) in combining several copies of active ingredients in one DDS to further enhance tumor targeting and cytotoxicity.

The final set of studies in this dissertation is based on the evaluation of synthesized drug delivery systems or polymeric prodrugs in the treatment of metastatic ovarian cancer in order to prove the efficacy of the proposed system in the treatment of all the stages of ovarian cancer. The primary cell-culture techniques are used in these studies. Ascites cells obtained from peritoneum of an ovarian cancer patient are used for the studies. To further prove our hypothesis, Pharmacokinetic studies are also performed on nude mice bearing metastatic ascites tumor.

In conclusion, the studies discussed in this dissertation address the various issues in pharmaceutical therapeutics and are aimed at resolving some of the problems with focus on development of a novel drug delivery system for efficient anticancer drug delivery.

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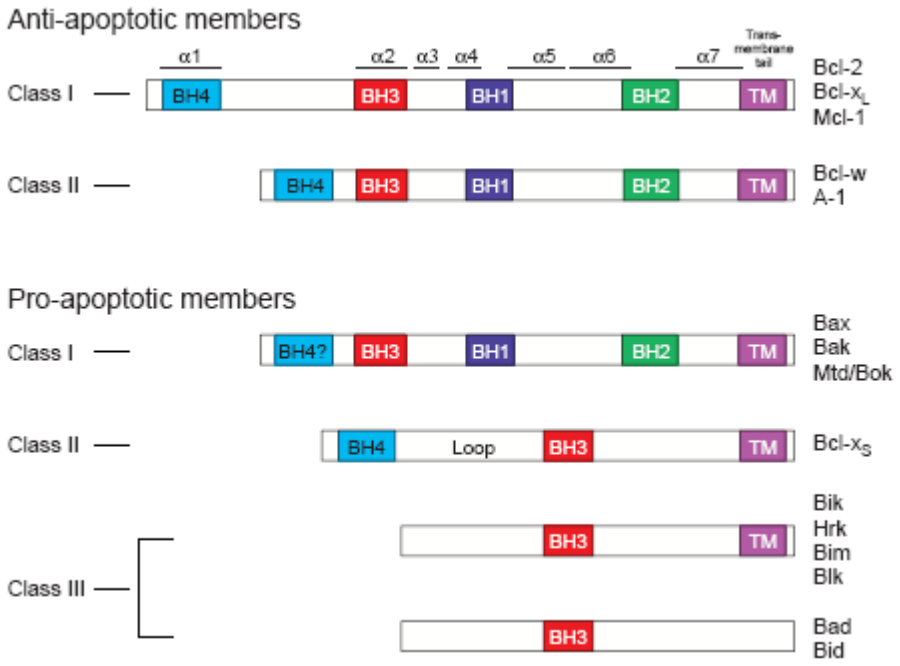

Fig. 1.1 Classification of the Bcl-2 family of proteins based on domain organization. The general architecture of the proteins is shown (not to scale); Bcl-2 homology (BH) domains and transmembrane (TM) domains are labeled (Reproduced from Ref. [14]).

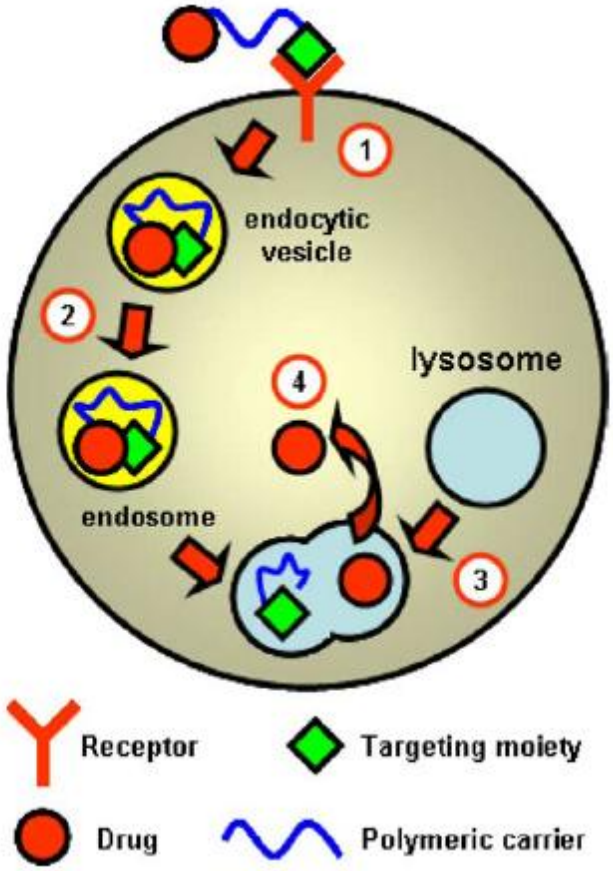

Fig. 1.2. Mechanism of receptor-mediated endocytosis exhibited by LHRH conjugates (Reproduced from Ref. [13]).

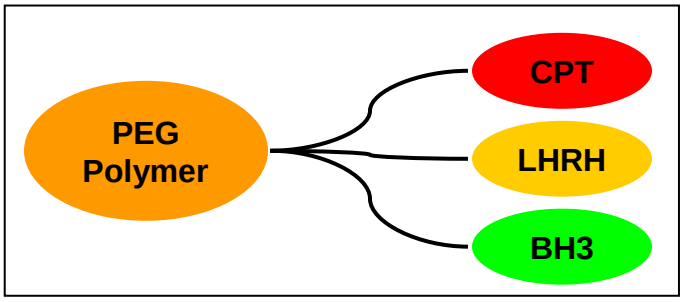

Fig. 1.3 Four tier Drug delivery system/ polymeric prodrug composed of a carrier (PEG), targeting moiety (LHRH), an anticancer drug/ apoptotic agent, Camptothecin and an inhibitor of antiapoptotic cellular resistance (BH3).

2. BACKGROUND AND SIGNIFICANCE.

2.1. OVARIAN CANCER.

Cancer is a disease in which certain body cells don't function right, divide very fast, and produce too much tissue that forms a tumor (1).

Ovarian cancer is a disease produced by the rapid growth and division of cells within one or both ovaries—reproductive glands in which the ova, or eggs, and the female sex hormones are made. The ovaries contain cells that, under normal circumstances, reproduce to maintain tissue health. When growth control is lost and cells divide too much and too fast, a cellular mass or tumor is formed. If the tumor is confined to a few cell layers, for example, surface cells, and it does not invade surrounding tissues or organs, it is considered benign (1,2, 3, 4).

If the tumor spreads to surrounding tissues or organs, it is considered malignant, or cancerous. When cancerous cells break away from the original tumor, travel through the blood or lymphatic vessels, and grow within other parts of the body, the process is known as metastasis (4).

Ovarian cancer is the fifth leading cause of cancer deaths in women, the leading cause of death from gynecological malignancy, and the second most commonly diagnosed gynecologic malignancy (1). According to the American Cancer Society, there is no true test for ovarian cancer.

The exact cause is usually unknown. The disease is more common in industrialized nations, with the exception of Japan. In the United States, females have a 1.4% to 2.5%

(1 out of 40-60 women) lifetime chance of developing ovarian cancer. Older women are at highest risk (1, 2). More than half of the deaths from ovarian cancer occur in women between 55 and 74 years of age and approximately one quarter of ovarian cancer deaths occur in women between 35 and 54 years of age.

The risk of developing ovarian cancer appears to be affected by several factors. The more children a woman has, the lower is her risk of developing ovarian cancer. Early age at first pregnancy, older ages of final pregnancy and the use of low dose hormonal contraception have also been shown to have a protective effect (2).

Types of ovarian cancer.

Ovarian cancer is not a single disease. There are actually more than 30 types and subtypes of ovarian malignancies, each with its own histopathologic (diseased tissue) appearance and biologic behavior (2, 3). Because of this, most experts group ovarian cancers within three major categories, according to the kind of cells from which they were formed:

1. Epithelial tumors arise from cells that line or cover the ovaries;
2. Germ cell tumors originate from cells that are destined to form eggs within the ovaries; and
3. Sex cord-stromal cell tumors begin in the connective cells that hold the ovaries together and produce female hormones.

In addition, some tumors that are adjacent to ovarian tissues may be viewed as ovarian cancer. For example, extraovarian peritoneal carcinoma (intraperitoneal

carcinomatosis)—cancer of the membrane lining the walls of the pelvic cavity next to the ovaries—is a tumor that is thought of and treated as if it were advanced ovarian cancer. The prognosis or probable outcome of the disease, in patients with this condition is not very different from that in women with advanced ovarian carcinoma (2, 4).

Treatment

There are three main forms of treatment for ovarian cancer:

1. Surgery to remove cancerous tissue.
2. Chemotherapy to destroy cancer cells using strong anti-cancer drugs. It is commonly used as follow-up therapy to surgery.
3. Radiotherapy to destroy cancer cells by high-energy radiation exposure. However, this method is rarely used in the treatment of ovarian cancer in United States.

There are also many combinations of these treatment methods and it is usually worthwhile to get a second opinion about treatment before entering into a specific program. Treatment depends upon a number of factors (e.g., stage and grade of the disease, the histopathologic type, and the patient's age and overall health.

2.2. CURRENT CHEMOTHERAPEUTICS IN THE TREATMENT OF OVARIAN CANCER.

Ovarian cancer originates in tissue exclusive to the ovary, including surface epithelial cells, germ cells, and the sex cord-stromal cells. Most epithelial ovarian tumors are

benign, including serous adenomas, mucinous adenomas, and Brenner tumors. Malignant epithelial ovarian carcinomas comprise 85%-90% of all ovarian cancer.

The primary route of treatment usually consists of surgery followed by chemotherapy with a platinum agent, carboplatin and a taxane, typically taxol.

2.2.1. FIRST-LINE CHEMOTHERAPY.

In general, chemotherapy is offered to patients with stage Ic disease or above, (disease confined to one or both ovaries with positive peritoneal washings or ascites). The most effective treatment in epithelial ovarian carcinoma is platinum-based chemotherapy, first introduced in the form of cisplatin in the mid-1970s. An improvement in efficacy was demonstrated in the mid-1990s with the addition of paclitaxel to the platinum regimes (4, 5).

The usual regime is of 6*3-weekly cycles of carboplatin area under the concentration curve (AUC) 5–7.5 given over 1 h plus paclitaxel 175 mg/m^2given over 3 h.

The mechanism of action of the platinum coordination complexes (cisplatin, carboplatin, oxaliplatin) and most alkylating agents is that they form strong electrophilic intermediates that act via nucleophilic substitution reactions to form inter- and intrastrand DNA crosslinks. Although toxicity among these agents varies, most side effects also are attributed to nucleophilic substitution reactions, e.g., cisplatin toxicity (nephrotoxicity, neurotoxicity, ototoxicity) is attributable to protein sulfhydryl binding and inactivation of Thiol-containing enzymes (5).

The mechanism of action of Paclitaxel is that it interferes with the normal function of microtubule breakdown by inhibiting the depolymerizariton of tubulin subunits in metaphase of cell-cycle (4).

This destroys the cell's ability to use its cytoskeleton in a flexible manner. Specifically, paclitaxel binds to the β subunit of tubulin. Tubulin is the "building block" of microtubules, and the binding of paclitaxel locks these building blocks in place. The resulting microtubule/paclitaxel complex does not have the ability to disassemble. This adversely affects cell function because the shortening and lengthening of microtubules (termed dynamic instability) is necessary for their function as a mechanism to transport other cellular components.

Recent clinical data suggest that docetaxel, a semi synthetic taxane with a superior preclinical profile, is at least equally effective to paclitaxel with significant differences in its side-effect profile. Docetaxel has shown a response rate of 28% in platinum-refractory patients and activity in paclitaxel-refractory patients (23%response rate) (3, 4).

2.2.2. RECURRENT DISEASE/ SECOND LINE TREATMENT.

Ovarian cancer that relapses after first-line treatment is incurable; although most ovarian tumors initially respond to chemotherapy, eventually the disease becomes resistant to treatment. However, the judicious use of chemotherapy can result in improvements in both survival and quality of life for patients with recurrent disease. Even though there is no standard for recurrent ovarian cancer, but the question arises is when to start the second line of treatment (6).

Most women with ovarian carcinoma have high serum concentrations of the antigen CA-125 at the time of diagnosis, and this antigen is also a sensitive marker of relapse with a median lag time of 3–9 months before disease becomes evident macroscopically. However, there is no definite correlation between the serum levels of CA-125 and relapse in patients who do not show any symptoms for the disease.

When proceeding with the second line treatment of ovarian cancer, there are several compounds that have shown activity in relapsed ovarian cancer. Some of these are summarized below (6, 7, 8, 9).

Topotecan. Topotecan is a topoisomerase I inhibitor with proven antitumor efficacy in a number of malignancies, including ovarian cancer. It has become an established treatment for relapsed ovarian cancer and has demonstrated good response rates in both platinum-sensitive and –resistant tumors

Phase I /II studies have confirmed that weekly topotecan dosing, using either a 24-h or a 30-min intravenous infusion, produces substantially less myelosuppression than the standard schedule (1.5 mg/m^2 daily for 5 days every 21 days).

Pegylated liposomal doxorubicin

Liposomal entrapment of cytotoxic agents offers the possibility of greater efficacy through changes in biodistribution and pharmacokinetic profile, and there is some evidence that liposomes can be preferentially taken up by tumor cells. Polyethylene glycol coating of liposomes (pegylation) is a further refinement that prolongs liposome integrity and inhibits uptake by the reticuloendothelial system.

The mechanism of action of Doxorubicin is that it interacts with DNA by intercalation and inhibition of macromolecular biosynthesis. This inhibits the progression of the

enzyme topoisomerase II, which unwinds DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication.

Endocrine agents

Endocrine therapy has long been used for patients with refractory ovarian cancer. The estrogen receptors are expressed in 40% of ovarian carcinomas, however, till date; there is no correlation between number of receptors and efficacy of tamoxifen.

The overall response rate to progestagens and antioestrogens is low (10–15%), but a large GOG study reported an 18% response rate, including a 10% complete remission rate, for tamoxifen administered at a dose of 20 mg twice daily by mouth. Therefore, tamoxifen or progestagen therapy can be a useful option for patients who cannot tolerate cytotoxic regimens or in whom they have failed.

There are several other second line therapies for relapsed ovarian cancer, such as, Altretamine, Etoposide, Gemitacibine, Vinorelbine and Capecitabine.

2.3. METASTASIS/ MALIGNANCY IN OVARIAN CANCER.

Epithelial ovarian cancer is the sixth most frequent form of cancer in women worldwide and the fourth most frequent cause of cancer death among women in both the United States and the United Kingdom. At the same time it is the second most common gynecologic malignancy and the most frequent cause of death from gynecologic cancer in the developed countries. At the time of diagnosis the majority of patients will present

with advanced disease (FIGO stage III-IV) because the disease is often asymptomatic in its early stage (11).

Despite the fact that it is one of the most chemosensitive cancers, with response rate to platinum-containing regiments of greater than 60% (4) and intravenous paclitaxel greater than 80% (5), the prognosis remains poor with a 5-year survival rate of approximately 15%- 20% in stage III and less than 5% in stage IV patients (6). The largely unchanged mortality rate from ovarian cancer reflects its late clinical appearance. Two-thirds of the patients are diagnosed with stage III or IV disease, commonly associated with the accumulation of ascitic fluid in the peritoneal cavity (11, 12).

Many diseases are complicated by the accumulation of free fluid within the peritoneal cavity i.e. the onset of ascites. The most common cause of ascites is liver cirrhosis, but in about 20% of cases there is an extrahepatic cause. Malignant ascites is a manifestation of end stage events in a variety of cancers and is associated with significant morbidity (11, 12).

It accounts for about 10% of all cases of ascites and usually caused by ovarian, endometrial, breast, oesophageal, gastric, colorectal, lung, pancreatic, hepatobiliary and primary peritoneal carcinomas (12).

Ascites is a common and distressing complication of human abdominal cancer, including ovarian cancer. The collection of intraperitoneal fluid in a patient with ovarian cancer is most likely due to intraperitoneal spread of disease and if neoplastic cells are identified, the term malignant ascites is used (12).

CHARACTERISTICS OF MALIGNANT ASCITES

Malignant ascites is characterized by positive cytology of malignant cells, large number of white blood cells and a higher lactate dehydrogenase level (12, 13). Interestingly, the main ascitic fluid protein-levels are high in patients with peritonitis carcinomatosa, as are ascites albumin concentrations (13). The data show intraperitoneal protein and albumin accumulation in malignant ascites. Fluid accumulation occurs if lymphatic drainage of peritoneal cavity is compromised or if net filtration is increased, overwhelming the lymphatic capacity. In malignant ascites, fluid accumulation is the result of filtration minus drainage.

2.4. APOPTOSIS.

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death (which is why apoptosis is often referred to as cell suicide) (14).

2.4.1. APOPTOSIS IN HEALTH AND DISEASE

Apoptosis occurs during the normal development of multicellular organisms and continues throughout adult life. The combination of apoptosis and cell proliferation is responsible for shaping tissues and organs in developing embryos.

Apoptosis is also an important part of the regulation of the immune system. T lymphocytes are cells of the immune system that are responsible for destroying infected or damaged cells in the body. They mature in the thymus, but before they can enter the bloodstream they are tested to ensure that they are effective against foreign antigens and are also not reactive against normal, healthy cells. Any ineffective or self-reactive T-cells are removed through the induction of apoptosis (14).

Problems with the regulation of apoptosis have been implicated in a number of diseases. Cancer is a disease that is often characterized by too little apoptosis. Cancer cells typically possess a number of mutations that have allowed them to ignore normal cellular signals regulating their growth and become more proliferative than normal. Under normal circumstances damaged cells will undergo apoptosis, but in the case of cancer cells mutations may have occurred that prevent cells from undergoing apoptosis. In these cases there is no check on the cellular proliferation and consequently the disease can progress to the formation of tumors. In many cases these tumors can be difficult to kill as many cancer treatments rely on damaging the cells with radiation or chemicals and mutations in the apoptotic pathway often produce cells that are resistant to this type of attack. Understanding how apoptosis is regulated in cancer is therefore of major interest in the development of treatments for this disease (14, 15).

Apoptosis is also important for normal placental development. During pregnancy trophoblast cells from the placenta invade the uterine environment in order to remodel the maternal blood vessels and help establish and maintain a successful pregnancy. Strict control over cell proliferation and apoptosis is required to achieve this. In some cases this process can be compromised and excessive apoptosis of the trophoblast cells is thought to

be implicated in the failure to fully remodel the maternal environment that is observed in complications of pregnancy such as preeclampsia (14, 15).

2.4.2. ROLE OF MITOCHONDRIA IN APOPTOSIS

Mitochondria play an important role in the regulation of cell death. They contain many pro-apoptotic proteins such as Apoptosis Inducing Factor (AIF), Smac/DIABLO and cytochrome C. These factors are released from the mitochondria following the formation of a pore in the mitochondrial membrane called the Permeability Transition pore, or PT pore. These pores are thought to form through the action of the pro-apoptotic members of the bcl-2 family of proteins, which in turn are activated by apoptotic signals such as cell stress, free radical damage or growth factor deprivation. Mitochondria also play an important role in amplifying the apoptotic signaling from the death receptors, with receptor recruited caspase 8 activating the pro-apoptotic bcl-2 protein, Bid (14, 15).

2.4.3. ROLE OF BCL-2 PROTEINS

The bcl-2 proteins are a family of proteins involved in the response to apoptosis. Some of these proteins (such as bcl-2 and bcl-XL) are anti-apoptotic, while others (such as Bad, Bax or Bid) are pro-apoptotic. The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic bcl-2 proteins. When there is an excess of pro-apoptotic proteins the cells are more sensitive to apoptosis, when there is an excess of anti-apoptotic proteins the cells will tend to be more resistant. An excess of pro-apoptotic bcl-2 proteins at the surface of the mitochondria is thought to be important in the formation of the PT pore (15, 16).

The release of cytochrome C from the mitochondria is a particularly important event in the induction of apoptosis. Once cytochrome C has been released into the cytosol it is able to interact with a protein called Apaf-1. This leads to the recruitment of pro-caspase 9 into a multi-protein complex with cytochrome C and Apaf-1 called the apoptosome. Formation of the apoptosome leads to activation of caspase 9 and the induction of apoptosis (16).

2.4.4. CASPASES AND APOPTOSIS

The caspases are a family of proteins that are one of the main executors of the apoptotic process. They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis (**Fig. 2.1**).

Induction of apoptosis via death receptors typically results in the activation of an initiator caspase such as caspase 8 or caspase 10. These caspases can then activate other caspases in a cascade. This cascade eventually leads to the activation of the effector caspases, such as caspase 3 and caspase 6. These caspases are responsible for the cleavage of the key cellular proteins, such as cytoskeletal proteins, that leads to the typical morphological changes observed in cells undergoing apoptosis (15, 16).

2.4.5. THE APOPTOTIC PROCESS

Upon receiving specific signals instructing the cells to undergo apoptosis a number of distinctive changes occur in the cell. A family of proteins known as caspases is typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular components that are required for normal cellular function including structural

proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus.

Apoptotic cells display distinctive morphology during the apoptotic process, and this can be seen in **Figure 2.2**. Typically, the cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton (A). The breakdown of chromatin in the nucleus often leads to nuclear condensation and in many cases the nuclei of apoptotic cells take on a "horse-shoe" like appearance (B).

Cells continue to shrink (C), packaging themselves into a form that allows for their removal by macrophages. These phagocytic cells are responsible for clearing the apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death. In order to promote their phagocytosis by macrophages, apoptotic cells often undergo plasma membrane changes that trigger the macrophage response. One such change is the translocation of phosphatidylserine from the inside of the cell to the outer surface. The end stages of apoptosis are often characterized by the appearance of membrane blebs (D) or blisters process. Small vesicles called apoptotic bodies are also sometimes observed (14, 15, 16).

2.5. MULTIDRUG RESISTANCE IN TUMORS.

Many chemotherapeutic drugs target cancer cells at multiple molecular and cellular levels. Resistance to chemotherapy emerges as an important factor that negatively influences the efficiency of cancer treatment. This is especially seen in epithelial tumors including breast, ovarian and lung carcinoma despite utilization of multi-drug regiments. There are various mechanisms responsible for cancer cell chemoresistance, such as

modification of drug-target interactions, decreased uptake or increased elimination of active molecule and apoptosis defects (17, 18).

CELLULAR MECHANISMS OF DRUG RESISTANCE

A wide range of metabolic or structural properties within tumor cells may lead to drug resistance. The identified mechanisms include:

- decreased drug uptake,
- increased drug efflux,
- increased repair of DNA damage induced by chemotherapy and
- Reduced ability to undergo apoptosis.

In addition, because cancer cells are heterogeneous, more than one mechanism of drug resistance may be present in any particular case.

Classical multidrug resistance

Many drugs are substrates for membrane-based proteins (from the ATP-binding cassette family) that actively pump drugs out of cells (**Fig. 2.3**). The expression of these proteins can cause multidrug resistance (MDR) towards numerous anticancer drugs, including the taxanes. Two important MDR-associated proteins are P-glycoprotein and MDR-associated protein (MRP) which actively pump the drug out of the cells (17, 18, 43).

This characteristic action of the P-gp efflux pump is a serious problem to the effectiveness of chemotherapeutic agents and hence is the focus of major research.

Many agents that can modulate the function of efflux pumps have been identified, including calcium channel blockers, calmodulin antagonists, steroids, protein kinase C inhibitors, immunosuppressive drugs, antibiotics and surfactants. However, the main

concern with the use of these agents was the elevated level of toxicity at which they exhibit their inhibitory effect.

One of the most studied PgP modulator is the cytotoxic agent, Valspodar, a derivative of cyclosporine D. Even though Valspodar lacks the immunosuppressive effects and is a more potent MDR reversing agent than cyclosporine, it has its own share of flaws.

The newer batch of MDR modulators aim at specifically inhibiting the efflux pump of concern. The use of combinatorial chemistry and structure –activity relationship may help overcome the problems associated with current MDR-modulators (18, 19).

2.6. WATER-SOLUBLE POLYMERIC DRUG CARRIERS.

Polymer conjugation is of increasing interest in pharmaceutical chemistry for delivering drugs of simple structure or complex compounds such peptides, enzymes and oligonucleotides. In the past, drugs mainly with antitumoral activity have been coupled to natural or synthetic polymers with the purpose of increasing their blood permanence time, taking advantage of the increased mass that reduces kidney ultrafiltration (20).

The initial concept of the “magic bullet” presented by Paul Ehrlich, described the need to specifically direct drugs to their specific site of action. The cellular uptake of macromolecules occurs via endocytosis while concentration-gradient driven diffusion incase of small molecules is of limited relevance.

A further step in the design of the conjugates penetrating the cells by endocytosis is the introduction of spacer between the drug and the polymer. The spacer is generally cleavable by lysosomal proteases but not by blood enzymes. This allows the drug to be liberated by the lysosomal enzyme form the polymer matrix once inside the cell.

An example of such conjugates is that of the N-(2-hydroxypropyl) methacrylamide (HPMA) polymer and Doxorubicin directed towards the tumor cells. In these studies, Doxorubicin was conjugated with HPMA in order to protect the labile drug from degradation and to slow down its renal ultrafiltration. Additionally, the high molecular weight of the conjugate caused localization within the tumor as the vascular epithelial cells in these areas is already fenestrated. This is known as Enhanced Permeability and Retention (EPR) effect (20, 21).

Another component of the bioconjugates is the addition of targeting moiety. The targeting moiety is attached to the polymer via covalent bond and acts as a means to direct the macromolecule towards the specific site of action (20, 21).

Usually, these targeted systems utilize the incorporation of targeting moiety to interact with receptors present on the surface of specific cells. A major advantage is the lack or decrease in side –effects generated by the drug.

The high-molecular weight of the bioconjugates coupled with the site directed targeting moiety, determined its intracellular as well as the extracellular route, which involves uptake due to receptor mediated endocytosis followed by lysosomal degradation of the spacer and the release of the drug (21).

Advantages of bioconjugation of low molecular weight drugs with the polymers.

- The shielding effect of the polymer that conveys a relevant protection from chemical or enzymatic degradation to the conjugated drug.
- The reduction in renal excretion, due to the large volume of the macromolecular conjugate.

- In some cases, polymer coupling was demonstrated to promote a targeted delivery of drugs to body sites characterized by an increased capillary permeability as, for example, inflamed tissues. This phenomenon is also at the basis of the so-called ‘enhanced permeability and retention’ (EPR) effect. EPR allows the specific localization of a drug at the level of cancer tissue due to the higher permeability of blood capillaries in that area, accompanied by a reduced lymphatic drainage. Both these phenomena permit the accumulation of the drug–polymer at the level of the tumor tissue through an ultrafiltration process.
- Besides the modification of the pharmacokinetic profiles, the macromolecular characteristic of the bioconjugates is responsible for the exploitation of a totally new pathway for the drug’s entrance into the cell that can only be based on adsorption- or receptor- mediated endocytosis.

The bioconjugation also referred to as ‘polymer therapeutics’ describes several distinct classes of agents, including polymeric drugs, polymer–drug conjugates, polymer–protein conjugates and polymeric micelles to which drug is covalently bound. They are also considered as new chemical entities by Regulatory authorities. Over the last decade, more than 10 water-soluble polymer-drugconjugates also visualised as macromolecular prodrugs have entered phase I/II clinical trials as i.v. administered agents. These include six conjugates based on N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers and, more recently, aseries of PEG and polyglutamic acid conjugates (**Table 2.1**) (21).

2.7. POLY (ETHYLENE GLYCOL) CONJUGATES.

Conjugation of biomolecules with polyethylene glycol (PEG), a process known as pegylation, is now an established method for increasing the circulating half-life of protein and liposomal pharmaceuticals. Polyethylene glycols are nontoxic water-soluble polymers that, owing to their large hydrodynamic volume, create a shield around the pegylated drug, thus protecting it from renal clearance, enzymatic degradation, and recognition by cells of the immune system (22, 23).

Poly(ethylene glycol) (PEG), also known as **poly(ethylene oxide)** (PEO) or polyoxyethylene (POE), are the most commercially important polyethers. PEG, PEO or POE refers to an oligomer or polymer of ethylene oxide. It is a linear or branched polyether with the chemical formula, $HO\text{-}(CH_2\text{-}CH_2\text{-}O\text{-})_n\text{-}H$. PEG is commonly manufactured by the aqueous ionic polymerization of ethylene oxide, where a range of polymers with different molecular weights can be generated. Low molecular weight (<1000Da) PEGs are viscous and colorless liquids, whereas higher-molecular weight PEGs are waxy in nature. PEG polymers are non-toxic, dissolve in water as well as organic solvents and are approved by FDA for human intravenous, oral and dermal applications. The polymers are also safely excreted from the body by renal or hepatic pathways (23).

PEGylation–PEG prodrugs

Prodrug design comprises an area of drug research that is concerned with the optimization of drug delivery. A prodrug is a biologically inactive derivative of a parent drug molecule that usually requires an enzymatic transformation within the body in order to release the active drug, and has improved delivery properties over the parent molecule (22, 24). Too rapid breakdown of the prodrug can lead to spiking of the parent drug and

possible toxicity, while too slow a release rate will compromise the drug's the efficacy. In the case of PEG conjugates it is clear that the solubility of the prodrug will almost always exceed that of the original drug, usually overcoming any existing aqueous insolubility and thus increasing the possibility of more effective drug delivery.

Recently, there have been many studies carried out on therapeutic effects of PEG-CPT copolymer. CPT has the unique structural elements of a lactone ring and a 38 alcohol (20-OH), both of which are requirements for activity (topoisomerase I inhibition).

The biggest drawback to the use of this potent drug is that camptothecin is virtually insoluble in water.

One such study carried out by Greenwald et al. validated the effects of ester prodrugs of CPT. Using a PEG prodrug delivery strategy, they reported that CPT can be solubilized as a non-ionic α-alkoxyester conjugated to PEG carboxylic acid with a molecular weight of 40 000. CPT's solubility as the 20-camptothecin PEG 40 000ester prodrug form was approximately 2 mg/ml in water, and is dramatically greater than that of CPT (0.0025 mg/ ml, water). PEG–CPT has been shown to hydrolyze in vivo and gradually release native CPT (24).

2.8. ANTICANCER DRUG-CAMPTOTHECIN.

Camptothecin is a plant alkaloid present in wood, bark, and fruit of the Asian tree Camptotheca acuminata. The US National Cancer Institute screening programme identified camptothecin as a drug with potential antitumor activity in 1966.

Camptothecin has a five-ring structure and 20S chiral carbon is required for its activity, and also noted a dynamic equilibrium between the closed ring lactone and open-ring carboxylic acid forms (**Fig 2.4**). The closed ring lactone has most cytotoxic activity. At

neutral and alkaline pH, equilibrium favors the essentially inactive carboxy-acid form. In 1985, the mechanism of action of Camptothecin was found out to be the suppression of Topoisomerase I. The drug reversibly induces single-strand breaks, thereby affecting the cell's capacity to replicate. Camptothecin stabilizes the so-called cleavable complex between topoisomerase 1 and DNA. These stabilized breaks are fully reversible and non-lethal. However, when a DNA replication fork collides with the cleavable complex, single-strand breaks are converted to irreversible double-strand breaks. Apoptotic cell death is then mediated by caspase activation. Inhibition of caspase activation shifts the cells from apoptosis to transient G1 arrest followed by cell necrosis.

Identification of topoisomerase 1 as a viable target for antineoplastic treatment, and elucidation of its inhibition as the mechanism of action of camptothecin, led to renewed efforts to develop soluble analogues of camptothecin. There are two analogues being used for the treatment of variety of tumors including cervix and colorectal cancer (25, 26, 27).

Topotecan

Topotecan (9-[(dimethylamino) methyl]-10-hydroxycamptothecin) was the first camptothecin analogue to be approved for clinical use by the US Food and Drug Administration (FDA). It is water-soluble because of its side-chain at carbon 9 of the A ring (figure). Topotecan is proven to be effective in many tumor types, including adenocarcinomas of the ovary and colon, tumors of the central nervous system, and sarcomas.

The pivotal study in the 1996 FDA approval of topotecan for relapsed ovarian cancer was a randomized phase 3 trial by ten Bokkel Huinink and colleagues. They assessed

topotecan versus paclitaxel in patients with recurrent ovarian cancer after a platinum-containing regimen or who had not responded to a regimen containing platinum. Topotecan was administered at 1·5 mg/m2 daily for 5 days, repeated every 21 days. Hycamtin (Topotecan.HCl) is a chemotherapeutic agent approved by the US Food and Drug Administration (FDA) for the treatment of metastatic carcinoma of the ovary after failure of initial or subsequent chemotherapy.

Irinotecan

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin) was the first water-soluble semi-synthetic derivative of camptothecin to enter clinical trials. After initial development in Japan, the drug has been licensed to several different pharmaceutical firms and has undergone a truly global development plan. Irinotecan became commercially available in Japan in 1994, where its approved indications were cancers of the lung (small-cell and non-small-cell), cervix, and ovaries. Irinotecan was approved in Europe in 1995 as a second-line agent for colon cancer. Irinotecan was approved in the USA in 1996 for treatment of advanced colorectal cancer refractory to fluorouracil.

A unique characteristic of irinotecan is its bulky dipiperidino side-chain linked to the camptothecin molecule via a carboxyl-ester bond (figure). This side chain, although providing necessary solubility, leads to a substantial reduction in anticancer activity. Cleavage of the side-chain by carboxylesterases—found mainly in the liver and gastrointestinal tract—forms the metabolite SN-38 (7-ethyl-10-hydroxycamptothecin). SN-38 is as much as 1000-fold more potent in inhibition of topoisomerase 1 than irinotecan and is thus the predominant active form of the drug. One major drawback

associated with Irinotecan is the side-effect, diarrhea but the dose-limiting toxic effect of irinotecan found out to be was myelosuppression (25).

Other analogues have also been developed such as 9-aminocamptothecin and glycoconjugates of Camptothecin involving the attachment of Polyethylene glycol to enhance the stability of lactone-ring stability and improve the solubility of Camptothecins.

2.9. TARGETING STRATEGIES.

Targeting of anticancer drugs to tumor provides several advantages over non-targeted ones. The main advantages are the prevention of side-effects on healthy tissues and enhancement of drug uptake by the targeted cells (28).

Targeting a drug specifically to cancer cells inside the cells permits the internalization of drug substances by endocytosis and rug release in targeted organelles, for example, lysosomes, mitochondria and nucleus (28, 29). Then the targeted drug would act like a ‘magic bullet’ selectively killing the villain (tumor cells) and sparing the innocent (healthy cells).

There are two general approaches generally used for delivery of drug and they are, Passive and Active targeting.

2.9.1. PASSIVE TUMOR TARGETING.

Passive targeting includes three different approaches, 1) the enhance permeability and retention effect, 2) using special conditions in the tumor, 3) topical delivery directly to the tumor.

Enhanced permeability and retention (EPR) effect: The capillaries of the tumor vascular system has increased permeability for circulating macromolecules. This particular property of the solid tumors together with the limited lymphatic drainage from the tumor interstitium makes it possible for accumulation of the drug molecules. This phenomenon is known as Enhanced permeability and retention (EPR) effect. EPR effect can be observed with the macromolecules of molecular size greater than 50kDa. The low molecular weight drugs are usually coupled with high molecular weight carriers.

The main advantage of the EPR effect is that it provides accumulation of the drug inside the tumor and prevents the healthy tissues from the toxic effects of the drugs.

The major disadvantage associated with the passive targeting by EPR effect is that it can work only with solid tumors and not on spreading tumors or metastatic tumors.

The second passive approach is to utilizing the specific conditions in an organ bearing the tumor. These conditions include particular pH, certain enzymes in the tumor or the organ. The limitation to this approach is the targeting of the whole organ, not the tumor itself, posing the possibility of severe organ cytotoxicity (29, 30).

The third passive approach is local delivery of anticancer agents directly into the tumor. This technique can limit the side –effects on healthy tissues but the topical delivery for some tumors is not possible as they are difficult to access for local drug delivery.

2.9.2. ACTIVE TUMOR TARGETING.

Active targeting is usually achieved by adding to drug delivery system a targeting component that can provide preferential accumulation of the whole system or drug in the tumor bearing organ, in the tumor itself, intracellular organelles or intracellular

organelles. This approach takes advantage of the certain molecules that are over expressed on the plasma membranes of the targeted cells. This approach is based on specific interactions such as ligand-receptor, avidin-biotin, antibody-antigen, and lectin-carbohydrate.

Targeting specific organs.

Lectin-carbohydrate is one of the classical examples of such a kind of pair. Several Drug delivery systems utilizing lectins or carbohydrates have been developed to target organs. The approach has been most often used for targeted delivery to normal and malignant colon cells. The main drawback of targeting to entire organ is adverse side effects on intact cells.

Targeting cancer cells.

The most obvious way to target Drug delivery system DDS to cancer cells is to target antigens present on the plasma membrane of cancer cells. The interaction of a ligand with plasma membrane receptor enhances its intracellular uptake. The polymer-based DDS that target cancer cells using antibody are examples of such systems.

Targeting cellular organelles.

Mitochondria and nuclei are the two intracellular organelles that are considered to be the most important targets for anticancer drugs since they are critical role in apoptosis. Mostly, gene delivery systems are targeted to the nuclei components such as DNA, the enzymes involved in DNA replication, biosynthesis. In addition to direct targeting to the nuclei, anticancer DDS can also be designed to provide drug release in lysosomes after internalization of DDS by endocytosis providing indirect nuclear targeting.

Targeting intracellular molecules.

In addition to targeting sites and binding ligands, anticancer DDS can also be targeted to intracellular molecules. Such a type of targeting could also provide a synergic effect, increasing the efficacy of the main anticancer agent. This second target might include proteins responsible for drug efflux, for example P-gp or cellular detoxification enzymes or anti-apoptotic mechanisms.

2.10. RECEPTOR-MEDIATED ENDOCYTOSIS.

Endocytosis is a process where small invaginations in the plasma membrane form a new intracellular vesicle about 0.5 to 0.1μm in diameter (32).

Types of endocytosis.

The absorption of material from the outside environment of the cell is commonly divided into two processes: phagocytosis and pinocytosis.

- **Phagocytosis** (literally, cell-eating) is the process by which cells ingest large objects, such as cells which have undergone apoptosis, bacteria, or viruses. The membrane folds around the object, and the object is sealed off into a large vacuole known as a phagosome.

- **Pinocytosis** (literally, cell-drinking). This process is concerned with the uptake of solutes and single molecules such as proteins.

- **Receptor-mediated** endocytosis is a more specific active event where the cytoplasm membrane folds inward to form coated pits. These inward budding vesicles bud to form cytoplasmic vesicles.

Receptor-mediated endocytosis (RME) is a process by which cells internalize molecules (endocytosis) into a cell by the inward budding of plasma membrane vesicles containing proteins with receptor sites specific to the molecules being internalized.

In receptor-mediated endocytosis, an extracellular molecule or ligand is recognized by the specific cell receptor expressed on the plasma membrane. Interaction of ligand with the receptor leads to the engulfing of the plasma membrane inside the cells and formation of coated pit. The pit then pinches off from the plasma membrane and forms an endosome, a membrane limited transport vesicle with a Drug delivery system inside. When in the cells, the endosomes fuse with lysosomes. If the bond between targeting moiety and spacer is designed in such a way that lysosomal enzymes are capable to cleave it, the drug is released from the delivery complex and might exit the lysosome by diffusion (32.33).

Additionally, the rate at which receptor-mediated endocytosis takes place or ligand is internalized depends on the amount of corresponding receptor on the cell surface.

Receptor-mediated endocytosis generally occurs via clathrin-coated pits that constitute about two percent of surface of cells such as fibroblasts and hepatocytes. This region is considered to function as a signal for the localization of receptors.

The most common examples of such receptor-mediated endocytosis are the internalization of cholesterol by the low density lipoprotein (LDL) receptor and that of iron by transferrin receptor (33).

Cholesterol is a hydrophobic molecule and quite insoluble in water. Thus it cannot pass from the liver and/or the intestine to the cells simply dissolved in blood and ECF. Instead

it is carried in tiny droplets of lipoprotein. The most abundant cholesterol carriers in humans are the low-density lipoproteins or LDLs.

LDL particles are spheres covered with a single layer of phospholipid molecules with their hydrophilic heads exposed to the watery fluid (e.g., blood) and their hydrophobic tails directed into the interior. Over a thousand molecules of cholesterol are bound to the hydrophobic interior of LDL particles. One molecule of a protein, called apolipoprotein B-100 (Apo B-100) is exposed at the surface of each LDL particle (33).

The first step in acquiring LDL particles is for them to bind to LDL receptors exposed at the cell surface. These transmembrane proteins have a site that recognizes and binds to the apolipoprotein B-100 on the surface of the LDL. The portion of the plasma membrane with bound LDL is internalized by endocytosis. A drop in the pH (from ~7 to ~5) causes the LDL to separate from its receptor. The vesicle then pinches apart into two smaller vesicles: one containing free LDLs; the other containing now-empty receptors. The vesicle with the LDLs fuses with a lysosome to form a **secondary lysosome**. The enzymes of the lysosome then release free cholesterol into the cytosol. The vesicle with unoccupied receptors returns and fuses with the plasma membrane, turning inside out as it does so (exocytosis) (**Fig. 2.5**). In this way the LDL receptors are returned to the cell surface for reuse (33).

2.11. LUTEINIZING HORMONE RELEASING HORMONE AND RECEPTORS

The hypothalamic decapeptide gonadotropin releasing hormone (GnRH, GnRH-I), also called luteinizing hormone releasing hormone (LHRH), plays a key role in the regulation

of mammalian reproduction. It is released from the hypothalamus in a pulsatile manner and stimulates the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (36).

The receptor for GnRH (GnRH-R) is a member of the G protein-coupled receptor (GPCR) family and the binding of GnRH activates phospholipase C (PLC), resulting in the hydrolysis of phosphatidylinositol 4, 5-bisphosphate. The resulting generation of both inositol 1, 4,5-trisphosphate and diacylglycerol is able to mobilize Ca^{2+} from intracellular stores and activate protein kinase C, respectively , thereby mediating many of the biological effects of GnRH such as synthesis and release of FSH and LH from the pituitary gonadotrophs (35).

GPCRs are characterized structurally by an extracellular amino terminus and an intracellular carboxyl terminus linked by seven transmembrane-spanning helices, which themselves is joined by three extracellular loops and three intracellular loops. In general, the extracellular domains and/or transmembrane regions are involved in ligand-recognition, whereas cytoplasmic regions present sites for interactions with not only G proteins but also other proteins. GnRH-Rs are unique among the GPCR family, because they completely lack an intracellular carboxyl-terminal tail (35).

Recent studies have shown that 80% of ovarian cancer and 50% of breast cancer express the receptor to bind LHRH. There are two types of receptors which are expressed. One is high capacity /low affinity whereas the other one is low capacity/ high affinity. The LHRH receptor generally present on pituitary and Ovarian, breast cancer are high affinity/low capacity.

Since, the LHRH receptor is not expressed abundantly on ovarian tissue, but is present in cancer phenotype; the LHRH receptor is a strong candidate for targeted chemotherapy. Anticancer drugs, delivered to cells, linked to LHRH will enhance the delivery of drug by targeted delivery as well as faster uptake due to receptor-mediated endocytosis (35).

Many LHRH analogs have been synthesized with cytotoxic compounds, such as methotrexate, cisplatin etc.

Currently many LHRH agonists and antagonists are used for the treatment of various types of cancer. The examples of LHRH agonists are Leuprolide, Buserelin and Nafaralin, whereas that of LHRH antagonists is Abarelix and Cetorelix.

2.12. LHRH AND LHRH RECEPTOR STRUCTURE AND FUNCTION.

2.12.1. LHRH STRUCTURE AND FUNCTION.

To date, there are 25 forms of luteinizing hormone - releasing hormone (LHRH) found in a wide range of vertebrates, carrying out multiple functions. The most ubiquitous one is LHRH II, isolated from chicken brain, whereas hypothalamic one is LHRH I. LHRH I is the hypothalamic decapeptide that acts on cells of human anterior pituitary to stimulate the release of FSH and LH (36).

The NH_2 and COOH terminals of the LHRH decapeptide (pGlu-His-Trp-Ser-Tyr- Gly-Leu-Arg-Pro-Gly.NH_2) have been shown to be highly conserved and critically important for the binding and activation for the LHRH receptor (**Fig. 2.6**). The incorporation of an arginine in position 8 is required for high-affinity binding to the LHRH receptor (36).

The biologically active conformation of GnRH is believed to contain a bend in the middle portion of the molecule and is bent about the flexible glycine position 6. The amino acids

Trp, His and pGlu (COOH terminus) are required for receptor binding and activation whereas the amino acids at the NH2 terminus, Arg, Pro and Gly are required for receptor binding (**Fig. 2.7**). Additionally, there is a prominent β-turn about Gly at position 6 and the NH2 and COOH termini in close proximity (36). Modifications in these residues of the LHRH peptide especially that of the amino terminus, result in LHRH analogs with agonistic and antagonistic properties.

2.12.2. LHRHR STRUCTURE AND FUNCTION.

The LHRHR is a member of a large family of G-protein coupled receptors (GPCR), comprising of seven transmembrane spanning domains. The typical characteristic of these types of receptors is their tendency to work via second messenger signaling pathways, including molecules such as diacylglycerol and calcium to exert activation or deactivation of proteins downstream (35,36).

The seven α helical membrane spanning domains have three extracellular regions and three intracellular loops. The general mechanism for these receptors involves the binding of the ligand to the extracellular domains, followed by a conformational change in the intracellular portions of the helices. This receptor activation then causes a propagation of the signal.

There are six main amino acids on the LHRHR which are thought to be critical in ligand-receptor binding. These amino acids, Asp (at position 98), Asn (at position 92), Lys (at position 121), Asp (at position 302) and possibly Trp (at position 279) and Trp (at position 101) are primarily close to the extracellular boundaries of the helices. These amino acids are conserved in all LHRH receptors for vertebrates. The exact mechanism

by which the binding of LHRH to its receptor is translated into receptor activation is still unknown (36).

2.13. THE BCL-2 PROTEIN FAMILY AND BH3 PEPTIDE.

The BCL 2 family of proteins plays an important role in moderating the cellular program of apoptosis and hence exhibit importance in the process of cell proliferation and cancer.

It consists of three distinct families: antiapoptotic members, such as BCL2 and BCLXL with BH1, BH2, BH3 and BH4 domains, proapoptotic members, such as BAK and BAX with sequence homology only at BH1, BH2 and BH3 domain. These BCL2 (BH) domains are critical for various aspects of their activities such as the induction or suppression of cell death and the ability to heterodimerize with other family members.

The ratio between anti-apoptotic and multidomain pro-apoptotic Bcl-2 family members determine the cellular susceptibility to death stimulation (38, 40).

The BH3 domain is critical for heterodimerization with other BCL2 family proteins and is essential for promoting cell death activity. A conformation change that exposes the BH3 domain in BAX has been shown during the induction of apoptosis (**Fig. 2.8**).

The pro-apoptotic “BH3 domain-only” proteins of the Bcl-2 family (e.g. Bid and Bad) transduce multiple death signals to the mitochondrion. They interact with the anti-apoptotic Bcl-2 family members and induce apoptosis by a mechanism that requires the presence of at least one of the multidomain pro-apoptotic proteins Bax or Bak.

The BH3 domain serves as the ligand for binding to the receptor domain in antiapoptotic members. NMR and X-ray crystallography of the BCL-XL has indicated that BH1-4 domains correspond to α helices 1-7. The helices of BH1-3 are closely juxtaposed to form a hydrophobic pocket (38, 39, 40).

The BH3 domain serves as the minimal ‘death domain’ in the proapoptotic members of the BCL2 family and is therefore a novel pharmacological approach for manipulation of cell’s susceptibility to death and tumorigenesis (40).

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Product	Status	Payload	Indication
Therapeutic antibodies			
Rituxan®	Market	–	Non-Hodgkin's Lymphoma CD20 +ve
Herceptin®	Market	–	HER2 + ve breast cancer
Campath	Market	–	B-cell Chronic Lymphocytic
Avastin	Market	–	Leukaemia
Antibody-drug conjugates			
Mylotarg®	Market	calicheamicin	Acute Myeloid Leukaemia CD33 +ve
Radioimmunotherapeutics			
Tositumomab® CD20	Market	[^{131}I]iodide	Non-Hodgkin's Lymphoma
Zevalin® CD20	Market	[^{90}Y]ttrium	Non-Hodgkin's Lymphoma
Liposomes			
Daunoxome®	Market	daunorubicin	Kaposi's Sarcoma
Doxil®/Caelyx®	Market	doxorubicin	Kaposi's Sarcoma, Ovarian cancer
Depocyt-lipidic formulation	Market	cytarabine	Intrathecal therapy of lymphomatous meningitis
Polymer-protein conjugates			
Zinostatin Stimalmer® (SMANCS)	Market	neocarzinostatin	Local administration – hepatocellular carcinoma
Oncaspar® PEG-L-asparaginase	Market	asparaginase	Acute Lymphoblastic Leukaemia
PEG-intron™ PEG-α-interferon 2b	Market	α-interferon 2b	Hepatitis C, also in clinical development in cancer, multiple sclerosis, HIV/AIDS
Neulasta™ PEG-GCSF	Market	GCSF	Neutropenia associated with cancer chemotherapy
Polymer-drug conjugates			
CT-2103, XYOTAX™ Polyglutamate-paclitaxel	Phase II/III	paclitaxel	Particularly lung and ovarian cancer
PK1; FCE28068 HPMA copolymer-doxorubicin	Phase II	doxorubicin	Particularly lung and breast cancer
PK2; FCE28069 HPMA copolymer-doxorubicin-galactosamine	Phase I/II	doxorubicin	Hepatocellular carcinoma
AP5280 HPMA copolymer platinate	Phase II	platinate	Cancer
AP5346 HPMA copolymer platinate	Phase I	platinate	Cancer
CT-2106 Polyglutamate-camptothecin	Phase I	camptothecin	Cancer
PROTHECAN™ PEG-camptothecin	Phase II	camptothecin	Cancer
Polymeric micelles			
NK911 PEG-aspartic acid-doxorubicin micelle	Phase I	doxorubicin	Cancer

Table 2.1 Examples of antibodies and drug delivery systems used in cancer therapy (Reproduced from Ref. [21])

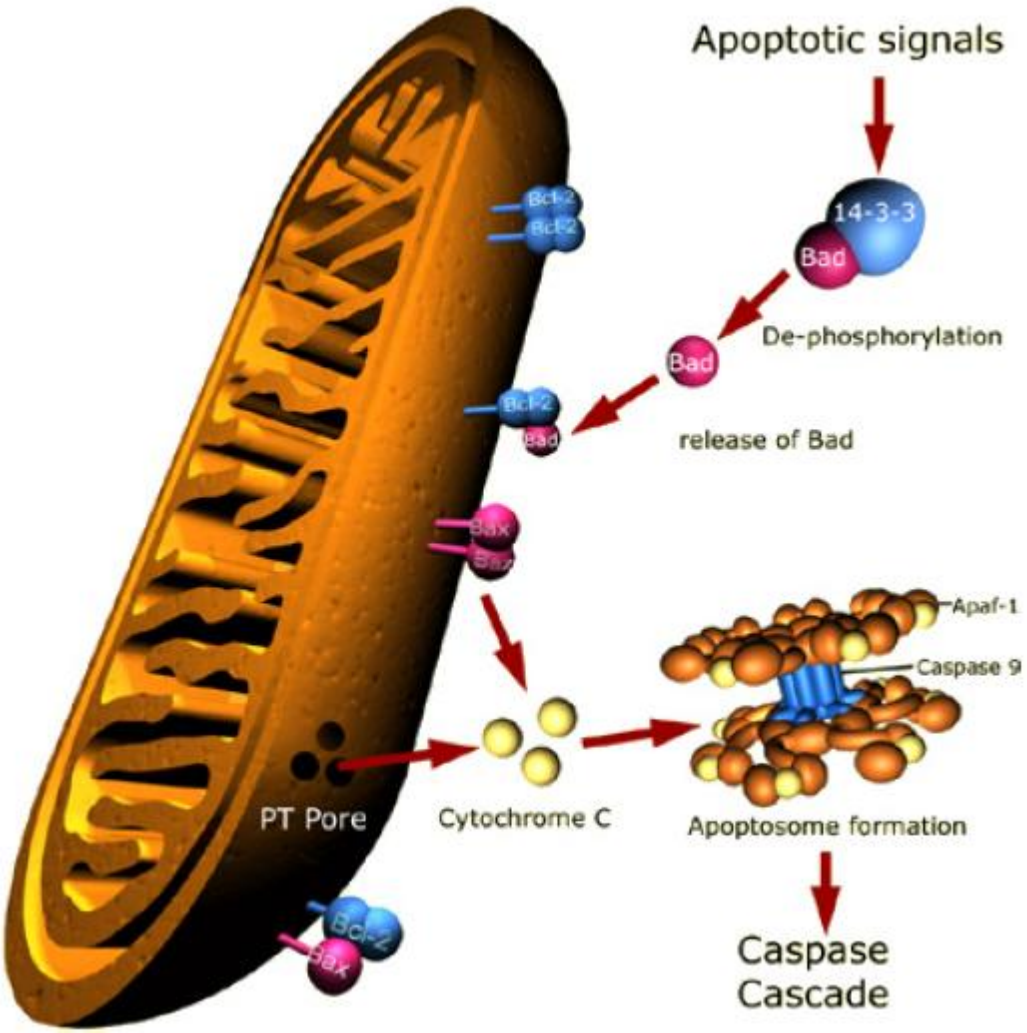

Fig. 2.1. Illustration of main apoptotic signaling pathways involving mitochondria (Reproduced from Ref. [14]).

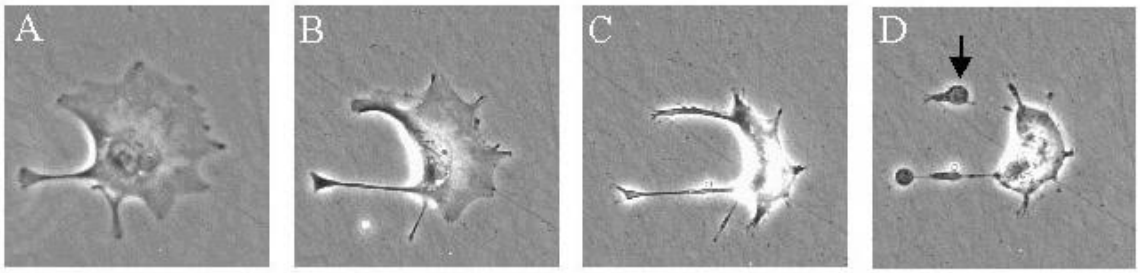

Fig.2.2. Typically, the cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton (A). The breakdown of chromatin in the nucleus often leads to nuclear condensation and in many cases the nuclei of apoptotic cells take on a "horse-shoe" like appearance (B). Cells continue to shrink (C), packaging themselves into a form that allows for their removal by macrophages. These phagocytic cells are responsible for clearing the apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death. In order to promote their phagocytosis by macrophages, apoptotic cells often undergo plasma membrane changes that trigger the macrophage response. One such change is the translocation of phosphatidylserine from the inside of the cell to the outer surface. The end stages of apoptosis are often characterized by the appearance of membrane blebs (D) or blisters process. Small vesicles called apoptotic bodies are also sometimes observed (D, arrow) (Reproduced from Ref. [14]).

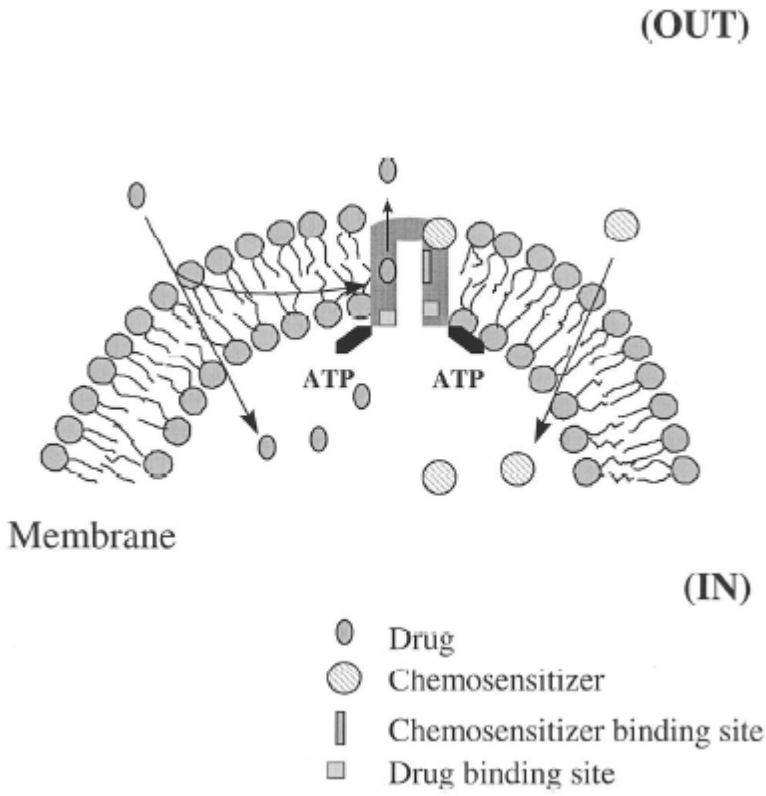

Fig.2.3. Function of the P-GP pump. The model illustrates a protein which uses ATP energy to actively efflux drug substrate across the plasma membrane (Reproduced from Ref. [43]).

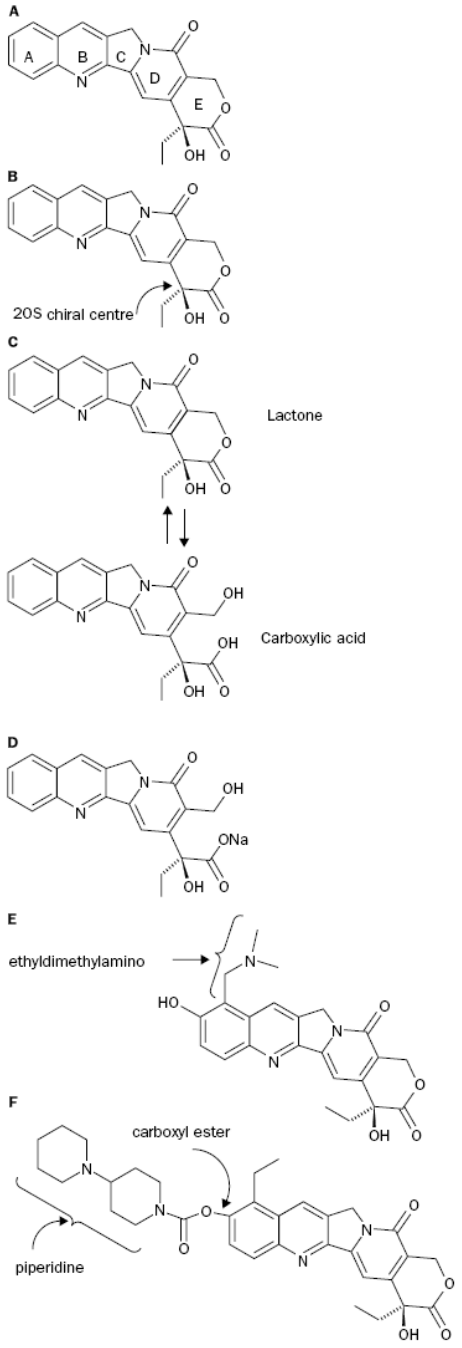

Fig. 2.4. Structures of camptothecin class of drugs (A) Camptothecin. (B) 20S chiral centre. (C) Dynamic equilibrium of camptothecin. (D) Camptothecin sodium salt. (E) Topotecan. (F) Irinotecan (Reproduced from Ref. [25]).

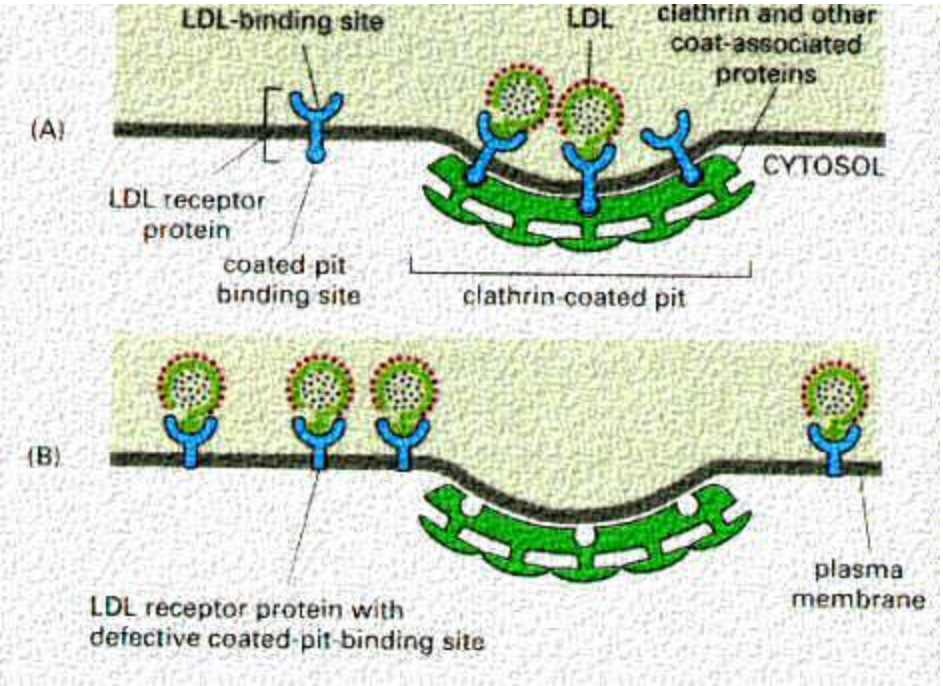

Fig.2.5. Receptor-mediated endocytosis of LDL (low-density lipoproteins) (Reproduced from Ref. [33]).

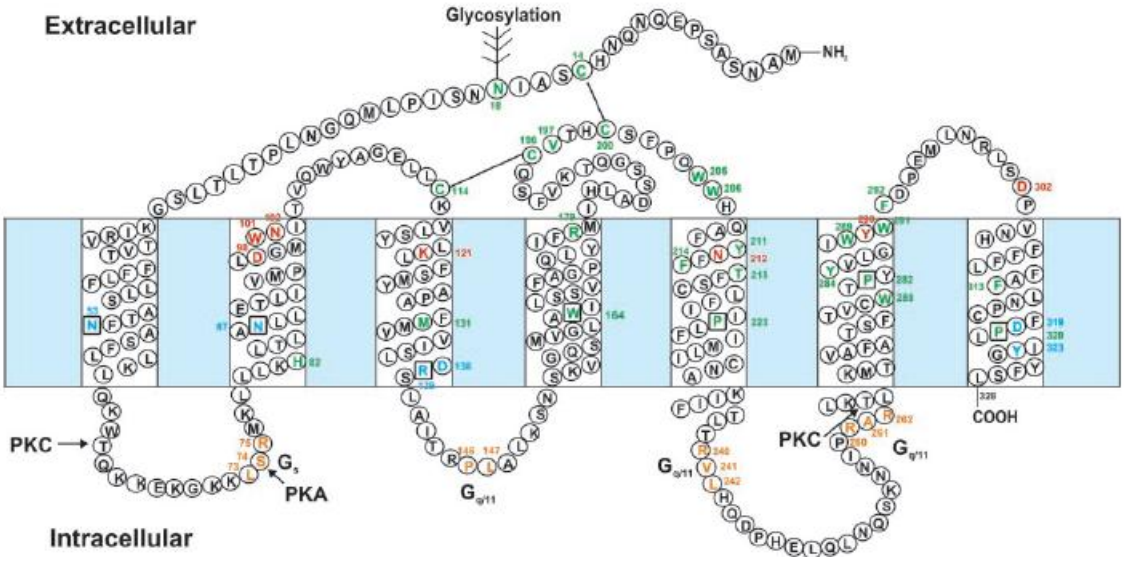

Fig. 2.6. The seven transmembrane spanning domains of the LHRHR (Reproduced from Ref. [36]).

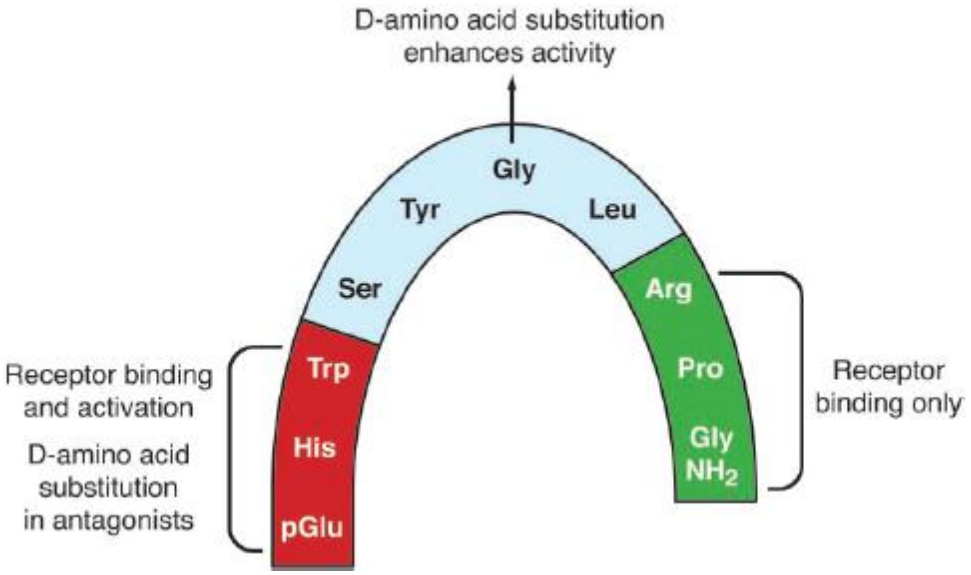

Fig.2.7. The structure-activity relationship of LHRH (Reproduced from Ref. [36]).

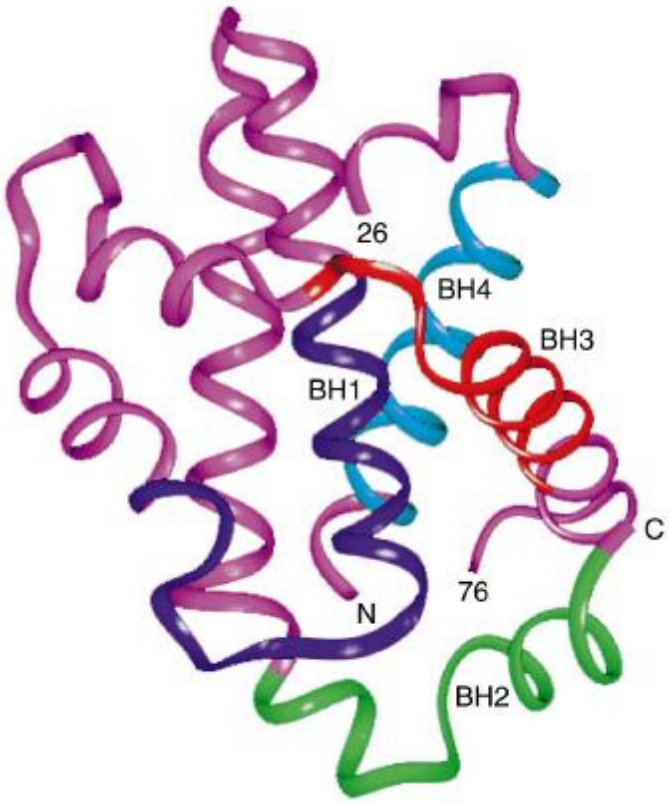

Fig.2.8. Ribbon representation of the averaged, minimized NMR structure of Bcl-xL 25 without the C-terminal transmembrane domain. Bcl-homology (BH) domains BH1 (purple), BH2 (green), BH3 (red) and BH4 (cyan) are illustrated. Not shown in the figure is a large, unstructured loop between residues 26–76 in the protein (Reproduced from Ref. [38]).

3. SPECIFIC AIMS.

Specific aim 1. To design, synthesize, and evaluate in vitro and in vivo a novel targeted anticancer polymeric prodrug containing multiple copies of tumor targeting moiety (synthetic Luteinizing hormone-releasing hormone (LHRH) peptide, analog of LHRH) and anticancer drug (Camptothecin).

Cancer is one of the leading causes of death in the United States. Although localized primary solid tumors can be successfully removed surgically, the treatment of spreading tumors and tumor metastases requires extensive chemotherapy. However, two main obstacles limit the success of chemotherapy: (1) severe adverse side effects on healthy organs and (2) development of drug resistance. Many anticancer drugs used in chemotherapy require modifications to increase solubility, decrease adverse side effects, limit nonspecific activity, increase circulation time, modify biodistribution, and so on. A prodrug is an inactive precursor of a drug which is converted into the active drug in the targeted organ, tissue, or cell. Advantages of the prodrug approach include, but are not limited to, the prevention of systemic side effects, the increase in the bioavailability of the modified drug, and the possibility of using specific organ, tissue, or cellular conditions to convert the inactive prodrug into its active form. In most cases, a prodrug includes a drug bound to (or encapsulated in) a water-soluble carrier. The more advanced type of prodrug or drug delivery system (DDS) might include a targeting moiety and supplementary active ingredients in addition to the carrier and drug. The carrier combines all components of the DDS together and provides the required characteristics of the whole DDS, i.e., solubility, molecular mass, etc. A targeting moiety enforces the specific

delivery of a drug to the targeted organ, tissue, or cell. Acting as a penetration enhancer, the targeting moiety also improves the cellular uptake of the entire DDS.

Since polyethylene glycol (PEG) polymer is widely used as a carrier for DDS, it is used in many biochemical, cosmetic, pharmaceutical, and industrial applications. It is also important that PEG polymers show low-antigen activity and in most cases decrease the antigenicity of active ingredients conjugated to them. Successful bioconjugation depends on the chemical nature, structure, molecular weight, steric hindrance, and reactivity of the biomolecule, as well as that of the polymer. In most prodrug conjugations, a variety of spacers can be incorporated between polymer and biomolecules, because they can offer a chemical flexibility and can be hydrolyzed to release the bioactive component. The suitability of a spacer molecule depends on its linearity, molecular weight, end functionality, charge, and reactivity. Current bioconjugation methodologies are limited to only one drug molecule per polymeric chain. On the other hand, polymers can deliver multiple drug molecules simultaneously to the cancer cells, thereby accumulating higher concentration of a drug and consequently increasing the efficacy of cancer treatment.

The first goal of this dissertation is to develop a polymeric prodrug consisting of multiple copies of an anticancer drug, Camptothecin and targeting agent, Luteinizing hormone-releasing hormone (LHRH) peptide which is able to simultaneously deliver several copies of anticancer drugs specifically to tumor.

Specific aim 2. To increase the efficacy of the chemotherapy by co-delivery of anticancer drug, targeting agent and suppressor of antiapoptotic cellular defense.

Most of the anticancer drugs exert a dual effect on cancer cells. Apart from provoking cell death mainly by activation of the central apoptotic signal, they also trigger antiapoptotic cellular defense primarily by inducing the overexpression of antiapoptotic members of the BCL2 protein family. In order to prevent the activation of cellular defense and enhance the apoptosis induction, we proposed to include in the same DDS an anticancer drug, Camptothecin with a synthetic analogue of BCL2 homology 3 (BH3) peptide or antisense oligonucleotides targeted to BCL2 mRNA. Our previous experimental data (5, 6, 7) showed the effectiveness of LHRH peptide as an effective tumor targeting moiety and BH3 peptide as a suppressor of antiapoptotic cellular defense. Nevertheless, the separate use of only one peptide in the DDS was not able to eliminate both of the main obstacles of chemotherapy: adverse side effects and activation of cellular resistance. LHRH peptide enhanced the uptake of DDS by cancer cells and limited the accumulation of an anticancer drug in healthy tissues but was not able to prevent the overexpression of antiapoptotic members of the BCL2 protein family. BH3 peptide substantially enhanced the cytotoxicity of traditional chemotherapeutic drugs but was unable to prevent their accumulation in healthy organs, thus increasing the risk of adverse side effects.

Hence, the next aim of this dissertation is to design and evaluate in vitro and in vivo a novel multifunctional targeted proapoptotic drug delivery system which provides for the simultaneous tumor targeting by LHRH peptide, cell death induction by an anticancer drug camptothecin (CPT), and suppression of cellular antiapoptotic defense by BH3 peptide.

Specific aim 3. **To evaluate pharmacokinetics and the efficiency of proposed targeted proapoptotic delivery system on the treatment of mice bearing human malignant ascites.**

When cancer spreads to other organs or areas of the body, it is called metastasis. In ovarian cancer, metastasis can occur in four ways.

- By direct contact or extension, it can invade nearby tissue or organs located near or around the ovary, such as the fallopian tubes, uterus, bladder, rectum, etc.
- By seeding or shedding into the abdominal cavity, which is the most common way ovarian cancer spreads. Cancer cells break off the surface of the ovarian mass and drop to other structures in the abdomen such as the liver, stomach, colon or diaphragm.
- By breaking loose from the ovarian mass, invading the lymphatic vessels and then traveling to other areas of the body or distant organs such as the lung or liver.
- By breaking loose from the ovarian mass, invading the blood system and traveling to other areas of the body or distant organs. This type of metastasis is rare in ovarian cancer.

Ovarian cancer may spread to the lining of the abdominal cavity and lead to the buildup of fluid inside the abdomen, called Ascites. Ovarian cancer may cause symptoms such as swelling of the abdomen, pain, irregular bowel movements or difficulty breathing when fluid places pressure on the lungs.

To confirm the feasibility of use of the targeted proapoptotic anticancer drug delivery system in the treatment of metastatic ovarian cancer, biodistribution of the DDS will be

studied in primary cell-culture using malignant ascites and in nude mice bearing xenografts of both human ovarian primary tumors and malignant ascites.

4. To design, synthesize, and evaluate in vitro and in vivo a novel targeted anticancer polymeric prodrug containing multiple copies of tumor targeting moiety (synthetic Luteinizing hormone-releasing hormone (LHRH) peptide, analog of LHRH) and anticancer drug (Camptothecin).

4.1. Introduction

Current cancer therapy usually involves intrusive processes including application of catheters to allow chemotherapy, initial chemotherapy to shrink any cancer present, surgery to then remove the tumor(s) if possible, followed by more chemotherapy and radiation. The purpose of the chemotherapy and radiation is to kill the tumor cells as these cells are more susceptible to the actions of these drugs and methods because of their growth at a much faster rate than healthy cells, at least in adults. Research efforts to improve chemotherapy over the past 25 years have led to an improvement in patient survival but there is still a need for improvement. Many anticancer drugs used in chemotherapy require modifications to increase solubility, decrease adverse side effects, limit nonspecific activity, increase circulation time, and modify biodistribution. Current research areas include development of carriers to allow alternative dosing routes, new therapeutic targets such as blood vessels fueling tumor growth and targeted therapeutics that are more specific in their activity (1, 2, 3, 4). In all cases, the effectiveness of the treatment is directly related to the treatment's ability to target and to kill the cancer cells while affecting as few healthy cells as possible. The degree of change in the patient's quality of life and eventual life expectancy is directly related to this targeting ability of the treatment. Various types of drug delivery systems have been developed to modify the

existing chemotherapeutic agents in order to enhance their efficacy in the treatment of different kinds of cancer.

The development of targeted anticancer drug delivery systems have further led to advanced drug modifications and provide for the so-called “advanced targeted prodrug approaches. Targeting is especially important in circumstances where a localized tumor is removed surgically, and chemotherapy is prescribed as a follow-up preventive against potential metastases. Cancer targeting is usually achieved by adding to the DDS a ligand moiety specifically directed to certain types of binding sites on cancer cells. Several different targeting moieties were examined, including sugars, lectins, receptor ligands, and antibodies and their fragments. Mostly, such systems contain a nanocarrier or a water-soluble carrier conjugated with a targeting moiety and an anticancer drug. Because of such conjugation, or encapsulation into a carrier, therapeutic compounds are said to form a “prodrug,” which is inactive during the delivery to the site of action and is converted into an active drug at the targeted organ, tissues, or cells. Recently, we found that the receptors for luteinizing hormone-releasing hormone (LHRH) are overexpressed in breast, ovarian and prostate cancer cells (5, 6, 7, 8). Since, LHRH receptors (LHRHRs) are not expressed detectably in most visceral organs in normal physiological conditions, we have taken advantage of this differential receptor expression and used a modified LHRH peptide as a targeting moiety on DDS to enhance drug uptake by the cancer cells and reduce the relative availability of the toxic drug to normal cells. Polyethylene glycol (PEG) polymer is widely used as a carrier for DDS (9). Polyethylene glycol is a water-soluble nonionic polymer approved by the Food and Drug Administration for pharmaceutical applications. Because of its nontoxic character, it is widely used in many

biochemical, cosmetic, pharmaceutical, and industrial applications. It is also important that PEG polymers show low-antigen activity and in most cases decrease the antigenicity of active ingredients conjugated to them (10). Successful bioconjugation depends on the chemical nature, structure, molecular weight, steric hindrance, and reactivity of the biomolecule, as well as that of the polymer. In most prodrug conjugations, a variety of spacers can be incorporated between polymer and biomolecules, because they can offer a chemical flexibility and can be hydrolyzed to release the bioactive component (11, 12). The suitability of a spacer molecule depends on its linearity, molecular weight, end functionality, charge, and reactivity. Current bioconjugation methodologies are limited to only one drug molecule per polymeric chain. On the other hand, polymers can deliver multiple drug molecules simultaneously to the cancer cells, thereby accumulating higher concentration of a drug and consequently increasing the efficacy of cancer treatment. In the present proposal, we hypothesized the use of citric acid (CA) as a multivalent spacer that allows conjugation of substantially higher number of copies of active ingredients. Our main objective was to design, synthesize and evaluate a novel water-soluble polymeric targeted multivalent anticancer prodrug, which is able to simultaneously deliver several copies of anticancer drugs specifically to tumor. The described system consisted of up to three copies of each targeting moiety (synthetic LHRH peptide, analog of LHRH) and anticancer drug [camptothecin (CPT)] conjugated to bis-carboxyl functional PEG polymer via multivalent spacer. However, the design of the developed delivery system is not limited to only three copies of active ingredients per one molecule of PEG carrier.

4.2 Materials and Methods

4.2.1. Cell Line.

The experiments were carried out on human ovarian (A2780) cancer cell lines. The human ovarian carcinoma A2780 cell line was obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO), supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Cells were grown at 37°C in a humidified atmosphere of 5% CO2 (v/v) in air. All of the experiments were performed on cells in the exponential growth phase.

4.2.2. Synthesis and characterization of targeted anticancer drug delivery system with several copies of LHRH peptide and CPT.

Synthesis of α,ω -Bis(2-Carboxyethyl) PEG-CPT Conjugates.

CPT, N,N-di-isopropyl-ethylamine, and 4-(methylamino)pyridine (DMAP) were obtained from Sigma Chemical Co., Atlanta, GA; bis(2- carboxyethyl) PEG molecular mass of 3000 Da polymer and fluorescein isothiocyanate (FITC) were obtained from Fluka (Allentown, PA). CPT is a quinoline-based indole alkaloid and is a close chemical entity of aminocamptothecin, CPT-11 (generic name, irinotecan), DX-8951f, and topotecan, found in the bark of the Chinese camptotheca tree and the Asian nothapodytes tree. Rhodamine red succinimidyl ester and Hoechst 33258 dye were purchased from Invitrogen- Molecular Probes (Carlsbad, CA). The selection of molecular weight of PEG polymer to design DDS was based on the following considerations. One the one hand, an increase in the molecular weight of the carrier improves drug pharmacokinetics and enhances its accumulation in the tumor whereas on the other hand, an increase in

molecular weight of polymer substantially limits drug bioavailability and cytotoxicity. Based on our previous studies (5,6,7), we selected PEG polymer with a molecular mass of 3000 Da, which shows only moderate decrease in bioavailability and cytotoxicity of CPT while improving drug pharmacokinetics. Such relatively low molecular weight polymer does not provide effective passive tumor targeting by the enhanced permeability and retention (EPR) effect. However, the inclusion of LHRH peptide as a targeting moiety in the delivery system ensures very effective tumor targeting and retention of CPT-PEG-LHRH conjugates (7).

α,ω,-Bis(2-Carboxyethyl) PEG-CA Conjugates (Compound 3).

α,ω,-Bis-PEG3000-CA conjugate was synthesized using a one-step procedure (Fig. 4.1.2). Citric acid (**compound 2 in Fig. 4.2 A**; CAS no. 77-92-9, 2-hydroxy-1, 2, 3-propanetricarboxylic acid) contains one hydroxyl and three carboxyl functional reactive groups for chemical conjugation. In brief, bis(2-carboxyethyl) PEG (compound 1 in Fig. 2B) (100 mg, 0.033 mM) and 2 mol of CA (12.8 mg, 0.066 mM) were dissolved in 5 ml of anhydrous dimethylformamide (DMF) and 20 ml of anhydrous dichloromethane (DCM). N-(3-Dimethylaminopropyl) - N-ethylcarbodiimide HCl (EDC.HCl, 13 mg, 0.0678 mM) was added to this solution as a coupling agent, and DMAP (4.03 mg, 0.032 mM) was used as a catalyst. The reaction solution was stirred continuously for 24 h at room temperature. The carbodiimide urea formed during the reaction was removed by filtration. The unreacted CA and EDC.HCl were removed by dialysis using Spectra/Por membrane (molecular mass cutoff, ~2000 Da) in DMF as a solvent. Further purification

of the **α ,ω,**-bis-PEG-CA conjugate was carried out using size-exclusion Sephadex G10 columns. The conjugate was dried under the vacuum at room temperature.

α,ω ,-Bis(2-Carboxyethyl) PEG-Citrate-CPT Conjugate.

α,ω-Bis(2-carboxyethyl) PEG3000-CA conjugate (compound 3 in Fig. 4.2 B) (molecular mass, 3382.24 Da, 50 mg, 0.0147 mM) and CPT (compound 4 in Fig 4.2C) (5.2 mg, 0.0147 mM) were dissolved in 5 ml of anhydrous dimethyl sulfoxide (DMSO) and 10 ml of anhydrous DCM. The reaction mixture was allowed to stir for 30 min. EDC.HCl (3 mg, 0.0156 mM) was added to the above solution as a condensing agent, and DMAP (2.0 mg, 0.016 mM) was used as a catalyst. The reaction was stirred continuously for 24 h at room temperature. The carbodiimide urea formed during the reaction was removed by filtration. The unreacted CPT and EDC.HCl were removed by dialysis using Spectra/Por dialysis membrane (molecular mass cutoff, ~2000 Da) in DMSO as a solvent. The conjugate was dried under vacuum at room temperature. In addition, polymer conjugates with two and three copies of CPT were prepared by using two and three molar ratios of CPT and EDC.HCl, respectively. 1H NMR spectra of **α,ω** -bis(2-carboxyethyl) PEG-CPT conjugates were recorded on Varian 400-MHz spectrophotometer using DMSOd6 as a solvent. 1) CPT protons correspond to δ 0.9 CH3 (t), δ 1.9 CH2 (m), δ 5.35 CH2 (s), δ 5.5 CH2 (s), δ 6.7 CH (s), -7.4 δ CH (s), δ 7.65 to 7.8 to 8.0 2H, m-CH, δ 8.2-OO-CH, O-CH (d), and 2) bis(2-carboxyethyl) PEG protons correspond from δ 3.2 to 3.8 broad peaks (**Fig. 4.3**).

α,ω,-Bis(2-Carboxyethyl) PEG-Citrate-CPT-LHRH Conjugate.

α,ω,-Bis(2-carboxyethyl) PEG3000-CA-CA-1CPT conjugate (molecular mass, 3729 Da, 50 mg, 0.0134 mM) and peptide LHRH-NH2 (18 mg, 0.013 mM) were dissolved in 3 ml

of anhydrous DMSO and 12 ml of anhydrous DCM. The reaction mixture was allowed to stir for 30 min. EDC.HCl (3.0 mg, 0.0134 mM) was added to the above solution as a condensing agent, and DMAP (1.0 mg, 0.008 mM) was used a catalyst. The reaction was stirred continuously for 48 h at room temperature. The carbodiimide urea formed during the reaction was removed by filtration. The unreacted CPT and EDC.HCl were removed by dialysis using Spectra/Por dialysis membrane (molecular mass cutoff, 2000 Da) using DMSO as solvent. The conjugate was dried under vacuum at room temperature. In addition, polymer conjugates with two and three copies of peptide LHRH-NH2 were prepared by using two and three molar ratios of LHRH-NH2 and EDC.HCl, respectively. Molecular weights of conjugates were recorded on MALDI/TOF (**Fig. 4.3A**), and the presence of LHRH peptide was analyzed by UV spectroscopy (**Fig. 4.3 B**).

4.2.3. α,ω,-Bis(2-Carboxyethyl) PEG-FITC Conjugate.

Hydroxyl groups of FITC were condensed with the carboxyl group of bis (2-carboxyethyl) PEG using EDC.HCl as coupling agent. In brief, bis- PEG (50 mg, 0.016 mM) and FITC (6.2 mg, 0.016 mM) were dissolved in 2 ml of anhydrous DMSO and 10 ml of anhydrous DCM. EDC.HCl (4 mg, 0.020 mM) was added as a condensing agent, and DMAP (2.0 mg, 0.016 mM) was used as a catalyst. The reaction was stirred for 24 h under subdued light. The reaction solution was filtered to remove the carbodiimide salt. The conjugate was washed with excess acetone three times to remove free FITC, and the conjugate was precipitated in diethyl ether. Furthermore, bis-PEG-FITC conjugate was purified by dialysis using Spectra/Por dialysis membrane (molecular mass cutoff, ~2000 Da) in DMSO as a solvent. The conjugate was further purified by size-exclusion G10 Sephadex column chromatography.

LHRH-Rhodamine Red Succinimidyl Ester Labeling

Synthetic analog of LHRH peptide was synthesized according to our design (5, 6) by American Peptide Company, Inc. (Sunnyvale, CA). Rhodamine red succinimidyl ester was covalently conjugated, with LHRH peptide having NH2 at the terminal. In brief, LHRH (5.0 mg, 0.0037 mM) and LHRH-NH2 (3 mg, 0.0039 mM) were dissolved in 2.0 ml of anhydrous DMF, and 4.0 ml of N, N-di-isopropyl-ethylamine was added to adjust alkaline pH and maintain amine group in nonprotonated form. The reaction was stirred for 2 h under subdued light. The conjugate was washed three times using acetone to remove free rhodamine red succinimidyl ester. The conjugate was further purified by size-exclusion G10 Sephadex column chromatography to remove nonconjugated LHRH and rhodamine red ester.

4.2.4. UV Analysis of LHRH and LHRH-Containing Conjugates

One milligram of standard LHRH-NH2, 3*CPT-PEG (no LHRH), 3*CPT-PEG-1*LHRH, 3*CPT-PEG-2*LHRH, and 3*CPT-PEG- 3*LHRH conjugates were dissolved in 1 ml of deionized water, and UV spectra were recorded from 250 to 450 nm **(Fig.4.4)**.

4.2.5. Fluorescence Measurements.

The concentration of CPT was estimated using fluorescence spectrophotometer (excitation 360 nm and emission 465 nm) with gain of 40 and number of flashes = 3. Varying concentrations of CPT were prepared using DMSO as a solvent. The fluorescence intensity was measured for each lowered dilution, and the standard plot was drawn for concentration in milligram per milliliter. Amount of CPT in bis (2-carboxyethyl) PEG-CA-CPT conjugates with one, two, and three copies of CPT was estimated using CPT standard plot **(Fig.5.5)**.

4.2.6. Molecular modeling.

Conformational structures for conjugates with one, two and three copies of CPT were explored using molecular modeling studies. Due to high molecular weight constraints, the three dimensional confirmation of polymers along with conjugated biomolecules is not being elucidated. It was thought worthwhile to build a molecular confirmation and evaluate energy minima's, stable confirmations and molecular dynamics for synthesized conjugates. The minima represented the energies (kcal) indicating degree of conformational freedom for 7 repeating units of bis PEG polymeric chain along with citric acid as a spacer. In addition, molecular dynamics confirmations were established for conjugates with one, and three copies of CPT molecules. **Figure 4.6A** shows the molecular dynamic confirmation for the bis PEG-CA conjugate containing three copies of CPT, whereas **Fig. 4.6**B represents distance and adjunct molecular alignments for the same conjugate. Distance for molecular dynamic structure for free CPT was 8.887 Å and 15.83 Å for bis PEG-CA-1CPT. In addition, bis PEG-CA with three CPT molecules was to be 13.74 Å, where as it was 12.23 Å with energy minima. On the other hand, bis PEG-1xCPT conjugate without citric acid spacer exhibits distance of 6.05 Å. The results indicate that the longer polymeric chains do collapse in presence of spacer. It was surprising to note that the distance decreased between the carbon molecules in the presence of three CPT molecules over to one CPT molecule in bioconjugate.

4.2.7. In vitro cellular uptake of 3xCPT-PEG and 3xCPT-PEG-3xLHRH conjugates.

To show that bis PEG-citric acid-CPT conjugates contained three copies of anticancer drug can be internalized by ovarian cancer cells; we examined the cellular uptake of

3xCPT-PEG conjugate using A2780 human ovarian carcinoma cells. As seen in Fig. 4.5, almost 20% of the applied bis PEG conjugate with three copies of CPT entered the cell within 5 min indicating rapid internalization of the drug. The cellular entry was further increased to 26% within 20 minutes and reached a plateau. Addition to this polymer of three copies of targeting moiety (synthetic analog of LHRH peptide) substantially enhances internalization of the conjugate. Final intracellular concentration of 3xCPT-PEG-3xLHRH conjugate was almost three times higher when compared with the concentration of conjugate without LHRH.

4.2.8. Cytotoxicity assay.

The cytotoxicity of CPT conjugates and free CPT was assessed by using a modified 3-(4, 5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide assay as described previously (5, 6, 7, 8).

4.2.9. In Vivo Antitumor Activity.

Previously developed mouse model of human ovarian carcinoma xenografts was used (5, 6, 7). In brief, A2780 human ovarian carcinoma cells ($2X10^6$) were transplanted s.c. into the flanks of female athymic nu/nu mice. When the tumors reached a size of approximately 1 cm3 (15–20 days after transplantation), mice were separately treated i.p. with saline (control), CPT, and 1*CPT-PEG, 2*CPT-PEG, 3*CPT-PEG, 1*CPT-PEG-1*LHRH, 2*CPT-PEG-1*LHRH, 3*CPT-PEG-1*LHRH, 3*CPT-PEG-2*LHRH, and 3*CPT-PEG-3*LHRH conjugates. The dose of all of the substances (10 mg/kg for the single injection) corresponded to the maximal tolerated dose of CPT. Equivalent CPT concentrations were 3, 3.8, and 5.6 mg/kg for conjugates containing one, two, and three copies of CPT, respectively. Maximal tolerated dose was estimated in separate

experiments based on animal weight changes after the injection of increasing doses of the drugs as described previously (5,6,7). Tumor size was measured at 6, 12, 18, 24, 36, 48, 72, and 96 h after the treatment. Changes in tumor size were used as an overall mark of antitumor activity.

4.2.10. Release of Camptothecin from the Camptothecin-PEG conjugates

The release of drug from the conjugates was assessed by HPLC method using esterase enzyme at pH 7.4. The standard curve of Camptothecin was obtained by special series of HPLC experiments and was further used for calculation of drug release from the conjugates. The data obtained are presented as a percentage of total bound camptothecin, which is released from the conjugates. Free camptothecin from hydrolytic reaction mass were quantified by HPLC. The HPLC system (Waters, Milford, MA, USA) employed a C5 Jupiter-300Å column (4.6 x 250 mm; 5µm; Phenomenex (Torrance, CA, USA).

Fourty µL was injected into the column and a gradient elution was used for separations. Solvent A consisted of 10% MeOH in H2O adjusted to pH 3.5 with formic acid. Solvent B consisted of 20% H2O (pH 3.5), 20% MeOH, and 60% acetonitrile. At a flow rate of 1.2 mL min^{-1}, the gradient was used: 0 min, 100% A; 5 min 85% A; 10 min, 75% A; 20 min, 20 % A; 25 min, 0 % A, held at 0% A for 27 min., 30 min 100 % A and held at 100% A for 37 min. Five minutes of equilibration at 100% A was performed before and after each injection. Free camptothecin was quantified using the external standard method, with quantification being based on peak area. Scanning by the PDA (photo diode array detector) occurred at 370 nm, while data acquisition and processing were performed by Waters Empower™ Chromatography Software.

4.2.11. Statistics.

All of the in vitro and in vivo experiments were performed in quadruplicate. The results are expressed as mean ± S.D. from four to eight independent measurements. Statistical analysis was performed as a one-way analysis of variance, and comparisons among groups were performed by independent sample t-test.

4.3. Results

4.3.1. Cellular Localization of LHRH Peptide and PEG Polymer after Incubation with Cancer Cells

Previously, we (5, 6, 7, 8) and others (10) have reported that receptors for LHRH are overexpressed in many types of cancer cells, including ovarian, breast, and prostate carcinoma, and are not expressed in healthy visceral organs. Although LHRH receptors were slightly expressed in healthy ovarian, breast, and prostate tissues, the expression of these receptors in tumors was substantially higher compared with normal tissues from the same organ of the same patient (7). This allows us to use LHRH peptide as a targeting moiety to deliver anticancer drugs to tumors that overexpress LHRH receptors. To show that LHRH peptide and PEG polymer can be used as a targeting moiety and a delivery vehicle, respectively, we studied intracellular distribution of LHRH peptide and PEG after incubation with A2780 human ovarian carcinoma cells expressing LHRH receptors. The distribution study of labeled LHRH and PEG (**Fig. 4.7**) showed that rhodaminelabeled LHRH peptide accumulated predominantly in the plasma membrane and part of the cellular cytoplasm adjacent to the outer cellular membrane. In contrast, FITC-labeled PEG polymer was almost equally distributed in the cellular cytoplasm and nuclei.

4.3.2. Synthesis and Characterization of Targeted Anticancer Prodrug with Several Copies of LHRH Peptide and CPT

Our ultimate goal was to synthesize a complex targeted anticancer prodrug with multiple copies of targeting moiety (synthetic analog of LHRH peptide) and anticancer drug (CPT) per one molecule of PEG (**Fig. 4.1**). In this study we used one, two, or three copies of each active component to show the feasibility of the proposed approach. However, conjugates containing one, two, and three copies of CPT substantially enhanced aqueous solubility of the drug. This solubility was 15.0, 10.0, and 6.5 mg/ml for conjugates with one, two, and three CPT copies, respectively. In contrast, free CPT is practically insoluble in aqueous solutions. The details of the synthesis are summarized below.

α,ω,-Bis(2-Carboxyethyl) PEG-CA Conjugate. The PEG polymer reported here was **α,ω**,-bis(2-carboxyethyl) PEG (molecular mass, ~3000 Da) and was a diacid with the formula HOOCCH2CH2CONH(CH2CH2O)nCH2CH2NHCOCH2CH2COOH. Two moles of CA were coupled (i.e., hydroxyl group of CA), with 1 mol of carboxyl-terminated bis-PEG using EDC.HCl as a coupling agent to form tricarboxylate groups at both terminals (Fig.4.2 A). Multifunctional CA was used as a spacer to decrease the steric hindrance and increase the reactivity of PEG-CA conjugate with CPT. The resulting bis-PEG-CA conjugate was purified using molecular mass cutoff dialysis membrane (molecular mass, ~2000 Da) and size-exclusion G10 Sephadex column. The bis- PEG-CA conjugate contained a total number of six carboxylate groups for further conjugation with multiple copies of CPT-OH and LHRH-NH2.

α,ω,-Bis(2-Carboxyethyl) PEG-CA-CPT Conjugate.

Hydroxyl group at the position 20 of CPT was covalently coupled with carboxyl groups of bis-PEG-CA to form a degradable ester bond. One, two, and three copies of CPT per bis-PEG-CA conjugate were obtained by reacting 1, 2, and 3 mol of CPT with 1 mol of polymer, respectively (Fig. 4.2 A). Likewise, the molar ratio of EDC.HCl and DMAP were varied. Polymer conjugate with one, two, and three copies of CPT showed substantially higher aqueous solubility compared with free CPT, which is not soluble in aqueous solutions. The formation of CPT-bis-PEG conjugates was confirmed by proton and 13C nuclear magnetic resonance (1H NMR and 13C NMR). The aromatic peaks in **Fig. 4.3** indicate the presence of CPT molecules along with broad peaks for bis-PEG, whereas 13C NMR confirmed the formation of ester bond between CPT and PEG-CA (data not shown). In addition, 13C NMR shows the peaks for free acidic groups in the conjugate. The amount of CPT per PEG in conjugates using proton integration method was found to be 30%, 38.12%, and 55.85% (w/w) for one, two, and three copies of CPT, respectively. The theoretical ratio for the conjugation of CPT for one, two, and three copies of CPT is 11%, 22%, and 33% (w/w), respectively. The increased conjugation ratio in reactions may be because of the presence of multiple carboxyl groups for conjugation of drug with polymer. The integration ratios for PEG peaks were decreased with increased number of copies of CPT. MALDI/TOF (PE Biosystems Voyager System 6080) showed increased molecular weight for CPT-PEG conjugate. Furthermore, the concentration of CPT in each conjugate was estimated using fluorescence spectroscopy at excitation of 360 nm and emission of 465 nm. We selected fluorescence spectroscopy for the measurement of CPT based on the following considerations. First, CPT has a wide range of UV absorbance wavelengths, and the detection limit of the drug absorbance is

relatively high. In contrast, the fluorescence measurements are more sensitive, even with low concentrations of CPT using emission of ~360 nm and excitation of ~465 nm. Second, we found that PEG itself does not interfere with the fluorescence measurements of CPT in the conjugates and is not detected in the emission and excitation range of wavelengths to that of CPT up to concentrations of 1.0 mg/ml. The detection limit of CPT by fluorescence spectroscopy was 0.0007 mM concentration.

α,ω,-Bis(2-Carboxyethyl) PEG-CA-CPT-LHRH Conjugate.

LHRH analog, Lys6-des-Gly10-Pro9-ethylamide (Gln- His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-NH-Et), having a reactive amino group only on the side chain of the lysine at position 6 was reacted with free carboxyl groups in **α,ω,-**bis(2- carboxyethyl) PEG-CA-CPT conjugate to form a nondegradable amide bond (**Fig. 4.2C**). The amount of LHRH to obtain one, two, and three copies per bis-PEG-CA-CPT conjugate was manipulated on a molar basis. Similarly, the molar ratios of EDC.HCl and DMAP were varied. The concentration of CPT in the each conjugate was estimated by fluorescence spectroscopy at excitation of 360 nm and emission of 465 nm. MALDI/TOF showed increased molecular weight for LHRH-PEG-CA-CPT conjugates (**Fig. 4.2C**).

4.3.3. Molecular Modeling.

Conformational structures for conjugates with one, two, and three copies of CPT were explored using molecular modeling studies. Because of high molecular weight constraints, the three dimensional conformation of polymers along with conjugated biomolecules is not being elucidated. It was thought worthwhile to build a molecular conformation and evaluate energy minima, stable conformations, and molecular dynamics for synthesized conjugates (**Fig. 4.6**). The minima represented the energies

(kcal) indicating degree of conformational freedom for seven repeating units of bis-PEG polymeric chain along with CA as a spacer. In addition, molecular dynamic conformations were established for conjugates with one and three copies of CPT molecules. **Figure 4.6A** shows the molecular dynamic conformation for the bis-PEG-CA conjugate containing three copies of CPT, whereas **Fig. 4.6B** represents distance and adjunct molecular alignments for the same conjugate.

Distance between the first and last carbon atom for molecular dynamic structure of free CPT was 8.887 and 15.83 Å for bis-PEG-CA-1CPT. In addition, distance for bis-PEG-CA with three CPT molecules was observed to be 13.74 Å, whereas it was 12.23 Å with energy minima. On the other hand, bis-PEG-1*CPT conjugate without CA spacer exhibits distance of 6.05 Å. The results indicate that the longer PEG polymeric chains do collapse in the presence of CA spacer. It was surprising to note that the distance decreased between the carbon molecules in the presence of three CPT molecules over to one CPT molecule in bioconjugate.

4.3.4. UV Analysis of LHRH and LHRH-Containing Conjugates.

To show the presence of targeting LHRH-NH2 peptide in the conjugate, UV spectra were recorded for LHRH-NH2 peptide alone, 3*CPT-PEG (no LHRH), 3*CPT-PEG-1*LHRH, 3*CPT-PEG-2*LHRH, and 3*CPT-PEG-3*LHRH conjugates (Fig. 4.4). The increase in UV absorbance at ~280 nm for conjugates with one, two, or three copies of LHRH peptide indicates higher amount of peptide incorporation in the conjugate.

4.3.5 Release of Camptothecin from the CPT-PEG conjugates.

The % CPT release from the conjugate consisting of one copy of CPT was found to be 35 % in 30 minutes when compared with the conjugate consisting of three copies of CPT, where it was found to be 60% **(Fig. 4.10)**.

4.3.6. In Vitro Cellular Uptake of 3*CPT-PEG and 3*CPTPEG- 3*LHRH Conjugates.

To show internalization of conjugates by A2780 ovarian cancer cells, we examined the cellular uptake of CPT, 3*CPT-PEG, and 3*CPT-PEG-3*LHRH conjugates using A2780 human ovarian carcinoma cells. As seen in Fig.4.1.5, almost 20% of the applied bis-PEG conjugate with three copies of CPT entered the cell within 5 min, indicating rapid internalization of the drug. The cellular entry was further increased to 26% within 20 min and reached a plateau. In contrast, free CPT was internalized by the cells significantly slower when compared with 3*CPT-PEG conjugate. Only approximately12% of the applied dose of CPT entered the cells within 30 min. Moreover, it should be noted that, because of very low aqueous solubility of CPT, the drug was first dissolved in 5% DMF and further added to PBS buffer having pH 7.4. Addition of three copies of targeting moiety (synthetic analog of LHRH peptide) to the above polymer substantially enhances internalization of the conjugate, whereas final intracellular concentration of 3*CPTPEG-3*LHRH conjugate was almost three times higher compared with the concentration of conjugate without LHRH. Therefore, conjugation of CPT to PEG substantially enhanced solubility and cellular availability of water-insoluble drug CPT, whereas LHRH peptide provided further increase in bioavailability of CPT.

4.3.7. Increasing of LHRH and CPT Copies per One Molecule of PEG Polymer Enhances Cytotoxicity and Antitumor Activity of Whole Prodrug

In Vitro Cytotoxicity.

Conjugation of CPT with PEG polymer resulted in high molecular weight complex and a decrease in its toxicity (increased IC50 dose) when compared with CPT alone (**Fig. 4.8**). The increase in number of CPT molecules in the conjugate from one to two or three per one molecule of PEG led to the increase in the toxicity of the whole system of 1.9 and 3.1 times, respectively. An addition of one molecule of LHRH (targeting moiety/penetration enhancer) led to the increase in the toxicity of CPT-PEG conjugate with one, two, or three copies of the drug of 5.3, 8.87, and 12.9 times, respectively, compared with 1*CPT-PEG conjugate. An increase in the number of LHRH copies consequently led to the further increase in the anticancer activity of conjugates. As a result, targeted 3*CPT-PEG-2*LHRH and 3*CPT-PEG-3*LHRH conjugates were 30 and 77 times more toxic against human ovarian carcinoma cells compared with nontargeted 1*CPT-PEG conjugate. Preliminary studies showed that simple mixing without conjugation of PEG with CPT alone or CPT and LHRH do not attenuate CPT toxicity in tumor cells.

In Vivo Antitumor Activity.

We analyzed the antitumor activity of the developed conjugates containing different copies of active ingredients on mice bearing xenografts of human ovarian tumor. Although, in general, in vivo data corroborate in vitro toxicity experiments, one substantial difference between these series was revealed. In contrast to the in vitro data where free CPT was more toxic than most conjugates (except conjugates containing three copies of CPT and two or three copies of LHRH), even non targeted polymeric CPTPEG conjugates with one to three copies of CPT were substantially more effective in terms of suppressing tumor growth (compare curves 2–4 with curve 1 on Fig. 6 with bars 1–4 on

Fig. 4.9). This observation supports our previous findings related to "passive tumor targeting" by high molecular weight polymeric drugs because of the EPR effect (6, 7, 8). Further enhancement antitumor activity was achieved by "active targeting" when one to three copies of tumor targeting moiety were added to the multicomponent delivery system (compare curves 6–9 with curves 2–4).

4.4. Discussion.

The use of synthetic analog of natural LHRH peptide as a targeting moiety was based on the following main considerations. First, the receptor for this peptide was over expressed in several cancer cells, including ovarian, endometrial, breast, and prostate cancers (5, 6, 7, 8, 10, 11). Second, although these receptors are expressed in healthy ovarian, breast, and prostate tissues, expression of LHRH receptors in corresponding tumors is substantially higher compared with normal tissues from the same patient (5). Third, conjugation of LHRH peptide(s) to high molecular weight delivery system should enhance its uptake by cancer cells that overexpress LHRH receptors. In contrast to low molecular weight drugs, which are internalized inside the cells by diffusion, high molecular weight drugs are internalized by endocytosis (6). Endocytosis is a slow process and requires considerably higher drug concentration outside cells compared with diffusion.

Therefore, toxicity of high molecular weight drugs is substantially lower compared with their low molecular weight precursors. The rate of endocytosis is most likely the critical factor that limits cytotoxicity (in vitro) of high molecular weight CPT-PEG conjugates observed in the present study. In addition to targeting, moiety changes the mechanism of cellular uptake of prodrug to receptor-mediated endocytosis (5) and therefore enhances

its toxicity. Normal cells that do not express LHRH receptors on their surface will utilize LHRH-containing delivery system by endocytosis, whereas cancer cells that overexpress the receptor will do so by receptor- mediated endocytosis. Consequently, toxicity of tumor targeted DDS against cancer cells will be substantially higher compared with normal cells, limiting adverse side effects of drug to normal cells.

Based on the results of the present study, we expect that the difference in toxicities of 3*CPT-PEG-3*LHRH system between normal and cancer cells will be at least 70 times. In addition, high molecular weight of CPT-PEG-LHRH restricts its penetration through the blood-brain barrier, thus preventing possible adverse side effects on the brain and especially on the pituitary gland, which expresses LHRH receptor (6). It should be stressed that our data showed that PEG polymer itself provides passive targeting to solid tumor by the EPR effect, leading to the accumulation of high molecular weight substances in solid tumor (7, 8, 12, 13, 14). However, such passive targeting is attributed only to solid tumors and cannot provide targeting to spreading tumors and metastases. In contrast, LHRH targeting is equally applicable to solid tumor and a single cell. Therefore, proposed targeted prodrug has a potential against spreading tumors and metastases on the background of low toxicity to normal tissues. Such a wide range of targeting capabilities combined with high tumor toxicity and low toxicity to normal tissues is an essential characteristic of proposed novel multivalent delivery system that cannot be achieved by traditional anticancer approaches. Several targeting moieties can be added to a DDS using polymeric dendrimer approach (15, 16, 17).

However, such a type of DDS may have an excessive molecular weight, which in turn can limit cellular internalization of the whole system and therefore decrease its toxicity.

Our approach is based on the use of low molecular weight CA as a branched spacer that provides conjugation of multiple number of targeting moieties per one molecule of polymeric carrier with substantially lower molecular weight of the whole system compared with dendritic DDS. The polymeric conjugates are inactive in their prodrug form during systemic delivery to the tumor and must release the drug from the delivery system to provide their anticancer activity. Therefore, the conjugates containing CPT molecules have been intentionally designed to possess an ester bond between the spacer and CPT. It is known that such a bond between PEG conjugate and CPT is hydrolyzed or broken by cellular enzymes such as esterase inside the cellular endosomes, leaving free drug for its action (9, 10).

On the other hand, the bond between the LHRH and PEG is an amide bond, which is more stable for the hydrolysis and in presence of enzymes. Our data showed that PEG polymer is internalized by cancer cells and distributed uniformly inside cells in the cytoplasm and nuclei. Although we did not analyze the pharmacokinetics of the studied compounds in vivo, based on our previous investigations of similar PEG polymer conjugates, one can expect substantial improvements in the pharmacokinetics with the increase in the molecular mass of the delivery system. Free low molecular weight of model compound was almost completely eliminated from the blood within the first 8 h after a single injection. At the same time, the level of the compound in the tumor was very low. The increase in the molecular weight of the compound by conjugation with PEG polymer substantially prolonged its circulation time over 24 h and increased blood drug concentration. Significant amounts of the compound were observed in the tumor up to 48 h after single injection. In addition, the conjugation of CPT to PEG substantially

increases the stability of its active lactone form in the blood, preventing nonproductive premature biodegradation of the whole system (9, 10, 11, 17). Finally, all of these improvements substantially enhance antitumor activity of CPT after its conjugation with PEG polymer.

In the present investigation, we showed the possibility of combining in one targeted prodrug multiple copies of targeting moieties and anticancer drugs. For CPT, the maximal number of drug copies per one molecule of PEG conjugate was limited by the decrease in solubility of DDS when the number of CPT copies exceeded three. Addition of more copies of LHRH peptide seems not to improve the targeting. Similar targeted multifunctional approach can be applied to other drugs, targeting moieties, imaging agents, and other active components. It is also possible, using our approach, to combine several different drugs in one complex delivery system. Our novel approach is substantially different and involves the use of low molecular weight multifunctional spacer to obtain varying copies of the components. We hereby are proposing the use of CA as a spacer, which consists of three carboxyl and one hydroxyl groups. Therefore, it offers the choice of conjugation with compounds having –OH, –COOH, or –NH2 functionality. In addition, each branch of multivalent spacer can be connected to similar multivalent branched spacer, providing the possibility of increasing the number of active ingredients without substantial increase in the molecular weight of the whole system. Present in vivo data confirmed the enhanced antitumor activity of the developed delivery systems containing multiple copies of targeting peptide and anticancer drug. Our approach can form a basis for a novel class of targeted tumor prodrugs-multivalent DDS with branched multiarmed spacers.

4.5. Conclusions.

The proposed polymeric prodrug consisting of multivalent components demonstrated increased anticancer efficacy as compared to the free drug, Camptothecin.

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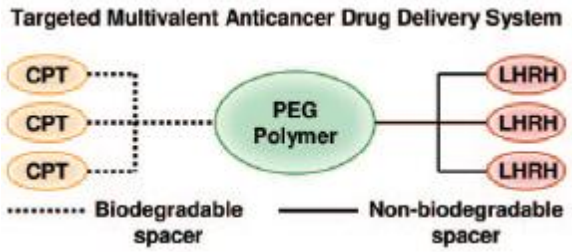

Fig. 4.1. Design of drug delivery system containing bis-PEG polymer as a carrier; one, two, or three copies of LHRH peptide as a targeting moiety; and one, two, or three copies of CPT as an anticancer drug.

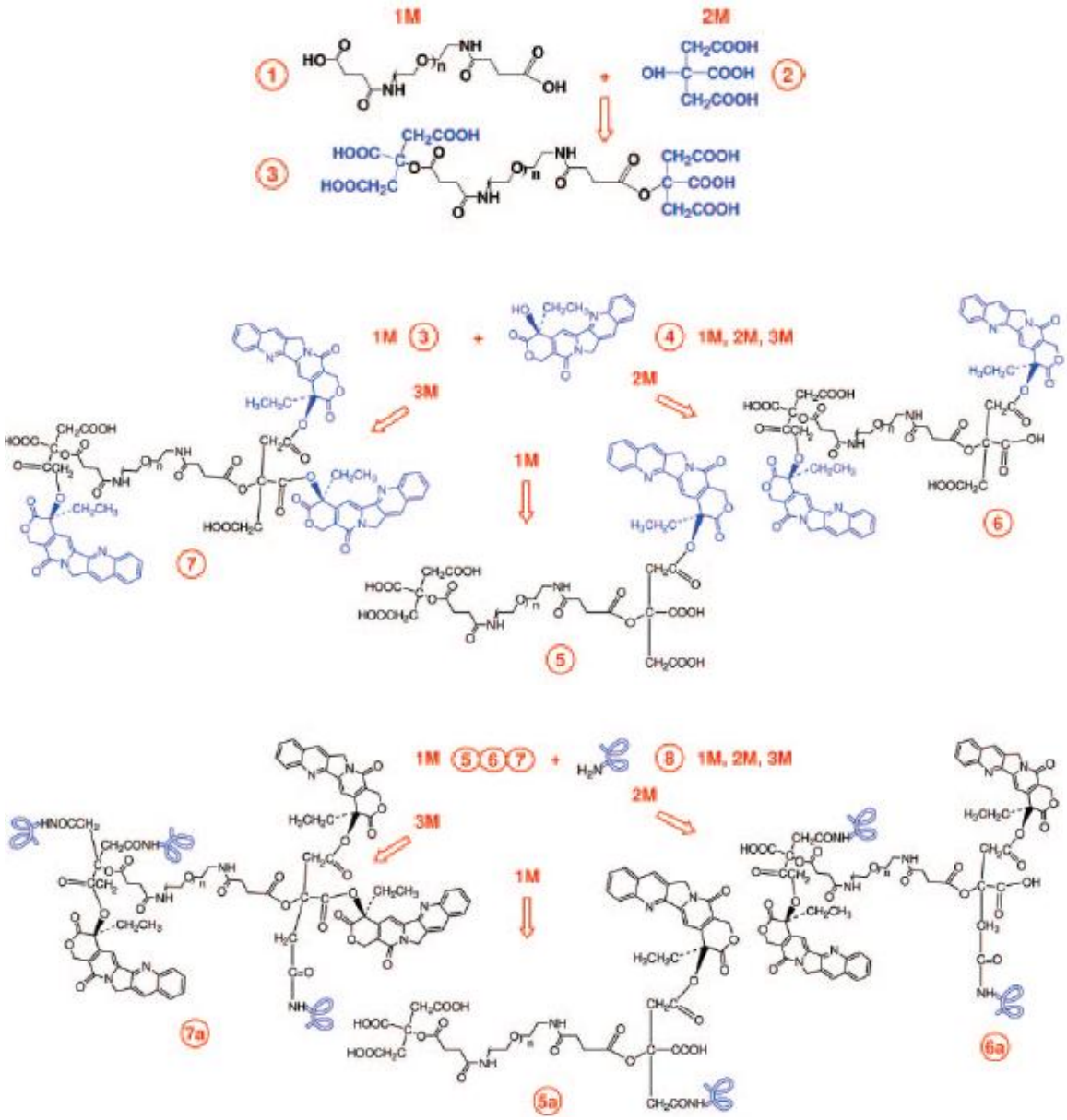

Fig. 4.2. Synthesis of targeted multivalent anticancer prodrug. **α,ω**,-bis-PEG3000-CA conjugate (3) was synthesized using one-step procedure by conjugation of 1 M bis(2-carboxyethyl) PEG (1) with 2 M CA (2). The bis-PEG-CA conjugate (3) was coupled with 1, 2, and 3 mol of CPT (4) to obtain **α,ω**,-bis(2-carboxyethyl) PEG-CA-CPT conjugates (c, 5, 6, and 7). Furthermore, 1, 2, and 3 mol of LHRH (8) were conjugated with c, 5, 6, and 7 to obtain **α,ω** -biscarboxyethyl) PEG-CA-CPT-LHRH conjugates (d, 5a, 6a, and 7a) having one, two, and three copies of CPT (4) and LHRH (8).

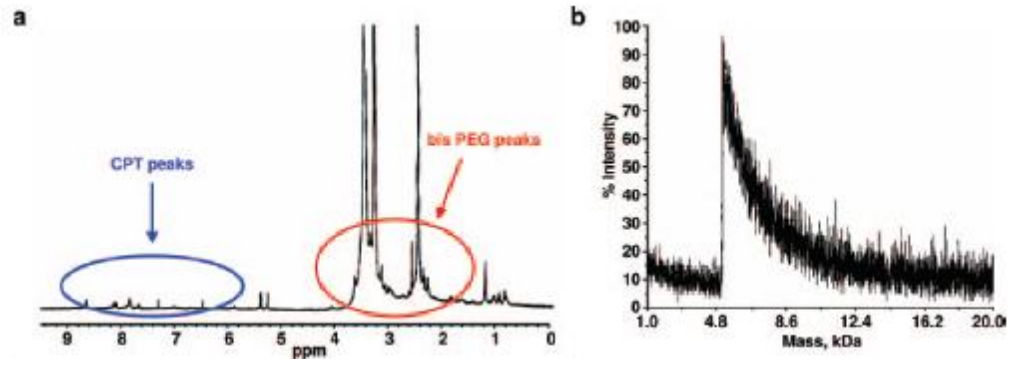

Fig. 4.3. Characterization of synthesized polymer conjugates. a, 1H NMR spectra for **α,ω**,-bis(2-carboxyethyl) PEG-CA-CPT conjugate in DMSOd6 400MHz. Proton from δ 3.2 to 3.8 broad peaks represents for bis(2-carboxyethyl) PEG, and peaks from δ 5.35 to 8.2 correspond to protons of CPT. b, MALDI/TOF for **α,ω**,-bis(2-carboxyethyl) PEG-CA-2*CPT-2*LHRH conjugate. Molecular mass for the conjugate was observed to be approximately 5363 Da. For bis-PEG-CA with one copy of CPT, molecular mass was estimated as 3413 Da (spectra not shown).

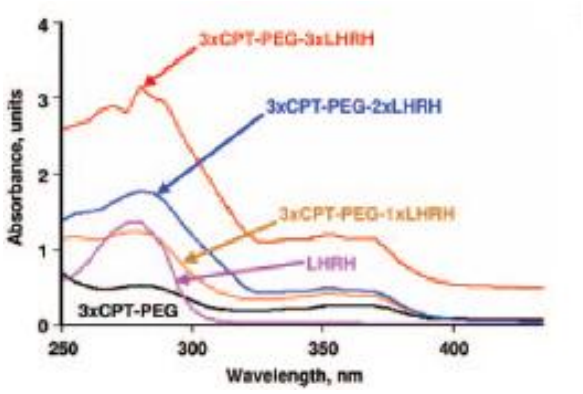

Fig. 4.4. UV spectra for LHRH and LHRH conjugates with bis-PEG-CPT. Free LHRH (standard) and conjugates containing one, two, and three copies of LHRH were dissolved in deionized water, and UV spectra were recorded. The increase in UV absorbance at ~280 nm for conjugates with one, two, or three copies of LHRH peptide indicates higher amount of peptide incorporated in the conjugate.

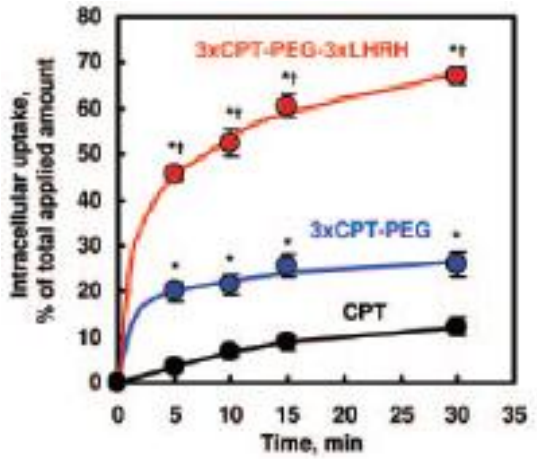

Fig. 4.5. Cellular entry of 3*CPT-PEG and 3*CPT-PEG-3*LHRH conjugates in A2780 ovarian carcinoma cells. Addition of LHRH peptide substantially enhanced cellular internalization of conjugate within 30 min compared with 3*CPT PEG conjugate.

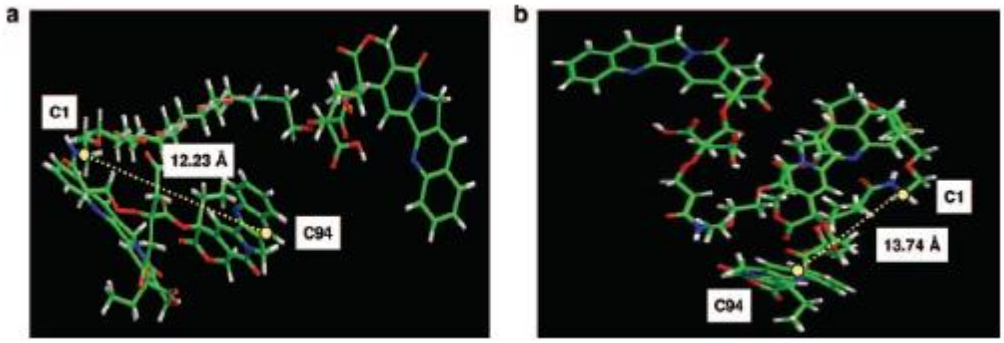

Fig. 4.6. Molecular modeling studies for seven repeating units of bis-PEG polymer with two copies of CA as a spacer attached with three copies of CPT. Polymeric structures were built in Chemdraw software and allowed to undergo conformational stabilization for energy minima (a) and molecular dynamics (b). The stabilized structures were exported to RasTop software to estimate the distances between first and last carbon atoms (here C1 and C94). The distance was measured to be 12.23 Å for energy-minimized bis-PEG-CA-3*CPT molecule, whereas it was 13.74 Å for molecularly dynamic structure. The above studies represent theoretical understanding of molecular conformations and structural stability of high molecular weight polymers covalently conjugated to multiple copies of drug molecules. , $P < 0.05$ compared with free CPT. †, $P < 0.05$ compared with 3*CPT-PEG.

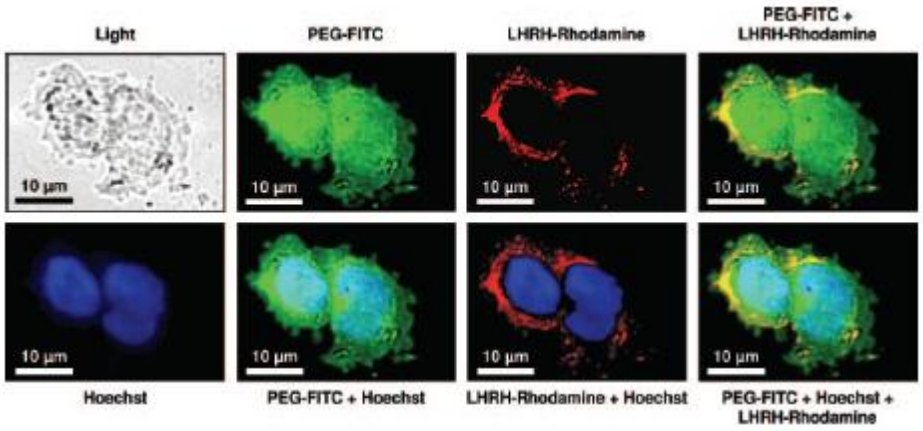

Fig. 4.7. Localization of LHRH and PEG in cancer cells expressed in LHRH receptors. Human ovarian carcinoma A2780 cells were incubated for 24 h with PEG polymer and LHRH peptide labeled with FITC (green fluorescence) and rhodamine (red fluorescence), respectively. In addition, cell nuclei were labeled with cell nuclear- specific dye (Hoechst 33258) with blue fluorescence. Fluorescence microscopy analysis showed that LHRH peptide localized in the plasma membrane and outer part of cellular cytoplasm, whereas PEG polymer of this size equally penetrated cellular cytoplasm and nuclei. Superposition of images allows for detecting of co localization of LHRH with PEG (yellow) and nuclear localization of PEG (cyan).

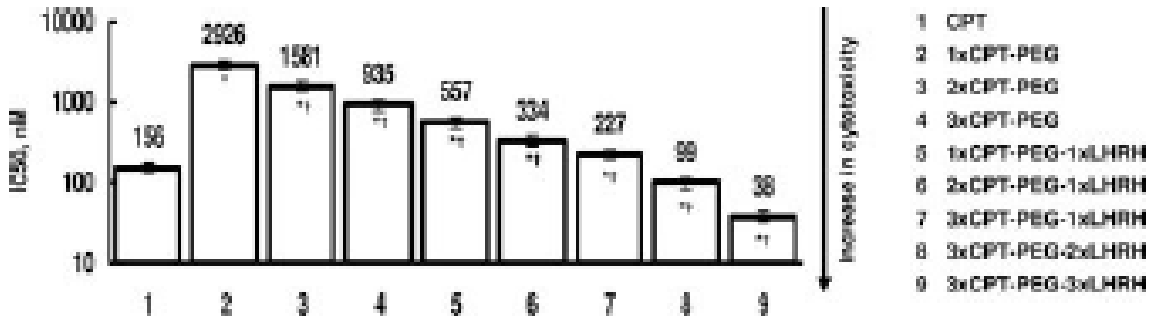

Fig.4.8. Inclusion of several copies of anticancer drug (CPT) and targeting moiety (synthetic analog of LHRH peptide) substantially enhances toxicity of prodrug toward human ovarian cancer cells. Human ovarian carcinoma A2780 cells were separately incubated with 45 different concentrations of each drug. IC50 dose (dose that kills 50% of cancer cells) was measured for each drug. Mean ± S.D. are shown. , $P < 0.05$ compared with free CPT. †, $P < 0.05$ compared with 1*CPT-PEG.

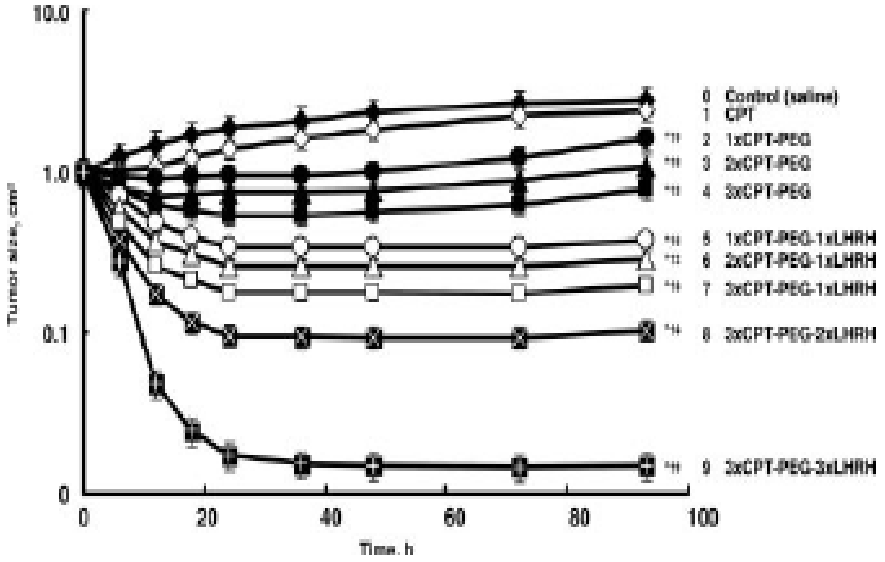

Fig.4.9. Inclusion of several copies of anticancer drug (CPT) and targeting moiety (synthetic analog of LHRH peptide) substantially enhances antitumor activity of prodrug toward xenografts of human ovarian tumor. A2780 human ovarian carcinoma cells were transplanted s.c. into the flanks of female athymic nu/nu mice. When the tumors reached a size of approximately 1 cm3 (15–20 days after transplantation), mice was treated once with each drug conjugate. The dose of all of the substances (10 mg/kg for the single injection) corresponds to the maximal tolerated dose of CPT. Equivalent CPT concentrations were 3, 3.8, and 5.6 mg/kg for conjugates containing one, two, and three copies of CPT, respectively. Tumor size was measured at 6, 12, 18, 24, 48, 72, and 96 h after the treatment. Mean ± S.D. are shown. , $P < 0.05$ compared with control. †,$P < 0.05$ compared with free CPT. ‡, $P < 0.05$ compared with 1*CPT-PEG.

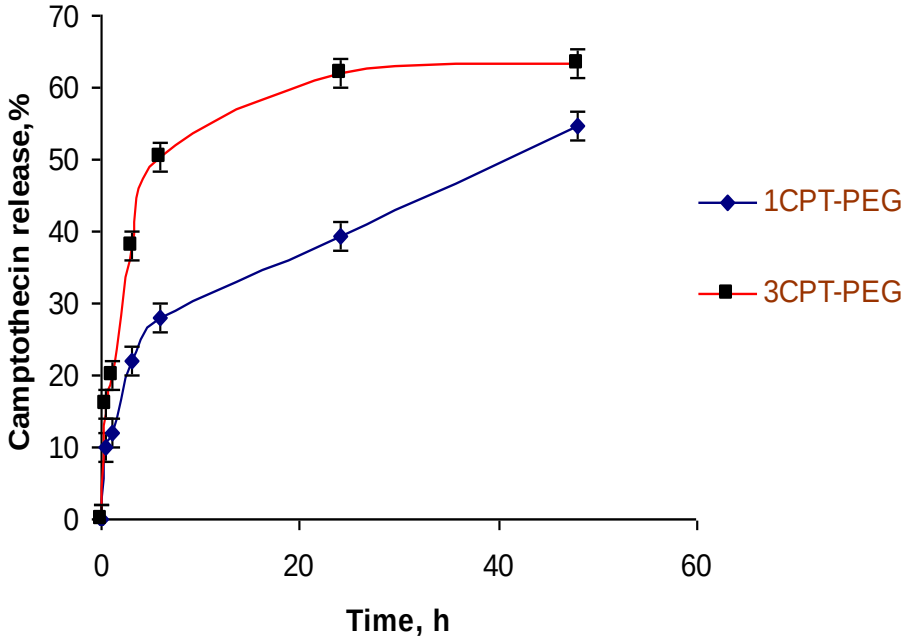

Fig. 4.10 Release profile of Camptothecin from the conjugates determined by HPLC after enzymatic hydrolysis using esterase as the enzyme at pH 7.4.

5. To increase the efficacy of the chemotherapy by co-delivery of anticancer drug, targeting agent and suppressor of antiapoptotic cellular defense.

5.1 Introduction

As it is known, that cancer is one of leading causes of death in the United States. Even though localized primary solid tumors can be successfully removed surgically, the treatment of spreading tumors or malignant tumors and tumor metastases requires high-dose chemotherapy. However, two main obstacles limit the success of chemotherapy: (1) severe adverse side effects on healthy organs and (2) development of multidrug resistance. The delivery of drugs as prodrugs specifically targeted to tumors and the suppression of cellular antiapoptotic defense can potentially be used to minimize the adverse side effects and suppress cellular resistance respectively.

A prodrug is an inactive precursor of a drug which is converted into the drug in the targeted organ, tissue, or cell (1-7). A prodrug remains inactive during its transfer to the site of drug action. Advantages of the prodrug approach include, but are not limited to, the prevention of systemic side effects, the increase in the bioavailability of modified drugs, and the possibility of using specific organ, tissue or cellular conditions to convert the inactive prodrug into its active form. In most cases, a prodrug includes a drug bound to (or encapsulated in) a water-soluble carrier. The more advanced type of prodrug, drug delivery system (DDS), might include a targeting moiety and supplementary active ingredients in addition to the carrier and drug. The carrier combines all components of the DDS together and provides the required characteristics of the whole DDS, i.e., solubility, molecular weight, size, etc. A targeting moiety enforces the specific delivery

of a drug to the targeted organ, tissue or cell. Acting as a penetration enhancer, the targeting moiety also improves the cellular uptake of the entire DDS.

Active ingredients can be included in a complex DDS to augment the action of a main drug. They may be represented by drugs with different mechanisms of action, or may provide an independent influence on cellular functions to promote effects of the drug. Frequently, separate components of the DDS are bound together by means of spacers. Such spacers play an important role by providing for the release of DDS components under certain conditions or at specific time-points. In most cases, a spacer that binds a targeting moiety to the carrier is made stable and non-degradable in body fluids and inside targeted cells. In contrast, spacers that connect a drug and other active components of the DDS to a carrier are usually biodegradable. The degradation of such spacers (or a bond between the spacer and a component) is normally caused by specific conditions inside the targeted organ, cell or cellular organelles, providing additional "targeting" to specific intracellular compartments or organelles, such as cellular cytoplasm, mitochondria, nuclei, etc (4,6,8,10). We already proposed the use of citric acid as a spacer that allows for the simultaneous binding of several copies of different active ingredients to one polymeric carrier, therefore forming a prodrug with multivalent components.

Two types of targeting of anticancer drugs to tumors are generally used: (1) passive and (2) active targeting (11). Passive targeting mainly utilizes the so-called Enhanced Permeability and Retention (EPR) effect (12, 14) responsible for the preferential accumulation of macromolecules in tumors. However, the passive targeting by the EPR effect is only efficient for the targeting of high molecular weight DDS to solid tumors

and cannot be used for the selective delivery of anticancer drugs to spreading tumors, metastases, and types of cancer that do not form tumors. Several types of active targeting are currently being used to provide for the specific delivery of therapeutics to cancer cells (11). The most effective and widely used approach is based on the coupling of a drug carrier with a targeted moiety specific to certain or a majority of cancer cells. Many different types of targeting moieties are being used in experiments and clinical trials. As we already proposed the use of slightly modified synthetic analog of Luteinizing Hormone Release Hormone (LHRH) for the targeting of DDS to cancer cells, our next approach was to include BH3 peptide as a suppressor of antiapoptotic cellular defense.

Most of the available anticancer drugs have a dual effect on cancer cells. They provoke cell death mainly by the activation of the central apoptotic signal but also trigger antiapoptotic cellular defense primarily by inducing the overexpression of antiapoptotic members of BCL2 protein family (11, 19). In order to prevent the activation of cellular defense and enhance the apoptosis induction, we proposed to include in the same DDS an anticancer drug together with a synthetic analog of BCL2 homology 3 (BH3) peptide or antisense oligonucleotides targeted to BCL2 mRNA.

Our previous experimental data showed the effectiveness of LHRH peptide as an effective tumor targeting moiety and BH3 peptide as a suppressor of antiapoptotic cellular defense. However, the separate use of only one peptide in DDS was not able to eliminate both main obstacles of chemotherapy: adverse side effects and activation of cellular resistance (15, 16, 17, 18). In fact, LHRH peptide enhanced the uptake of DDS by cancer cells and limited the accumulation of an anticancer drug in healthy tissues, but did not prevent the overexpression of antiapoptotic members of BCL2 protein family.

BH3 peptide substantially enhanced the cytotoxicity of traditional chemotherapeutic drugs, but was unable to prevent their accumulation in healthy organs, thus increasing the risk of adverse side effects. We hypothesize that only the simultaneous use of both LHRH and BH3 peptides in one drug delivery system will provide for a concurrent tumor targeting and a suppression of antiapoptotic cellular defense. Such multi-pronged attack on cancer will increase the efficacy of ovarian cancer therapy to an extent that cannot be achieved by individual components applied separately. The present experimental work is aimed at verifying the hypothesis and designing, synthesizing, and evaluating in vitro and in vivo of a novel multifunctional targeted proapoptotic drug delivery system which provides for the simultaneous tumor targeting by LHRH peptide, cell death induction by an anticancer drug camptothecin (CPT) and suppression of cellular antiapoptotic defense by BH3 peptide. The system also uses a novel multivalent branched spacer for combining several copies of active ingredients in one DDS in order to further enhance tumor targeting and cytotoxicity.

5.2. Materials and methods.

5.2.1. Cell Line.

The A2780 sensitive and A2780/AD multidrug resistant human ovarian carcinoma cell lines were obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center). Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Fisher Chemicals, Fairlawn, NJ). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 (v/v) in air. All experiments were performed on cells in the exponential growth phase.

5.2.2. Animal Tumor Model and Antitumor Activity.

An animal model of human ovarian carcinoma xenografts was used as previously described. (15, 16, 19). Briefly, A2780 sensitive or A2780/AD multidrug resistant human ovarian cancer cells (2×10^6) were subcutaneously transplanted into the flanks of female athymic nu/nu mice. When the tumors reached a size of about 0.3 cm^3 (15-20 days after transplantation), mice were treated intraperitoneally with saline (control), CPT, 1xCPT-PEG, 2xCPT-PEG, 1xCPT-PEG-1xLHRH, 1xCPT-PEG-1xBH3, 2xCPT-PEG-2xLHRH, 2xCPT-PEG-2xBH3, 1xCPT-PEG-1xBH3-1xLHRH and 2xCPT-PEG-2xBH3-2xLHRH. The dose of all of the substances (10 mg/kg for the single injection) corresponded to the maximum tolerated dose of CPT. Equivalent CPT concentrations were 3 and 3.8 mg/kg for conjugates containing one or two copies of CPT, respectively. The maximum tolerated dose of CPT was estimated in separate experiments based on the animal weight change after the injection of increasing doses of CPT as previously described (6, 15, 16). Tumor size was measured at 24, 48, 72 and 96 h after the treatment. Animal weight was measured every day. Changes in tumor size were used as an overall marker for antitumor activity as previously described (15, 16, 19).

5.2.3. Synthesis of Conjugates.

CPT, N,N-di-isopropyl-ethylamine, and 4-(methylamino)pyridine (DMAP) were obtained from Sigma Chemical Co., Atlanta, GA; and bis(2- carboxyethyl) PEG polymer was obtained from Fluka (Allentown, PA). LHRH analog, Lys6–des-Gly10–Pro9-ethylamide (Gln–His–Trp–Ser–Tyr–DLys–Leu–Arg–Pro–NH–Et) and BH3 (Ac-Met-Gly-Gln-Val-Gly-Arg-Gln-Leu-Ala-Ile-Ile-Asn-Arg-Arg-Tyr-Cys-NH2) peptides were synthesized according to our design by American Peptide Co. (Sunnyvale, CA)(15).α,ω

bis (2-carboxyethyl) PEG-citric acid conjugate was synthesized as previously described (6).

α,ω bis (2-carboxyethyl) PEG-citric acid-CPT Conjugates.

The conjugates were prepared by dissolving α,ω bis (2-carboxyethyl) PEG-citric acid conjugate (molecular mass, ~3382.24 Da, 50 mg, 0.0147 mM) and CPT (5.2 mg, 0.0147 mM) in 5 ml of anhydrous DMSO and 10 ml of anhydrous DCM. The reaction mixture was allowed to stir at room temperature for 30 min. EDC.HCl (3 mg, 0.0156 mM) was added to the reaction mixture which served as condensing agent and DMAP (2.0 mg, 0.016 mM) used as catalyst was also added. The reaction mixture was stirred at room temperature for 24 h. The carbodiimide urea formed during the reaction was removed by filtration. The unreacted EDC.HCl and CPT were also removed by using Spectra/Por dialysis membrane (molecular mass cut off, ~2000 Da) using DMSO as a solvent. The conjugate was dried under a vacuum at room temperature. Likewise, the conjugates with two copies of CPT were prepared by using two molar ratios of CPT and EDC.HCl, respectively.

α,ω bis (2-carboxyethyl) PEG-citric acid-CPT-LHRH conjugates.

α,ω,-Bis(2-carboxyethyl) PEG3000-CA-1CPT conjugate (molecular mass, ~3729 Da, 50 mg, 0.0134 mM) and peptide LHRH-NH_2 (18 mg, 0.013 mM) were dissolved in 3 ml of anhydrous DMSO and 12 ml of anhydrous DCM. The reaction mixture was allowed to stir for 30 min. EDC.HCl (3.0 mg, 0.0134 mM) was added to the above solution as a condensing agent, and DMAP (1.0 mg, 0.008 mM) was used a catalyst. The reaction was stirred continuously for 48 h at room temperature. The carbodiimide urea formed during the reaction was removed by filtration. The unreacted CPT and EDC.HCl were removed

by dialysis using Spectra/Por dialysis membrane (molecular mass cutoff, ~2000 Da) using DMSO as a solvent. The conjugate was dried under a vacuum at room temperature. In addition, polymer conjugates with two copies of LHRH-NH_2 peptide were prepared using two molar ratios of LHRH-NH_2 and EDC.HCl, respectively.

α,ω bis (2-carboxyethyl) PEG-citrate-CPT-LHRH-BH3 conjugates.

α,ω bis (2-carboxyethyl) PEG-citrate-CPT-LHRH conjugate (molecular weight ~ 6421 Da, 294 mg, 0.04578 mM) and BH3 peptide (106.20 mg, 0.04578 mM) were dissolved in 4 ml of DMSO and 10 ml of DCM. The reaction mixture was stirred for 30-40 minutes. EDC.HCl (8.74 mg, 0.045778 mM) was added to the above reaction mixture as a condensing agent and DMAP (1.0 mg, 0.08 mM) was used as a catalyst. The reaction mixture was stirred continuously for 48 h at room temperature. The carbodiimide urea formed during the reaction was removed by filtration. The unreacted CPT and EDC.HCl were removed by dialysis using Spectra/Por dialysis membrane (molecular mass cutoff, ~ 2000 Da) using DMSO as a solvent. The conjugate was dried under a vacuum at room temperature. The conjugate with two copies of BH3 peptide was prepared by using two molar ratios of BH3 and EDC.HCl, respectively (**Fig. 5.1**).

5.2.4. Synthesis of Cy5.5-PEG conjugates.

Bis-diamine PEG was purchased from NOF Corporation, Japan with a molecular weight 3,677 Da. Near-infrared fluorophore cyanine dye Cy 5.5 NHS ester (1.0 mg) was dissolved in 1.0 ml of DMF and bis PEG amine (9.0 mg) was separately dissolved in 1.0 ml of DMF. Triethanolamine (0.5 ml) was added to Cy 5.5 solution to activate the Cy5.5 NHS ester. Furthermore, PEG solution was added to Cy5.5 ester and stirred continuously for 4 h at room temperature. The reaction was protected from the light. Free Cy.5.5 was

removed by Spectra Pore dialysis membrane with cut off of 2,000 Da. In addition, further purification was carried out by Sephadex column G10. Conjugate was dried under the vacuum at room temperature.

5.2.5. Synthesis of Cy5.5-PEG-LHRH conjugate

The conjugate prepared as mentioned above resulted with one free amine at the terminal and was coupled with succinic acid (SA) to yield to form Cy-5.5-PEG-COOH (CP). Briefly, CP and SA were dissolved in 3.0 ml of an. DMSO and 5.0 ml of anhydrous DCM. The mole ratio of CP to SA was maintained to be 1:1. EDC.HCl was added as a coupling agent and DMAP was used as a catalyst. 0.4 ml of triethylamine base was added to remove the salt from the coupling agent. The reaction was stirred continuously for 24 hours at room temperature. The resulting solution was filtered to remove DCU and the filtrate was dialyzed extensively with anhydrous DMSO (dialysis membrane of molecular weight cut off ~ 2,000 Da) for 24 hours to remove unreacted SA and EDC.HCl. Furthermore, the conjugate was purified using size exclusion Sephadex G10 column. Conjugate was dried under the vacuum at room temperature. Additionally, LHRH-NH_2 was conjugated with resulting CP-SA conjugate to form an amide conjugate. LHRH-NH_2 and CP-SA conjugate were dissolved in 4.0 ml of an. DMSO and 6.0 ml of an. DCM. The mole ratio of LHRH NH_2 to CP-SA was maintained to be 1:1. EDC.HCl, was added as a coupling agent and DMAP was used as a catalyst. 0.4 ml of Triethylamine base was added to remove the salt from the coupling agent. The reaction was stirred continuously for 24 hours at room temperature. The resulting solution was filtered to remove DCU and the filtrate was dialyzed extensively with anhydrous DMSO (dialysis membrane of molecular weight cut off ~ 2,000 Da) for 24 hours to remove unreacted SA and

EDC.HCl. The conjugate was purified using size exclusion Sephadex G10 column and dried under the vacuum at room temperature.

5.2.6. Cellular Internalization of Polymers

To analyze cellular internalization of different molecular weight polymers, PEG polymers were labeled by fluorescein isothiocyanate (FITC) as previously described (6). Cellular internalization of FITC-labeled PEG polymers were studied by confocal microscopy in living cells at 37° C within 4.5 h.

5.2.7. Atomic Force Microscopy

Atomic force microscopy (AFM) imaging of PEG-conjugates was conducted in tapping mode which is generally described elsewhere (21). Briefly, 50 µl of the PEG-conjugates suspension in water was deposited on freshly cleaved mica, kept for 10 min at 100% humidity to achieve particles precipitation. After the removal of the excess water by gentle touch of the side of mica with the piece of filter paper, PEG-conjugates were dried under the gentle flow of nitrogen. The images were acquired in air with a Nanoscope III AFM Instrument (Veeco/Digital Instruments, Santa Barbara CA) using tapping mode etched OMCL-AC160TS silicon probes (Olympus Optical Co. Tokyo, Japan). The images were processed, and the measurements were performed with Femtoscan software v. 2.2.85(5.1) (Advanced Technologies Center, Moscow, Russia). For statistics, no less than 50 unobstructed PEG polymer conjugates were analyzed.

5.2.8. In Vivo Imaging

To assess the body distribution of PEG polymers, polymers were labeled with Cy5.5 as described above. In vivo body distribution of Cy5.5-labeled targeted and non-targeted conjugates was studied by the IVIS Imaging System (Xenogen, Alameda, CA) in

anesthetized animals according to the manufacturer instructions. Animals were anesthetized with Isoflurane using XGI-8 Gas Anesthesia System (Xenogen, Alameda, CA). Visible light and fluorescent images were taken and overlaid using the manufacturer's software to obtain a composite image.

5.2.9. Organ Distribution

Aliquots of PEG and LHRH-PEG conjugates were radiolabeled with tritium. Radiolabeling was done on LHRH-PEG-NH_2 or PEG-NH_2 with ^{3}H-Acetic anhydride in presence of 1% diisopropylethyl amine and methanol to get LHRH-PEG-NH-CO-CH_3 [^{3}H] and PEG-NH-CO-CH_3 [^{3}H] conjugates respectively. Tumor and organ (liver, kidney, spleen, heart, lung, brain and pituitary) distribution of radiolabeled conjugates has been studied in nude nu/nu mice. Twelve mice have been used in this experiment. Six mice were used as a control while the xenografts of human ovarian cancer were transplanted in the rest. When the tumors reached a size of about 1 cm^3 (15-20 days after inoculation), mice were treated intraperitoneally with 10 mg/kg of desired conjugates. The maximum tolerated dose was detected in separate experiments. Mice without tumor have received the same dose of conjugates.

5.2.10. Cytotoxicity

The cytotoxicity of all studied conjugates and free CPT was assessed using a modified MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (6, 15, 16, 19).

5.2.11. Protein Expression

The identification of BCL2 and Caspase 9 proteins was made by Western immunoblotting analysis and processed using scanning densitometry to quantify the expressed protein. To this end, harvested cells were lysed in RIPA buffer (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) using a needle and syringe. Following incubation on ice for 45 minutes, the cells were centrifuged at 10,000 g for 10 min. Protein content in the supernatant was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) and 50µg of protein was run on a 15% sodium dodecyl sulphate (SDS) polyacrylamide gel immersed in Tris/Glycine/SDS buffer (BioRad, Hercules, CA) for 90 minutes at 70V. Proteins were transferred to an Immobilon-P nitrocellulose membrane (Millipore, Bedford, MA) in a Tris/Glycine buffer (BioRad, Hercules, CA) for 90 minutes at 100V. The membrane was blocked in non-fat milk for 30 minutes at room temperature on a rotating shaker to prevent non-specific binding, washed and incubated overnight with anti-BCL2 rabbit primary antibody (1:250 dilution, Stress Gene Biotechnologies, Victoria State, BC, Canada), anti-Caspase 9 rabbit primary antibody (1:500 dilution, Stress Gen Biotechnologies, Victoria State, BC Canada) or anti-β-actin mice primary antibody (1:2000 dilution, Oncogene Research, San Diego, CA) at 4ºC. After further washing, the membrane was immersed in goat anti-rabbit and goat anti-mouse IgG biotinylated antibody (1:3000 dilution and 1:1000 dilution, respectively, BioRad, Hercules, CA) at room temperature for 1.5 h on a rotating shaker. Bands were visualized using an alkaline phosphatase color development reagent (BioRad, Hercules, CA). The bands were digitally photographed and scanned using the Gel Documentation System 920 (NucleoTech, San Mateo, CA). Beta-actin was used as an internal standard to

normalize protein expression. Band intensities of BCL2 protein and caspase 9 are expressed as the percentage of the β-actin band intensity, which was set at 100%.

5.2.12. Apoptosis

The apoptosis induction was analyzed by the measurement of the enrichment of histone-associated DNA fragments (mono- and oligo-nucleosomes) in homogenates of the tumor using anti-histone and anti-DNA antibodies by a cell death detection ELISA Plus kit (Roche, Nutley, NJ) as previously described (15, 16, 19).

5.2.13. Statistical Analysis

Data obtained were analyzed using descriptive statistics, single factor analysis of variance (ANOVA) and presented as mean value ± standard deviation (SD) from four to eight independent measurements in separate experiments.

5.3. Results

5.3.1. Cellular Internalization of Polymers.

To study cellular internalization of polymers with different molecular weight, we labeled poly (ethylene glycol) with FITC. Labeled polymers were visualized in living cells by a confocal microscope at 37º C within different time intervals. Two labeled PEG polymers with molecular weights of 3,000 and 20,000 Da were used. We found (**Fig. 5.2**) that intensive internalization of lower molecular weight polymer started 20 min after the beginning of incubation. Forty-five min after the beginning of the exposure, 3,000 Da FITC-labeled polymer distributed almost homogeneously within the cancer cell. Intensive fluorescence, comparable with fluorescence in the medium, was registered both in the cellular cytoplasm and nuclei. These data show that PEG polymer with molecular weight of about 3,000 Da easily penetrate cellular cytoplasm and nuclei. In contrast, the

internalization of PEG polymer with molecular weight of about 20,000 Da occurred much slower when compared with smaller polymer (3,000 Da). A considerable accumulation of polymer in the cellular cytoplasm was registered 2-4.5 h after the addition of the polymer into the medium (**Fig. 5.2**).

5.3.2. Imaging of DDS by the Atomic Force Microscope

To assess the shape, size and morphology of PEG conjugates we employed Atomic Force Microscopy (AFM), a technology that allows direct visualization of biopolymers such as DNA and proteins, and various drug delivery polymer particles at nanometer resolution (21). AFM imaging revealed the complex structure of conjugates visible as compact (30-50 nm) particles with distal protrusions (**Fig.5.3**). Although detailed organization of conjugates at the molecular level has to be further investigated, AFM images provide initial evidence of nano-sized dimensions and a predicted complex molecular structure of PEG conjugates.

5.3.3. Body Distribution of Targeted and Non-Targeted Polymers

To investigate the distribution of PEG polymers in mice, we labeled the polymers with near infrared dye Cy5.5. Conjugates were visualized in anesthetized alive animals using an IVIS imaging system. The system uses color coding of fluorescence with blue coding for low levels of fluorescence and red color for high levels. The results (**Fig. 5.4**) show that both targeted and non-targeted polymers provide for a relatively long circulation in the blood stream. Actually, a fairly high blood level of both conjugates was registered 72 h after the injection resulting in the comparable level of fluorescence (green-blue color of the composite image in **Fig. 5.4**, lower panel). However, body distribution and tumor accumulation of targeted and non-targeted conjugates substantially differed. Targeted

conjugate predominantly accumulated in the tumor, while the distribution of non-targeted PEG was more homogeneous. Qualitative imaging data are supported by a quantitative measurement of the PEG distribution using a radioactive conjugate labeling of the polymer (**Fig. 5.5**). The analysis of organ distribution of the radioactively labeled non-targeted and tumor-targeted conjugates shows preferential accumulation of LHRH-targeted conjugate in the tumor. It should be stressed, that significant fractions of targeted conjugate were accumulated in healthy ovaries of animals bearing subcutaneous xenografts of human ovarian tumor.

5.3.4. In vitro Cytotoxicity

Cytotoxicity data (**Fig. 5.6**) showed that the conjugation to the PEG polymer, inclusion in the anticancer drug delivery system a targeting moiety (LHRH peptide), and a suppressor of antiapoptotic cellular resistance (BH3 peptide) to the drug delivery system significantly increased cytotoxicity of CPT. Moreover, an increase in the number of copies of the drug and peptides per one molecule of the polymer led to further enhancement in the anticancer efficacy of Camptothecin.

5.3.5. Suppression of Antiapoptotic Cellular Defense and Enhancement of Apoptosis Induction

As expected, an incorporation of BH3 peptide into the CPT-containing DDS prevented the overexpression of BCL2 protein by the anticancer drug and even suppressed the level of this protein in ovarian tumor cells (**Fig. 5.7**). The suppression led to the inhibition of antiapoptotic cellular defense and, therefore, to the enhancement of the activation of Caspase 9 – the main initiator of apoptosis. In turn, Caspase activation initiated a proapoptotic signal and apoptosis itself (**Fig. 5.8**). It should be stressed that the proposed

DDS induced apoptosis in both drug sensitive and multidrug resistant ovarian tumors. However, apoptosis in resistant tumors was less pronounced.

5.3.6. Antitumor activity

The measurement of tumor size in mice bearing xenografts of sensitive and multidrug resistant human ovarian carcinomas showed that simultaneous tumor targeting by the LHRH peptide, cell death induction by CPT and suppression of antiapoptotic cellular defense by BH3 peptide led to high antitumor activity of the entire DDS (**Fig.5.9**). The system containing two copies of each CPT, LHRH and BH3 was the most effective in the suppression of tumor growth in both sensitive and multidrug resistant tumors.

5.4. Discussion.

The main goal of the present experimental work was to develop, synthesize and evaluate in vitro and in vivo a novel, highly effective anticancer drug delivery system. It was expected that this system will satisfy the following requirements. First, it should provide targeted delivery of active ingredients specifically to the tumor cells preventing drug accumulation in healthy organs and therefore limiting adverse side effects of the treatment. Second, this DDS should effectively induce cell death in cancer cells. Third, the system should prevent the development of cellular drug resistance after the treatment with an anticancer drug and fourth, the architecture of the DDS should allow for the easy addition of several copies of active ingredients per one molecule of the carrier.

To fulfill these demands, the targeted proapoptotic anticancer delivery system was designed, synthesized and evaluated. The system contains a carrier (PEG polymer) and one or two copies of each active component: an anticancer drug (CPT), a targeting moiety (LHRH peptide) and a suppressor of antiapoptotic cellular defense (BH3 peptide).

The carrier binds all components together providing for a high solubility of the entire DDS. The targeting moiety allows for tumor targeting and a specific uptake of the DDS by cancer cells through the interaction of targeting peptide with corresponding receptors overexpressed in cancer cells. The anticancer drug induces cell death and the suppressor of antiapoptotic cellular defense prevents the development of drug resistance in cancer cells.

We selected polymer with a molecular weight of 3,000 Da based on the following considerations. First, even without penetration enhancer (LHRH), PEG polymer of this mass easily penetrated through cellular plasma membrane, and distributed homogenously inside cancer cells. Although the high penetration ability, PEG polymers of a relatively small molecular weight sounds obvious, its easy penetration through the plasma membrane and homogenous distribution through the cancer cell including the cellular nucleus was first documented experimentally in the present study. Second, polymers of higher molecular weight exhibit pronounced passive targeting through the EPR effect (12, 13). Therefore, to ensure that specific tumor accumulation of targeted LHRH-PEG conjugates is due to the inclusion of LHRH peptide as a tumor targeting moiety and not as a result of the EPR effect, we used a relatively small PEG polymer (3,000 Da) as a carrier. The visualization of DDS with an atomic force microscope revealed a compact structure of PEG polymer with a size of about 30-50 nm conjugated with distant tree-arms citric acid multifunctional spacer containing globules of active ingredients (LHRH, CPT, BH3). The imaging confirms the predicted structure of synthesized polymers.

The data obtained in the present study about the body distribution of targeted and non-targeted polymers support previously reported data about the body distribution of similar

polymers obtained in our experiments by a different method (17). It is interesting that the average blood concentration (proportional to the registered fluorescence of labeled conjugates) were comparable for both targeted and non-targeted conjugates one and 72 h after the injection resulting in similar coloring of images. At the same time, tumor accumulation of the targeted conjugate was substantially higher in the tumor when compared with the accumulation of the non-targeted conjugate. This finding allows us to speculate that a considerable part of the injected dose of non-targeted conjugate was expelled from the organism within 72 h, while the targeted conjugate remained in the tumor and therefore was not eliminated from the body of the experimental animals. Such retention forms a prerequisite for higher antitumor activity and lowers the adverse side effects of the targeted conjugate.

The qualitative imaging data were supported by the quantitative measurement of conjugate accumulation using radioactive labeling of the conjugates. Experimental data showed that a significant part of the injected dose of targeted polymer accumulated in ovaries of experimental animals where the expression of LHRH receptors was also expressed, although in lower level when compared with ovarian tumor (15). This accumulation should not raise concerns about adverse effects of the LHRH-targeted conjugate on the female reproductive system. First, such adverse effects might not be taken into consideration because ovaries are usually surgically removed in females with ovarian and sometimes breast cancer (the main types of cancer that are prospective candidates for LHRH-targeted chemotherapy in females). Moreover, our previous data showed that similar treatment did not lead to the disturbances in the time-course of luteinizing hormone and reproductive functions in female mice (17). We also previously

showed that LHRH-targeted PEG conjugates did not impose pituitary toxicity probably due to the protection of the pituitary gland by the blood-brain barrier impermeable for the conjugate used.

We found that DDS which simultaneously combines a targeting moiety, an inhibitor of antiapoptotic cellular defense, and an anticancer drug possesses much higher cytotoxicity when compared with the free drug. Such enhancement in the toxicity reflects several mechanisms. First, conjugation of CPT to highly water-soluble PEG polymer substantially increases its aqueous solubility. Second, the interaction of the LHRH peptide with corresponding receptors initiates receptor-mediated endocytosis and therefore enhances the uptake of the entire DDS by cancer cells. Third, the suppression of antiapoptotic cellular defense by the BH3 peptide increases the cell death induction ability of the anticancer drug. The toxicity of the conjugates rose with the increase in the number of copies of LHRH and BH3 peptides conjugated with one molecule of PEG polymer. The number of copies of BH3 peptide and CPT is limited by the low solubility of the drug and the peptide. It is possible to attach more than two copies of LHRH peptide to one molecule of PEG polymer.

However, our preliminary data showed that further increases in the number of LHRH copies does not result in the additional increase of tumor targeting. Therefore, 2xCPT-PEG-2xBH3-2xLHRH configuration of the drug delivery system, most likely represents an optimal configuration of DDS providing for the highest possible levels of tumor targeting and cytotoxicity in ovarian cancer cells.

The analysis of in vivo apoptosis induction by different variants of developed DDS supports previously reported findings related to the suppression of cellular antiapoptotic

defense by BH3 peptide. The proapoptotic activity of BH3 peptide increased after its conjugation to the PEG polymer and with doubling the number of copies of the peptide in the DDS. In addition to this expected fact, we found that LHRH strengthened the ability of BH3 peptide to inhibit the synthesis of BCL2 protein and thus enhanced cell death inducing by the anticancer drug. At the same time, an increase in the number of LHRH copies in DDS containing CPT in the absence of the BH3 peptide led to the further overexpression of BCL2 protein. These phenomena may be explained by the increase in the bioavailability of the entire DDS and CPT in particular by LHRH peptide initiating receptor-mediated endocytosis in LHRH receptor-expressing tumor cells. If BH3 peptide was not included in the LHRH receptor-targeted DDS, higher bioavailability of CPT led to the higher compensative activation of cellular antiapoptotic defense when compared with non-targeted CPT-PEG conjugate. The inclusion of BH3 peptide into targeted DDS prevented such activation.

The synergism between CPT, LHRH and BH3 peptides included in one drug delivery system led to the higher antitumor activity of the entire multicomponent delivery system. Therefore, we were able to verify our hypothesis and show that simultaneous tumor targeting, apoptosis induction, and suppression of cellular antiapoptotic defense in tumor cells resulted in increase in the antitumor activity of entire complex to the level which cannot be achieved by individual components applied individually. In contrast to the existing drug delivery systems, the proposed DDS was effective in both sensitive and multidrug resistant tumors. However, in the present study, anticancer efficacy of the DDS was limited in P-glycoprotein-dependent multidrug resistant tumors. The suppression of such multidrug resistance should further enhance antitumor efficacy of DDS in multidrug

resistant tumors. The next generation of polymeric targeted proapoptotic DDS is being currently developed in our laboratory. DDS includes, in addition to LHRH and BH3 peptides, suppressors of P-glycoprotein-dependent multidrug resistance (similar to those previously used by us in liposomal delivery systems). Evaluation of such complex multifunctional anticancer drug delivery system is a task for the future investigations.

5.5. Conclusions

The simultaneous tumor targeting, apoptosis induction and suppression of cellular antiapoptotic defense in tumor cells resulted in an increase in the antitumor activity of the entire complex to a level which cannot be achieved by individual components applied separately. In contrast to the existing drug delivery systems, the proposed DDS was effective in both sensitive and multidrug resistant tumors.

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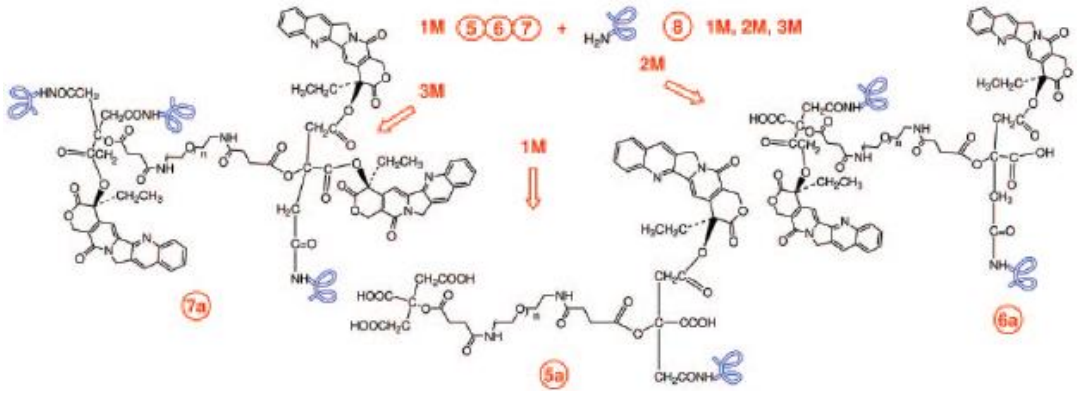

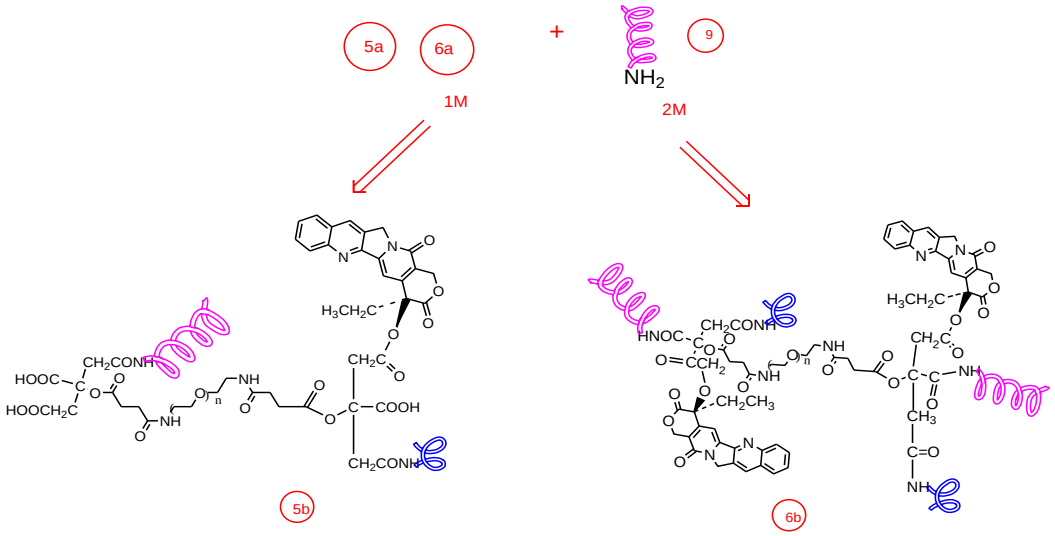

Fig. 5.1. Synthesis of CPT-PEG-LHRH-BH3 conjugates. α,ω,-bis(2-carboxyethyl) PEG-CA-CPT-LHRH conjugates (5a, 6a) were conjugated with 1 and 2 moles of BH3 (9) to obtain the conjugates containing one and two copies of CPT , LHRH and BH3.

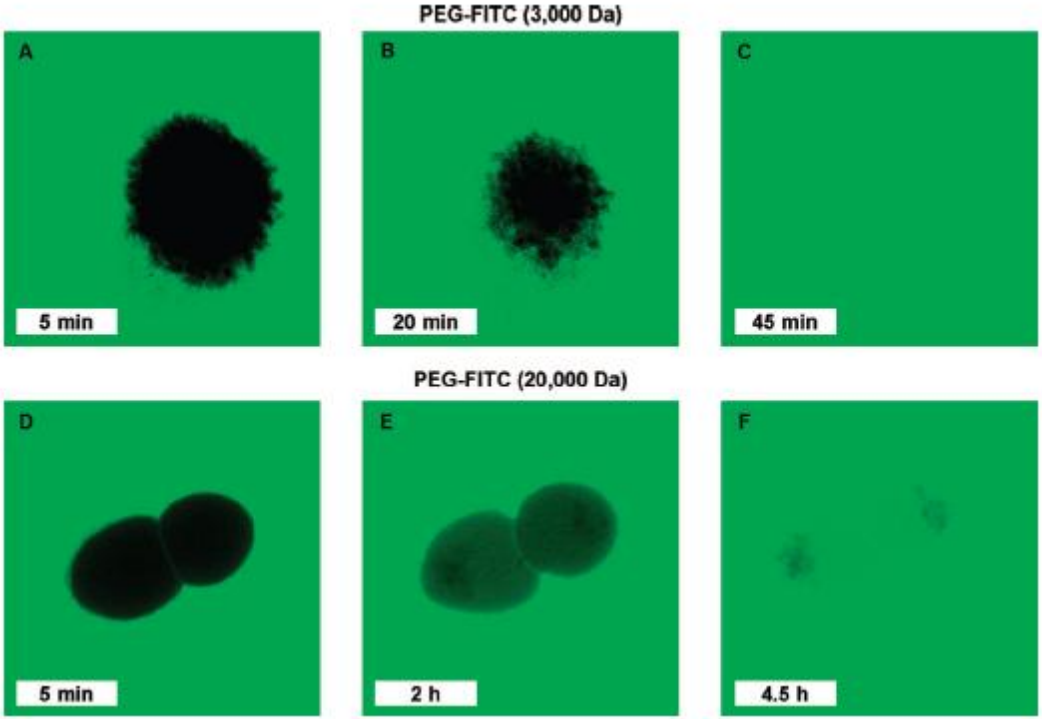

Fig. 5.2 Intracellular internalization of different molecular mass PEG polymers [(A–C) low- and (D–F) high-molecular mass polymers]. Images of FITC-labeled polymers were taken by a confocal microscope in living cells at 37 °C within different time points after the exposure.

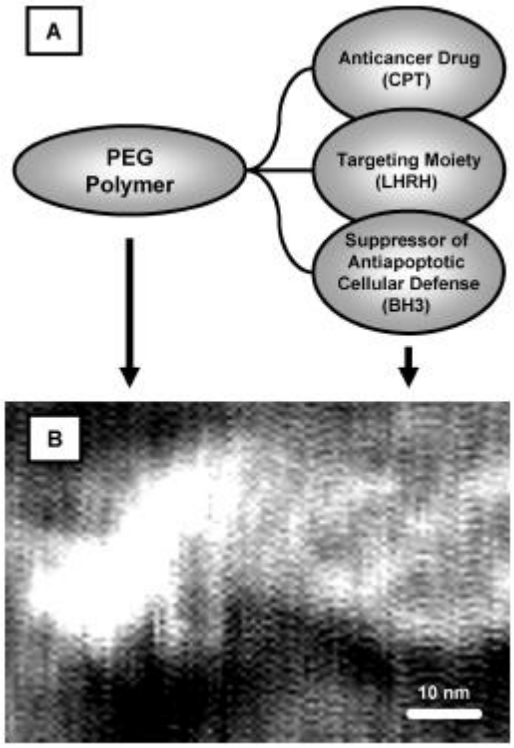

Fig. 5.3. Schematic structure (A) and representative atomic force microscope image (B) of PEG conjugates on a mica surface in tapping mode.

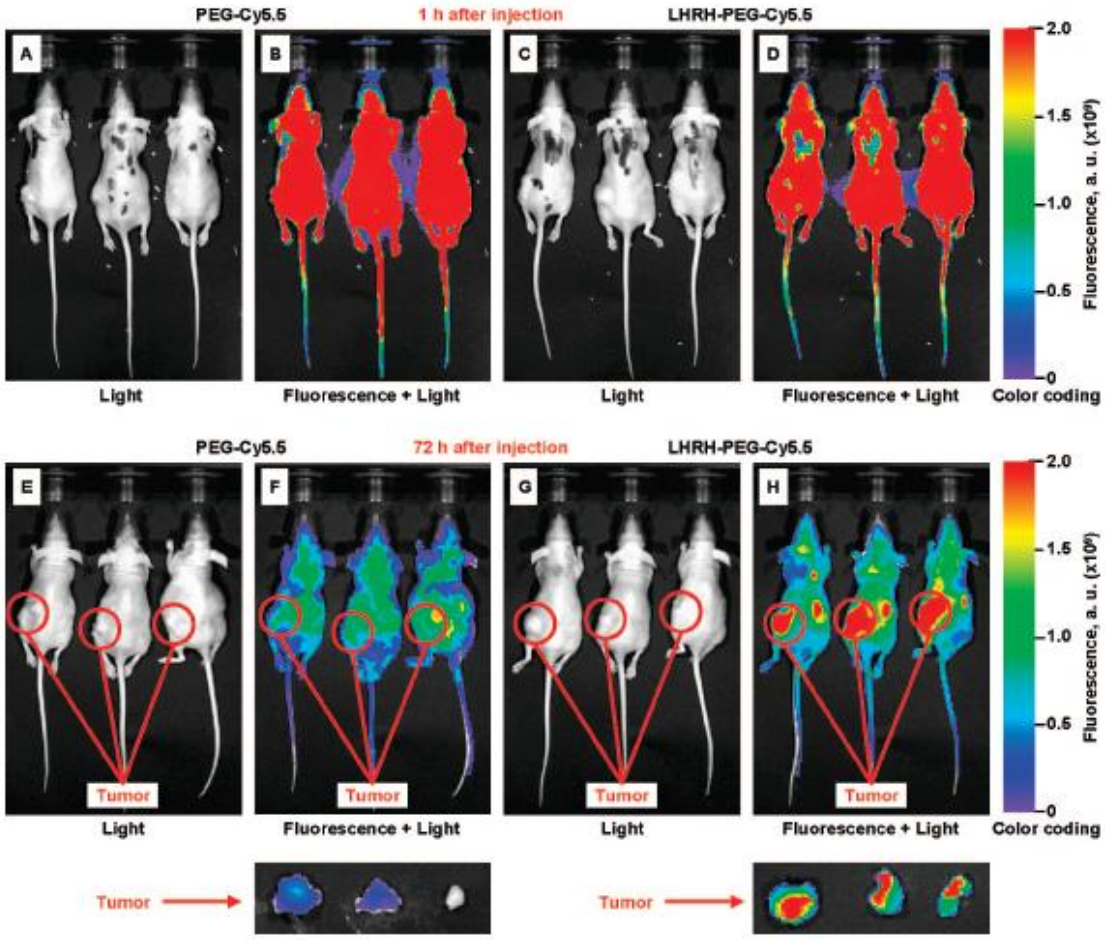

Fig. 5.4. Typical in vivo images of mice bearing xenografts of A2780 human ovarian carcinoma. Mice were injected with PEG (A, B, E, and F) and PEG–LHRH (C, D, G, and H) polymers labeled with near-infrared fluorophore cyanine dye (Cy5.5). Images were taken using the IVIS Imaging System (Xenogen) in anesthetized animals 1 and 72 h after injection of polymer. Tumors were excised 72 h after the treatment and imaged. Visible light (A, C, E, and G) and fluorescent images were overlaid to obtain composite images (B, D, F, and H).

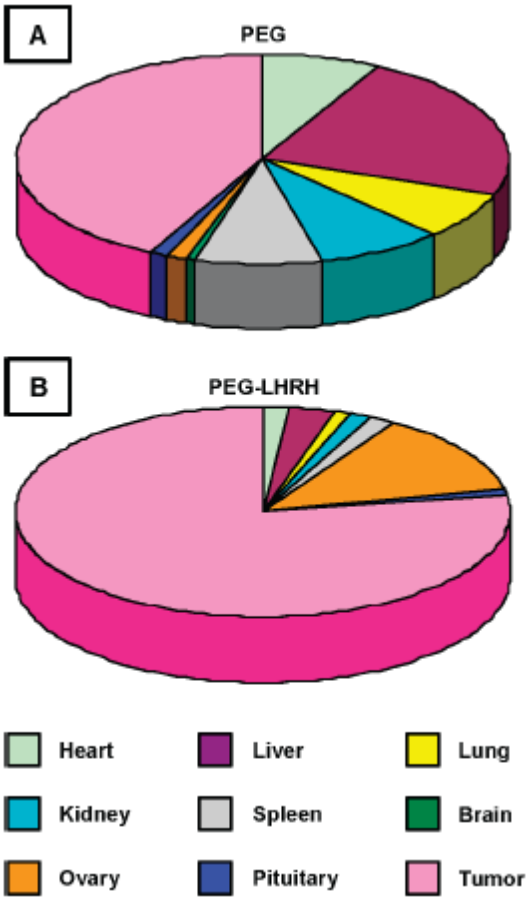

Fig. 5.5. Distribution of tritium-labeled PEG (A) and PEG–LHRH conjugate (B) in different tissues of mice bearing xenografts of A2780 human ovarian carcinoma. Radioactivity in each tissue is expressed as a percentage of the total.

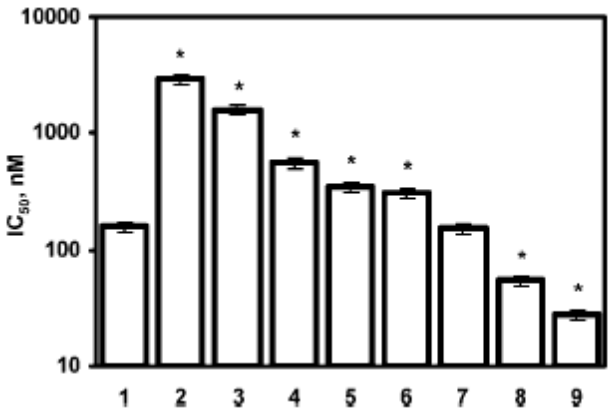

Fig. 5.6. Cytotoxicity of CPT (1), 1×CPT–PEG (2), 2×CPT–PEG (3), 1×CPT–PEG–1×LHRH (4), 1×CPT– PEG–1×BH3 (5), 2×CPT–PEG–2×LHRH (6), 2×CPT– PEG–2×BH3 (7), 1×CPT–PEG–1×BH3–1×LHRH (8), and 2×CPT–PEG–2×BH3–2×LHRH (9) in A2780 human ovarian carcinoma cells. Cells were incubated for 48 h with 45 different equivalent CPT concentrations. Means (SD are shown. An asterisk indicates that $P < 0.05$ when compared with the CPT value.

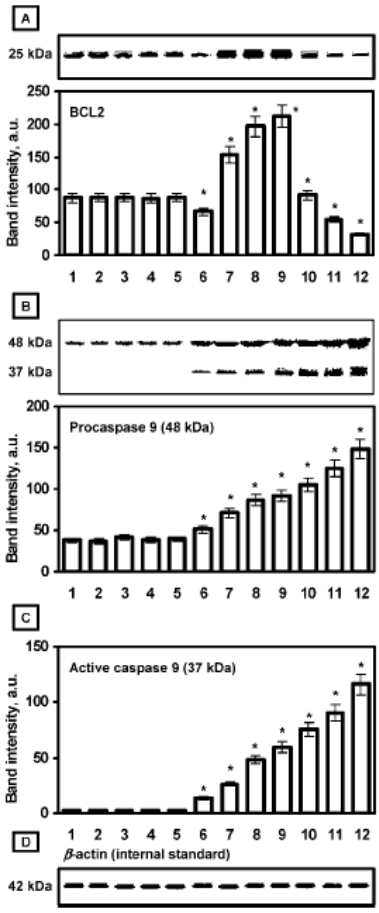

Fig. 5.7. Typical images of Western blots of BCL2 (A), caspase 9 (B and C), and β-actin (D, internal standard) proteins and densitometric analysis of bands in tumor tissues isolated from mice bearing xenografts of A2780human ovarian carcinoma. Mice were treated with the indicated formulations. Band intensities of BCL2 and caspase 9 proteins are expressed as the percentage of the β -actin band intensity, which was set to 100%. Means (SD are shown. An asterisk indicates $P < 0.05$ when compared with control: (1) control (saline), (2) LHRH, (3) BH3, (4) PEG, (5) PEG–1×LHRH, (6) PEG–1×BH3, (7) CPT, (8) 1×CPT–PEG, (9) 1×CPT–PEG–1×LHRH, (10) 1×CPT–PEG–1×BH3, (11) 1×CPT–PEG–1×BH3–1×LHRH, (12) 2×CPT–PEG–2×BH3–2×LHRH.

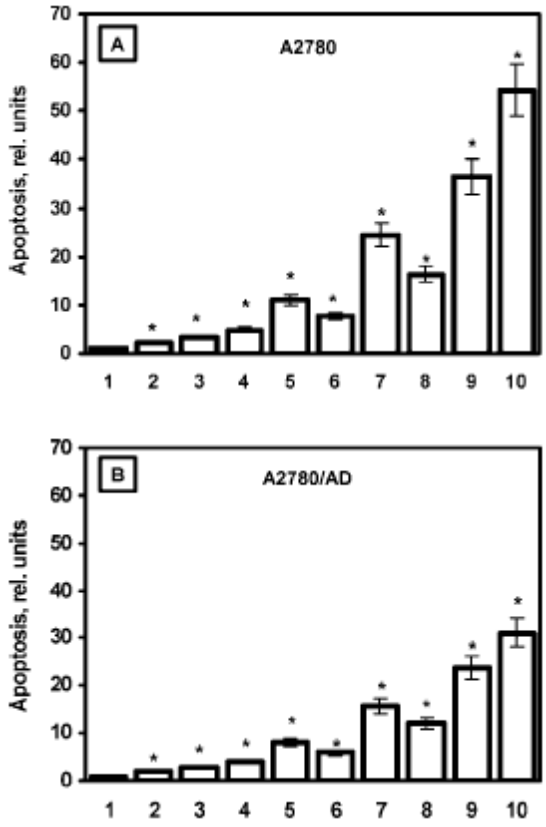

Fig. 5.8. Apoptosis induction in tumor tissues isolated from mice bearing xenografts of sensitive A2780 (A) and multidrug resistant A2780/AD (B) human ovarian carcinoma cells. Mice were treated with indicated formulations. The enrichment of histone-associated DNA fragments (mono- and oligonucleotides) in control mice was set to unit 1, and the degree of apoptosis was expressed in relative units. Mice were treated with the indicated formulations. Means ± SD are shown. An asterisk indicates $P < 0.05$ when compared with control: (1) control (saline), (2) CPT, (3) 1×CPT–PEG, (4) 2×CPT–PEG, (5) 1×CPT–PEG–1×LHRH, (6) 1×CPT–PEG–1×BH3, (7) 2×CPT–PEG–2×LHRH, (8) 2×CPT–PEG–2×BH3, (9) 1×CPT–PEG–1×BH3– 1×LHRH, and (10) 2×CPT–PEG–2×BH3–2×LHRH.

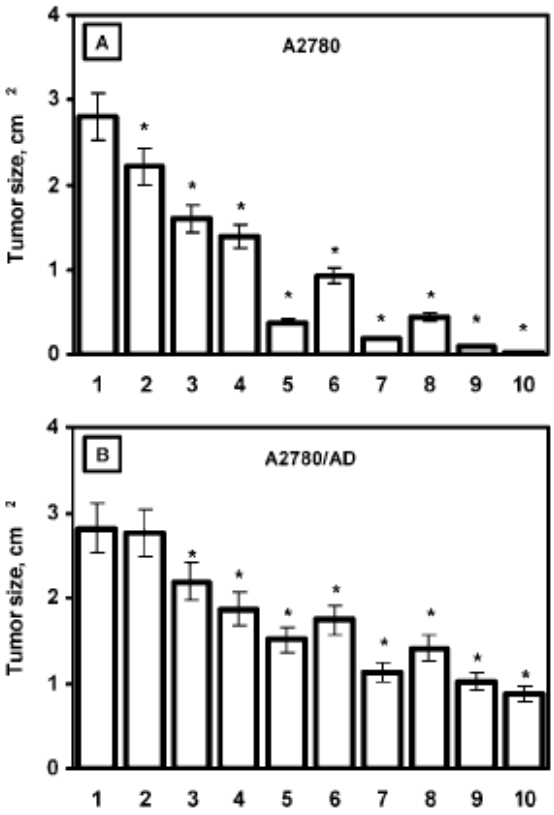

Fig. 5.9. Tumor size in mice bearing xenografts of sensitive A2780 (A) and multidrug resistant A2780/AD (B) human ovarian carcinoma cells. Mice were treated with the indicated formulations. Tumor size was measured 96 h after the treatment. Means ± SD are shown. An asterisk indicates $P < 0.05$ when compared with control: (1) control (saline), (2) CPT, (3) 1×CPT– PEG, (4) 2×CPT–PEG, (5) 1×CPT–PEG–1×LHRH, (6)1×CPT–PEG–1×BH3, (7) 2×CPT–PEG–2×LHRH, (8) 2×CPT–PEG–2×BH3, (9) 1×CPT–PEG–1×BH3– 1×LHRH, and (10) 2×CPT–PEG–2×BH3–2×LHRH.

6. To evaluate pharmacokinetics and the efficiency of proposed targeted proapoptotic delivery system on the treatment of mice bearing human malignant ascites.

6.1. Introduction

Ovarian cancer is the leading cause of human death from gynecologic malignances (1). The main problem in the early diagnostics of ovarian carcinoma is that this disease causes minimal and nonspecific symptoms during its initial stage. Consequently, most patients with ovarian carcinomas are diagnosed in the advanced stage of the disease which leads to the unfavorable prognosis. The main treatment choice for patients with ovarian cancer is aggressive “debulking” surgery followed by chemotherapy. However, intrinsic and acquired drug resistance of ovarian cancer substantially limits the efficiency of chemotherapeutic treatment (2). Several recent studies revealed that the major role in such resistance is playing by antiapoptotic defense of cancer cells that depends on the expression of BCL2 and BAX proteins (3-6). It was shown that overexpression of antiapoptotic BCL2 protein and a decrease in the expression of proapoptotic BAX proteins in cancer cells are the indicators of poor prognosis in chemotherapy of ovarian carcinoma. In order to overcome such resistance, high doses of chemotherapy are generally used (7, 8). However, the increase in the dose of chemotherapeutic drugs usually leads to the induction of high adverse side effects of the treatment on healthy organs. In order to suppress the resistance of cancer cells to chemotherapy and prevent adverse side effects of chemotherapy, we recently proposed a multifunctional multicomponent Polymer-Peptide-Drug Conjugates (PPDC) that include one or several copies of anticancer drug(s) conjugated to Poly(Ethylene Glycol) (PEG) carrier via

branched spacers together with one or several copies of Luteinizing Hormone-Releasing Hormone (LHRH) decapeptide and BCL2 Homology 3 domain (BH3) peptide for tumor targeting and suppression of cellular antiapoptotic defense, respectively (9). The proposed PPDC were tested *in vivo* and *in vitro* using established human ovarian and lung cancer cells and showed promising results in these models (10-16). However, these cell lines were cultured for a relatively long period of time after the isolation from patient tumor tissues. Consequently, these cells probably do not exactly reflect real conditions in patients' tumor cells. Therefore, one of the aims of the present study is to test proposed PPDC *in vivo* and *in vitro* using cancer models derived from the cells isolated from tumors obtained from patients with ovarian carcinoma.

Invasion and metastases of cancer cells along with the development of resistance to cancer therapies are the main causes of morbidity and mortality from ovarian cancer. Malignant ascites (the accumulation of fluid and cancer cells in the abdominal cavity caused by metastasising of cancer cells into the abdominal cavity) represent the most abundant adverse effects of advanced ovarian carcinoma. Generally, patients suffering from malignant ascites have a poor prognosis (17). Consequently, the development of novel therapeutics for treatment of malignant ascites is an important task for an effective chemotherapy of advanced ovarian cancer. In the present study, for the first time, we plan to answer four questions: (1) Are the malignant ascites more resistant to chemotherapy when compared to primary ovarian tumors and whether the mechanisms of drug resistance are similar in malignant ascites and primary tumor isolates? (2) Can LHRH peptide be used for effective targeting of cancer cells in malignant ascites as well as primary tumor cells? (3) Will the proposed PPDC be equally effective in treatment of

tumors derived from both primary tumor isolates and malignant ascites? (4) Are the mechanisms of apoptotic cell death induction by PPDC similar in both cases? The present paper is aimed at answering these questions and investigating novel multicomponent polymer-peptide-drug conjugates for treatment of primary and metastatic cancer.

6.2. Materials and Methods.

Materials and Synthesis of Prodrug-Conjugates.

Camptothecin (CPT), N,N-diisopropylethyl-amine, and 4-(methylamino)pyridine (DMAP) were obtained from Sigma Chemical Co. (Atlanta, GA); bis (2-carboxyethyl) PEG polymer and fluorescein isothiocyanate (FITC) were obtained from Fluka (Allentown, PA). LHRH analog, Lys6–des-Gly10–Pro9-ethylamide (Gln–His–Trp–Ser–Tyr–DLys–Leu–Arg–Pro–NH–Et) and BH3 (Ac-Met-Gly-Gln-Val-Gly-Arg-Gln-Leu-Ala-Ile-Ile-Asn-Arg-Arg-Tyr-Cys-NH2) peptides were synthesized according to our design (11-13) by American Peptide (Sunnyvale, CA). The sequence of native LHRH peptide, which is similar in human, mouse, and rat, was modified to provide a reactive amino group only on the side chain of a lysine residue, which replaced Gly at position 6 to yield the superactive, degradation-resistant-Lys-6-des-Gly-10-Pro-9-ethylamine LHRH analog (18). The system contains bis-poly(ethylene glycol) (PEG) polymer as a carrier, one or two copies of LHRH peptide as a targeting moiety, one or two copies of Camptothecin (CPT) as an anticancer drug and one or two copies of BH3 peptide as a suppressor of cellular antiapoptotic defense. The conjugates were synthesized as previously described (10, 14).

6.2.1. Cancer Cells.

Discarded anonymous pathological materials obtained from the Cancer Institute of New Jersey that do not allow identifying patient information were used to isolate cancer cells from tissues obtained from patients with ovarian carcinoma. Cancer cells were isolated from primary tumor tissue according to the standard protocol(19). The fluid with cancer cells was obtained from the peritoneum area of the patients with ovarian cancer. The samples were centrifuged for 20 minutes at 2000 g; the supernatant was discarded and cell pellets were consequently resuspended. The resuspended cells were cultured in RPMI media (Sigma, St. Louis, Mo) supplemented with fetal bovine serum (Fisher Chemicals, Fairlawn, NJ), 2.5 μg/ml insulin and 1.2 ml/100 ml penicillin-streptomycin (Sigma, St. Louis, Mo). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air. All experiments were performed on cells in the exponential growth phase.

6.2.2 Animal Model and In Vivo Antitumor Activity.

An animal model of human ovarian carcinoma xenografts was created as previously described (10, 11, 13, 20-22). Briefly, human ascitic cells ($2x10^6$) were subcutaneously transplanted into the flanks of female athymic nu/nu mice. When the tumors reached a size of about 0.3 cm^3 (15-20 days after transplantation), mice were treated intraperitoneally with saline (control), CPT, CPT-PEG-BH3-LHRH and 2xCPT-PEG-2xBH3-2xLHRH. The doses of CPT in formulations (10 mg/kg) were corresponded to the maximum tolerated dose of this drug. The maximum tolerated dose of CPT was estimated in separate experiments based on the animal weight change after the injection of increasing doses of CPT as previously described (10, 11, 13, 14). The animals were treated 6 times within three weeks and tumor size was measured by a caliper. Animal

weight was evaluated every day during the treatment period. Changes in tumor size were used as an overall marker for antitumor activity.

6.2.3. Cellular Internalization of Polymers.

To analyze cellular internalization of conjugates, PEG polymers were labeled by Fluorescein Isothiocyanate (FITC) as previously described[10, 14]. Cellular internalization of FITC-labeled PEG polymers was studied by confocal microscopy in living cells at 37° C within 4.5 h.

6.2.4. Cytotoxicity Assay.

The cellular cytotoxicity of CPT and CPT conjugates was assessed using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described(12, 23-26).

6.2.5. Gene Expression.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was used for the analysis of expression of genes encoding LHRH receptors (LHRHR), caspases 3 (CASP3) and 9 (CASP9), MDR1 and β_2-microglobulin (β_2-m). The pairs of primers used to amplify each type of gene were: 5'-CCCATCATTGCAATAGCAGG-3' and 5'-GTTCAAACTTCTGCTCCTGA-3' (MDR1); 5'-GGATTGTGGCCTTCTTTGAG-3' and 5'-CCAAACTGAGCAGAGTCTTC-3' (BCL2); 5'-TGACTGCCAAGAAAATGGTG-3' and 5'-CAGCTGGTCCCATTGAAGAT-3' (Caspase 9; 5'-ACCCCCACTGAAAAAGATGA-3' and 5'-ATCTTCAAACCTCCATGATG-3' (β_2-m). For the analysis of gene expression total cellular RNA was isolated using RNeasy kit (Qiagen, Valencia, CA) and QIA shredder micro spin homogenizer (Qiagen, Valencia, CA). First-strand cDNA was synthesized by

Ready-To-Go You- Prime First-Strand Beads (Amersham Pharmacia Biotech., Piscataway, NJ) according to manufacturer instructions with 2 mg of total cellular RNA and 100 ng of random hexadeoxynucleotide primer (Amersham Pharmacia Biotech., Piscataway, NJ). β_2-microglobulin (β_2-m) was used as an internal standard. PCR was carried out using a Thermocycler (Parkin Elmer, Waltham, MA) with the diluted first-strand reaction mixture, 1 unit of Taq DNA Polymerase (Promega Corporation, Madison, NJ), and 0.5 mM of specific primers in a final volume of 50 ml. The PCR regimen used was: 94^0C/4 min, 55^0C/1 min, 72^0C/1 min for 1 cycle; 94^0C/1min, 55^0C/50 sec, 72^0C/1 min for 28 cycles, 60^0C for 10 min. Gel electrophoresis was used for the separation of PCR products by submarine electrophoresis using agarose gel (Lonza Rockland, ME) at 4% w/v concentration in 0.5 3 TBE buffer: 0.0445 M Tris/Borate, 0.001 M EDTA, pH 8.3 (Research Organics, Cleveland, OH). The gels were digitally scanned and gene expression was calculated as a percentage of the internal standard (β_2-m).

6.2.6. Protein Expression.

The expression of Caspase 9 and BCL2 proteins was assessed by Western immunoblotting analysis and processed using scanning densitometry to quantify the expressed protein as previously described(22, 23). To this end, harvested cells were lysed in RIPA buffer (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) using a needle and syringe. Following incubation on ice for 45 minutes, the cells were centrifuged at 10,000 g for 10 min. Protein content in the supernatant was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) and 50µg of protein was run on a 15% sodium dodecyl sulphate (SDS) polyacrylamide gel immersed in Tris/Glycine/SDS buffer (BioRad, Hercules, CA) for 90 minutes at 70V. Proteins were transferred to an Immobilon-P

nitrocellulose membrane (Millipore, Bedford, MA) in a Tris/Glycine buffer (BioRad, Hercules, CA) for 90 minutes at 100V. The membrane was blocked in non-fat milk for 30 minutes at room temperature on a rotating shaker to prevent non-specific binding, washed and incubated overnight with anti-BCL2 rabbit primary antibody (1:250 dilution, Stress Gene Biotechnologies, Victoria State, BC, Canada), anti-Caspase 9 rabbit primary antibody (1:500 dilution, Stress Gen Biotechnologies, Victoria State, BC Canada) or anti-β-actin mice primary antibody (1:2000 dilution, Oncogene Research, San Diego, CA) at 4ºC. After further washing, the membrane was immersed in goat anti-rabbit and goat anti-mouse IgG biotinylated antibody (1:3000 dilution and 1:1000 dilution, respectively, BioRad, Hercules, CA) at room temperature for 1.5 h on a rotating shaker. Bands were visualized using an alkaline phosphatase color development reagent (BioRad, Hercules, CA). The bands were digitally photographed and scanned using the Gel Documentation System 920 (NucleoTech, San Mateo, CA). Beta-actin was used as an internal standard to normalize protein expression. Band intensities of BCL2 protein and caspase 9 were expressed as the percentage of the β-actin band intensity, which was be set at 100%. Similarly, the expression of BCL2, Caspase 9, P-gp and MRP2 was also measured in homogenates of tumor samples obtained from experimental animals.

6.2.7. Apoptosis. The apoptosis induction was analyzed by the measurement of the enrichment of histone-associated DNA fragments (mono- and oligo-nucleosomes) in homogenates of the tumor and other organs (liver, kidney, lung, heart and brain) using anti-histone and anti-DNA antibodies by a cell death detection ELISA Plus kit (Roche, Nutley, NJ) as previously described(13, 23, 25).

6.2.8. Pharmacokinetics studies.

An animal model of human ovarian carcinoma xenografts was used as described previously (12, 13). Female athymic nu/nu mice were injected with CPT and 1CPT-PEG-1LHRH-1BH3 conjugate. Animals were sacrificed at different time points, 15, 30, 45, 60, 120 and 180 minutes. The blood and different organs (kidney, liver, heart, lungs and spleen) were collected. Plasma was separated by centrifuging the blood samples for 10 min at 11,000g. Camptothecin was extracted from plasma samples by treating the samples with 400 µl of methanol and 20 µl of 1% phosphoric acid and subsequently analyzed by Reverse phase HPLC. The HPLC conditions used were, 360 nm and 465 nm as the fluorescence detector wavelengths, 1ml/min flow rate and 10-15 µl sample volume. The mobile phase was adjusted as flows: 40 % acetonitrile + 0.1 % trifluoro-acetic acid (TFA) in isocratic mode. The standard was prepared using DMSO and methanol in (3: 250 (v/v) ratio respectively.

6.2.9. Statistical Analysis.

Data obtained were analyzed using descriptive statistics, single factor analysis of variance (ANOVA) and presented as a mean value ± standard deviation (SD) from five independent measurements. We analyzed data sets for significance with Student's/test and considered *P* value of less than 0.05 as statistical significant.

6.3. Results

6.3.1. Tumor-targeted proapoptotic polymer-anticancer drug-peptide conjugates. We constructed a complex multifunctional polymer-peptide-drug conjugates for effective treatment of primary and aggressive metastatic ovarian tumors (Scheme 1, A). The conjugates contain one or two copies of an anticancer drug, targeting moiety, and a

suppressor of antiapoptotic cellular defense. In the present study, we used Camptothecin as an anticancer drug, conjugating one or two CPT molecules to one polymer molecule. The detection of the expression of plasma receptors to LHRH peptide in tissues of a primary tumor and malignant ascites from patients with metastatic advanced ovarian carcinoma showed that these receptors are overexpressed in primary and metastatic (malignant ascites) cancers (Fig. 1, A, B). Moreover, the measurement of LHRH expression in cancerous and normal ovarian tissues obtained from the same patient showed that these receptors are expressed substantially more pronounced in cancerous tissue when compared with normal ovarian tissue. Consequently, we used LHRH peptide as a targeting moiety to direct an entire conjugate/Prodrug specifically to cancer cells and enhance its penetration and intracellular uptake. The fluorescent microscopy analysis of cells incubated with FITC-labeled LHRH-polymer conjugates showed that in fact such conjugate effectively penetrated into cancer cells isolated from tumor tissues obtained from patients with ovarian carcinoma and distributed both in the cytoplasm and nuclei of the cells after 24 h long incubation period (Fig. 1, C). Consistently, based on our previous findings(10-12, 14, 23), in the present study, we used one or two copies of BH3 peptide as a suppressor of cellular antiapoptotic defense and one or two copies of LHRH peptide as a targeting moiety.

6.3.2. Apoptosis induction in vitro.

The ability of different polymer-peptide-drug conjugates to induce apoptosis was studied in experiments using cells isolated from tissues obtained from patients with primary ovarian carcinoma (Fig. 6.2, A). The cells were separately incubated with different drug-peptide-conjugate formulations and expression of two major caspases involved in

apoptosis induction (caspase 9 and 3), as well as the rate of apoptotic cell death were studied (Fig. 6.2, B-C). It was found that incubation of cells isolated from primary tumor with free, non-conjugated CPT led to the activation of caspase 9 and slight, but statistically significant ($P < 0.05$) induction of apoptosis (compare bars 2 and 1 in Fig.6.2, B and C). Conjugation of CPT to PEG polymer slightly increased expression of caspases 9 and 3 and apoptosis (bar 3 in Fig. 6.2, B and C). Further conjugation of CPT-PEG with LHRH to some extent enhanced the ability of CPT to induce apoptotic cell death by activating caspases 9 and 3 (bar 4 in Fig.6.2, B and C). An addition of proapoptotic BH3 peptide to the non-targeted polymer-drug conjugate dramatically (more than 3-times when compared with control) augmented the expression of caspases and apoptosis induction (bar 5 in Fig.6.2, B and C). Targeting of this proapoptotic conjugate to cancer cells by LHRH peptide significantly ($P < 0.05$) increased the expression of caspases 9 and 3 (on 17% and 34%, respectively when compared with non-targeted conjugate, compare bars 6 and 5 in Fig. 2, B) and apoptosis induction (more than 2-times, compare bars 6 and 5 in Fig. 6.2, C). Finally, doubling the number of copies of CPT, LHRH and BH3 peptides substantially enhanced apoptosis-inducing ability of the conjugate. It led to a further significant ($P < 0.05$) increase in the expression of caspases and more than 2-times increase in apoptosis induction (compare bars 7 an 6 in Fig. 6.2, C). As a result, the degree of apoptosis was augmented more than 15 times when compared with control (compare bars 7 and 1 in Fig. 6.2, C).

6.3.3. Activation of cell death signaling pathways and suppression of cellular antiapoptotic defense in vivo.

The ability of developed conjugates to induce a cell death signal and suppress cellular antiapoptotic defense was studied on mice bearing xenografts of cells isolated from human malignant ascites. This tumor model represents a much more aggressive type of cancer when compared with tumor produced by inoculation of cells isolated from primary tumors of patients with ovarian carcinoma. The expression of P53 (a central cell death signal), BCL2 (an antiapoptotic defense), BAX (a major pro-apoptotic protein from the BCL2 family), APAF1 (apoptotic protease activating factor 1), caspase 9 (CASP9, a major apoptosis initiator), caspase 3 (CASP3, a main apoptosis executor) was studied by RT-PCR using β_2-microglobulin (β_2-m) as an internal standard. In addition, a pro-apoptotic BAX/BCL2 ratio was calculated and the cleavage of active form of caspase 9 from pro-caspase 9 was confirmed by Western blotting. The results of this series showed the following. Free, non-conjugated CPT led to the activation of both pro-apoptotic signal (P53, BAX) and antiapoptotic defense (BCL2). As a result, only a moderate activation of APAF1 and caspases were registered (compare bars 2 and 1 in Fig 3, A and B). Conjugation of CPT to tumor-targeted proapoptotic polymer-peptide complex (CTT-PEG-LHRH-BH3) substantially enhanced pro-apoptotic cell death signal and activated caspases more efficiently (bar 3 in Fig. 3, A and B). Doubling the number of active components in the conjugate (2xCPT-PEG-2xLHRH-2xBH3) further enhanced proapoptotic signal and suppressed antiapoptotic defense after a single (bar 4 in Fig. 6.3, A and B) and multiple (bar 5 in Fig. 6.3, A and B) treatments.

6.3.4. Apoptosis induction and adverse side effects in vivo.

Apoptosis induction was analyzed in the tumor and other organs of animals bearing xenografts of human cancer cells isolated from malignant ascites from patients with

ovarian carcinoma (Fig. 6.4). The degree of apoptosis induction in the tumor was proportional to the antitumor effect of tested formulations, while that in healthy organs reflects adverse side effects. It was found that free CPT activates apoptosis not only in the tumor, but also in the heart, lung, liver, spleen, and kidney. This indicates wide unfavorable organ distribution of the free drug and its severe adverse side effects inducing cell death in healthy organs. Incorporation of CPT into the targeted proapoptotic polymer conjugate containing one copy of each active component (CPT, LHRH and BH3) led to significant enhancement of apoptosis induction in the tumor. However, considerable side effects leading to a substantial induction of cell death in healthy organs were still preserved. Including two copies of each CPT, LHRH and BH3 in one polymeric conjugate (2xCPT-PEG-2xLHRH-2xBH3) not only augmented cell death in the tumor but substantially limited adverse side effects of the treatment on healthy organs. Repeating treatment with this conjugate further enhanced cell death induction in the tumor while preventing adverse side effects in normal healthy tissues.

6.3.5. Antitumor effect of the conjugates.

The efficiency of tested formulations to suppress tumor growth was investigated in two aggressive models of human tumor xenografts. In the first series of the experiments, cancer cells were isolated from solid tumor tissues of patients with primary ovarian carcinoma and were subcutaneously injected in the flanks of nude mice. In the second series of the experiments, the cells were isolated from malignant ascites of patients with ovarian carcinoma and were used for creation of subcutaneous mouse model. The size of the tumor was measured every day using a caliper and tumor volume was calculated. It was found that cells isolated from metastatic tumor (malignant ascites) demonstrated

more aggressive tumor growth when compared with cancer cells isolated from primary tumor. In fact, the volume of untreated tumors produced by primary tumor isolates increased within 14 days from 0.5 to 2000 cm^3 (the maximal tumor size allowed for such experiments by the animal protocol approved by the Institutional Animal Care and Use Committee). In contrast, cells isolated from malignant ascites demonstrated more aggressive growth (approximately 1.4 times faster) reaching the same volume of subcutaneous tumor within 10 days. Consequently, proposed polymer-peptide-drug conjugates were substantially more effective in primary tumor isolates model when compared with model developed using malignant ascites. At the same time, the tendency in antitumor activity of tested formulations was preserved. Free non-conjugated CPT demonstrated lowest activity; PEGylated CPT was more efficient than free CPT; and tumor-targeted proapoptotic peptide-drug conjugate (CPT-PEG-LHRH-BH3) was more effective when compared with non-targeted conjugate. A doubling in the number of copies of all active components of delivery system led to significant increase in antitumor efficiency of the proposed conjugate. It should be stressed, however, that even conjugates with 2 copies of each active component were substantially less effective in tumor models created using malignant ascites when compared with that produced by primary tumor isolates. In fact, while 2xCPT-PEG-2xLHRH-2xBH3 conjugate led to a substantial tumor regression in primary tumor model, a similar conjugate just stabilized the tumor volume and prevented its growth when the tumor was created using cells isolated from human malignant ascites.

6.3.6. Pharmacokinetic studies.

Plasma concentrations and the area under the curve for the targeted DDS consisting of PEG bound CPT are approximately 100-fold higher than those of unconjugated CPT or CPT alone, injected intraperitoneally at an equivalent dose **(Fig 6.6)**. The plasma half-life of DDS ranges from 17 -20 h and is significantly greater than that of CPT alone (45 min).

6.4. Discussion

In the previous chapter we proposed a complex multifunctional tumor-targeted proapoptotic Polymer-Peptide-Drug Conjugates (PPDC)(10). Such PPDC contains a nanocarrier and one or several copies of anticancer drug, tumor-targeting peptide and proapoptotic peptide. A nanocarrier combines all components together providing an aqueous solubility of an entire complex. Several nanocarriers have been tested for such conjugates including linear polymers with branched spacers, liposomes, different modifications of dendrimers, etc (13-15, 27). An anticancer drug acts as a cell death inducer while a proapoptotic peptide acts as a suppressor of cellular antiapoptotic defense. By suppressing such defense, a proapoptotic peptide enhances cell death induction by an anticancer drug. Tumor targeting moiety plays a dual role in this PPDC. First, it directs an entire complex specifically to tumor cells thereby preventing the accumulation of such high toxic complex in normal tissues and consequently avoiding its severe adverse side effects on healthy organs. Secondly, targeting moiety enhances cellular uptake by cancer cells that expressed plasma membrane receptors specific to targeting peptide. We proposed and tested LHRH peptide as tumor targeting moiety(10, 12-16, 23, 27). In the present study, we used a linear PEG polymer as a carrier, one or two copies of Camptothecin, BH3 peptide and LHRH peptide as a cell death inducer, tumor targeting moiety and suppressor of cellular antiapoptotic defense, respectively.

Preliminary data showed that further increase in the number of copies of BH3 peptide or CPT substantially decreases the solubility of an entire complex limiting its bioavailability and toxicity. Previous detailed investigation of such conjugates showed that an entire complex acts as a prodrug, inactive and stable during its journey in the systemic circulation. This prodrug converts into a combination of active anticancer drugs and suppressors of cellular antiapoptotic defense after their cleavage from the conjugate via the enzymatic degradation inside cancer cells (14, 28).

Previously, we tested such a system *in vitro* and *in vivo* against established A2780 sensitive and A2780/AD multidrug resistant human ovarian cancer cell lines and their mice xenografts (10, 12). In the present investigation, we used ovarian cancer cells isolated from primary tumor and metastatic malignant ascites from patients with ovarian carcinoma. In contrast to well-established cancer cells which were passed many times in standard *in vitro* conditions losing their cancerogenous activity, these cells were processed only once after isolation in order to obtain a sufficient number of cancer cells to carry out *in vitro* and *in vivo* experiments. Therefore, these cells can be considered as those almost identical to patients' tumor or ascitic cells. In addition, such cells provide considerable variations in the expression of studied genes and proteins, ability to grow as xenografts of solid tumor in mice and resistance to chemotherapy giving a more reliable experimental model of clinical ovarian cancer and its intraperitoneal metastases. Moreover, cells isolated from patients' malignant ascites demonstrated substantially higher success rate in establishing of mice tumor xenografts and faster tumor growth. All cells isolated from primary tumors and malignant ascites overexpressed plasma membrane receptors for targeting LHRH peptide and successfully internalized our PPDC

containing both one and two molecules of each active component conjugated to one molecule of a carrier peptide.

Both proposed variants of PPDC (CPT-PEG-LHRH-BH3 and 2xCPT-PEG-2xLHRH-2xBH3) demonstrated similar mechanisms of cell death induction by apoptosis in primary tumor isolates and cells from malignant ascites. These mechanisms included activation of central cell death signal by the anticancer drug. At the same time, BH3 peptide suppressed the activity of cellular antiapoptotic defense by interfering in the activity of its major player – BCL2 protein. It is known that the suppression of BCL2 leads to the leakage of cytochrome c from mitochondria and the formation of "apoptosome" – a combination of APAF1, procaspase 9 and cytochrome c (29, 30). The activation of apoptosome by the cytochrome *c* cleaves procaspase into active caspase, which in turn activates a cascade of caspase-executors of apoptosis (31). The present study showed that all these steps are involved in apoptosis induction by all tested PPDC in primary tumor isolates and malignant ascites. However, the degree of activation of cell death signal, caspase-dependent signaling pathways of apoptosis and ultimately antitumor effect of tested PPDC were significantly different. First, the antitumor activity of peptide-drug conjugates increased with doubling of number of copies of each active component conjugated to one molecule of the nanocarrier. Secondly, the efficiency of the suppression of tumor growth by the same PPDC was significantly less pronounced in cells isolated from malignant ascites when compared with those originated from primary tumors. One possible explanation of this phenomenon is the overexpression of MDR1 gene encoding P-glycoprotein in cancer cells isolated from malignant ascites (Fig.6.1C). In contrast, cells isolated from primary tumor tissues do not demonstrate overexpression

of MDR1 gene (Fig6.1B). It is well known that expression of P-glycoprotein increases the resistance of cancer cells often leading to multidrug resistance (32, 33).

In conclusion, the present study clearly showed differences between cancer cells isolated from primary solid tumor and metastatic malignant ascites from patients with ovarian carcinoma. First, the cells isolated from malignant ascites demonstrated higher tumorogeneric activity in mice bearing xenografts of human ovarian carcinoma. Secondly, malignant ascites were found to be more resistant to chemotherapy even if such chemotherapy was accompanied by the suppression of cellular antiapoptotic defense. Therefore, the proposed PPDC should be further modified in order to suppress multidrug resistance in metastatic cancer. Nevertheless, the proposed multicomponent polymer-peptide-drug conjugates demonstrated significantly higher antitumor activity in both types of studied tumors when compared with free drug and polymer-drug conjugate.

6.5. References

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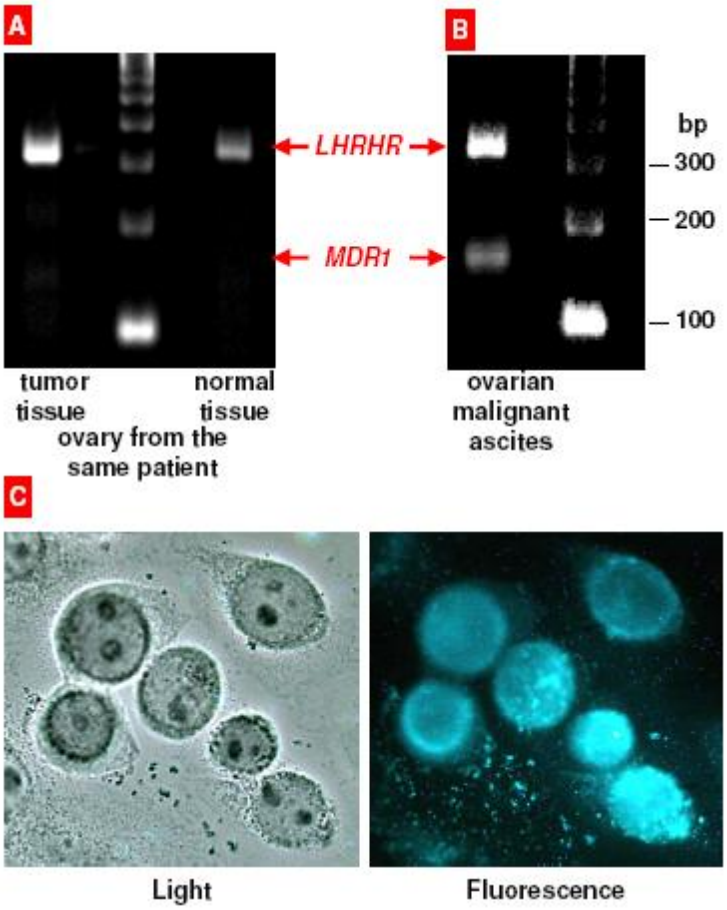

Fig. 6.1. Typical images of gel electrophoresis of RT-PCR products of gene encoding LHRH receptors (*LHRHR*) and Pglycoprotein (MDR1) in tissues obtained from patients with primary ovarian tumor (A) and intraperitoneal metastases (malignant ascites, B); typical images of primary tumor isolates incubated 24 h with FITC-labeled LHRH-PEG polymerconjugates (C).

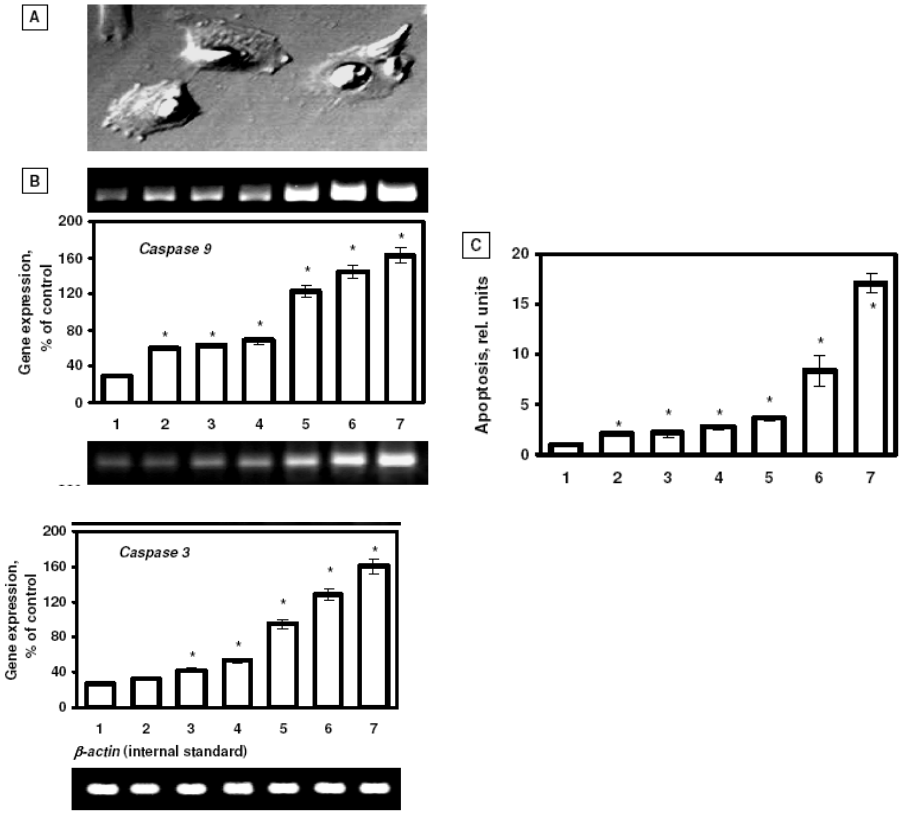

Fig.6.2. Gene expression and apoptosis induction in primary tumor isolates. Typical microscopy image of cancer cells isolated from primary tumor tissue samples obtained from patients with ovarian carcinoma (A). Typical images of gel electrophoresis of the RT-PCR products and quantitative analysis of expression of *Caspases 9 and 3* genes (B) and apoptosis induction (C) in primary tumor isolates treated with the indicated formulations. Band intensities of studiedgenes are expressed as the percentage of the b-*actin* (internal standard) band intensity, which was set to 100%. The enrichment of histone-associated DNA fragments (mono and oligonucleotides) in control (1) was set to 1 unit, and the degree of apoptosis was expressed in relative units. 1 – Untreated Control; 2 – CPT; 3 – CPT-PEG; 4 – CPT-PEGLHRH; 5 – CPT-PEG-BH3; 6 – CPT-PEG-LHRH-BH3; 7 – 2xCPT-PEG-2xLHRH-2xBH3. Means ± SD are shown. * $P < 0.05$ when compared with control.

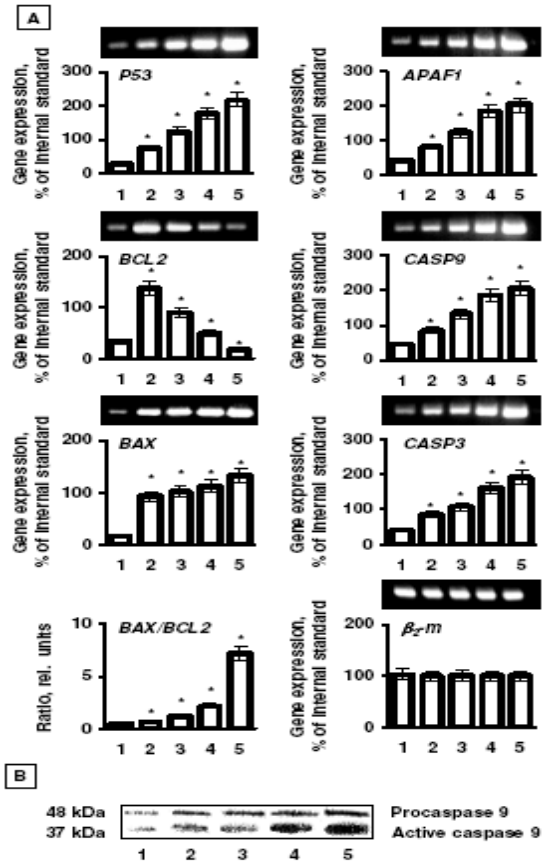

Fig.6.3. Gene and protein expression in tumor samples from mice bearing xenografts of cancer cells isolated from malignant ascites obtained from patients with metastatic ovarian carcinoma. Mice were treated with the indicated formulations. Typical images of gel electrophoresis of the RT-PCR products and quantitative analysis of gene expression (A). Typical image of Western blot (B). Band intensities of studied genes are expressed as the percentage of the b2-*microglobulin* (b2-*m*, internal standard) band intensity, which was set to 100%. The *BAX/BCL2* gene expression ratio is expressed in relative units. 1 – Untreated control; 2 – CPT (after a single treatment); 3 – CPT-PEGLHRH-BH3 (after a single treatment); 4 – 2xCPT-PEG-2xLHRH-2xBH3 (after a single treatment); 5- 2xCPT-PEG- 2xLHRH-2xBH3 (after 6 injections within 3 weeks). Means ± SD are shown. * $P < 0.05$ when compared with control.

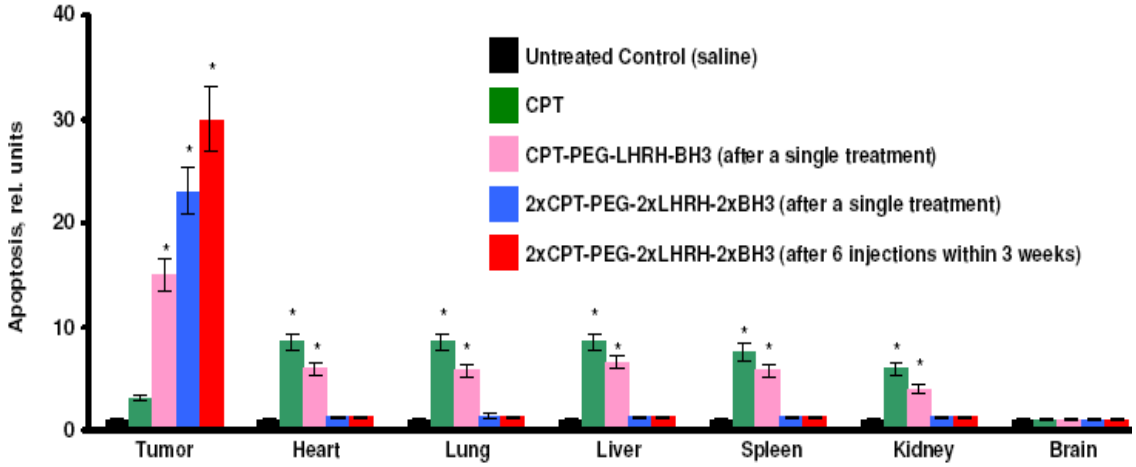

Fig.6.4. Apoptosis induction in tumor samples from mice bearing xenografts of cancer cells isolated from malignant ascites obtained from patients with metastatic ovarian carcinoma. Mice were treated with the indicated formulations. The enrichment of histone-associated DNA fragments (mono and oligonucleotides) in control (1) was set to 1 unit, and the degree of apoptosis was expressed in relative units. Means ± SD are shown. * $P < 0.05$ when compared with control.

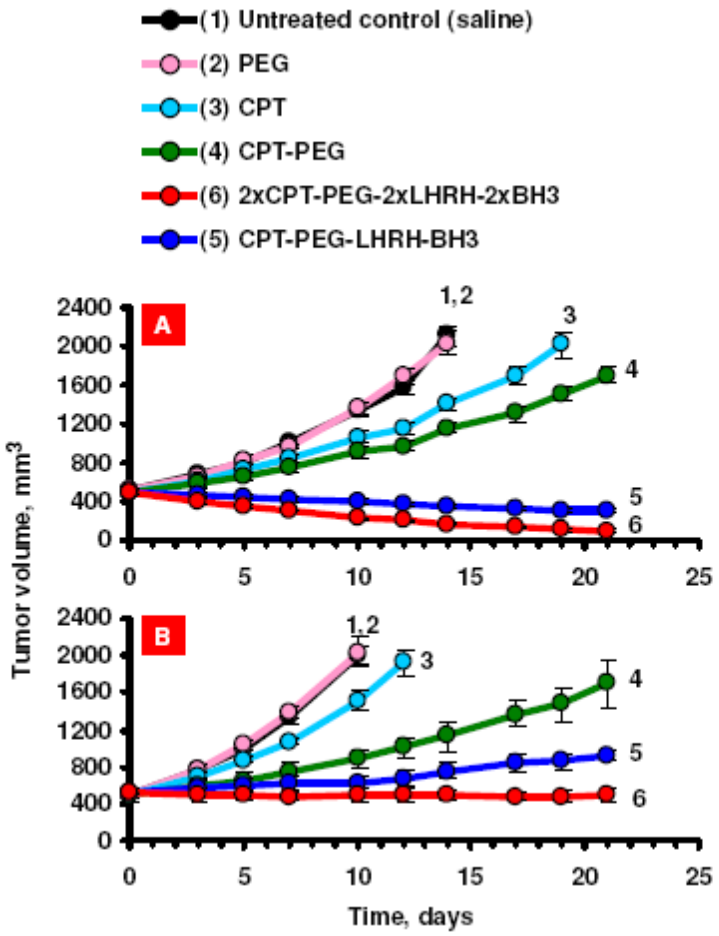

Fig.6.5. Tumor volume in mice bearing xenografts of cancer cells isolated from primary tumor (A) or malignant ascites (B) obtained from patients with primary and metastatic ovarian carcinoma. Mice were treated with the indicated formulations. Means ± SD are shown. * $P < 0.05$ when compared with control.

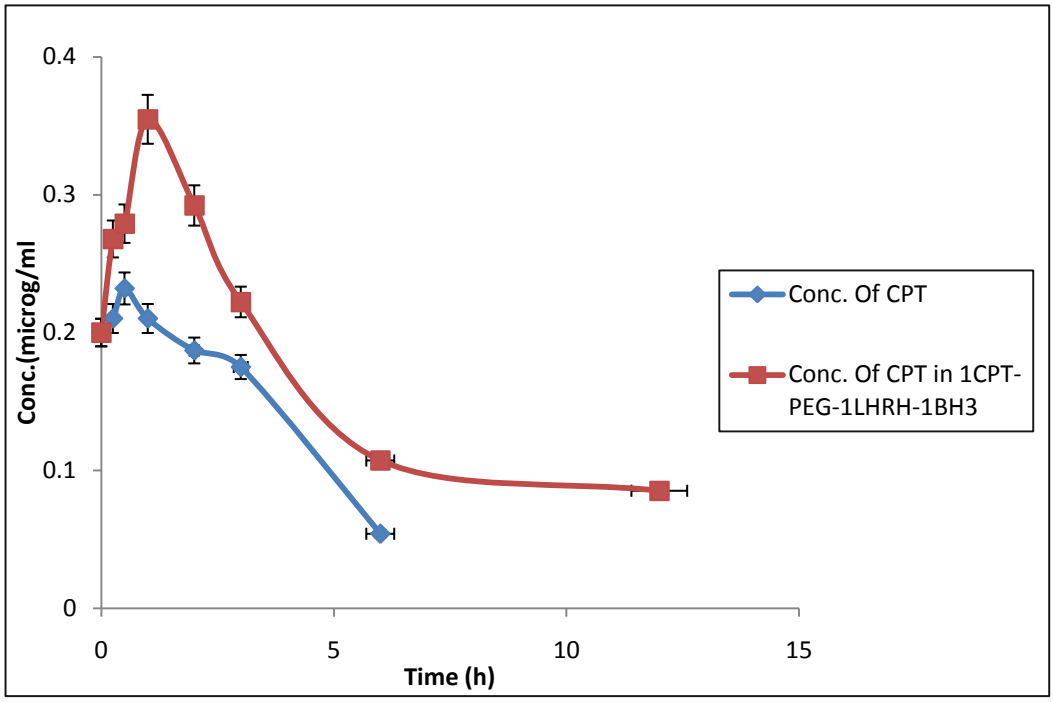

Fig. 6.6. Plasma profile of CPT (standard) and 1CPT-PEG-LHRH-BH3 estimated in ascetic tumor bearing nude mice after intraperitoneal administration.

Formulation	Cmax (µg/ml)	Tmax (min)	AUC (min.µg/ml)	MRT (min)
CPT solution	0.212	45	3.739	37.6
CPT-PEG-LHRH-BH3 conjugate	0.8742	55	20.5703	144.79

Table 6.1 Pharmacokinetic parameters recorded for CPT and for CPT in CPT-PEG-LHRH-BH3 conjugate in plasma after i.p. administration to mice bearing human ovarian malignant ascites.

7. Conclusions.

The studies in this dissertation addressed the fundamental issue existing in the therapeutics of cancer today. Chemotherapeutic agents, effective, however fail to avoid the onset of adverse effects on healthy tissues when administered in higher doses. To counteract this disadvantage, this project aimed at design of multicomponent drug delivery system consisting of multiple copies of anticancer drug and targeting moieties, which would overcome these side-effects while assuring the cell death of tumor cells.

The results of first set of experiments showed that upto three copies of anticancer drug, CPT and targeting agent, LHRH can be successfully delivered to human ovarian cancer cells. Our synthetic methodology allowed us to synthesize the polymeric DDS consisting of one, two and three copies of CPT and LHRH each respectively.

The second set of studies were aimed at the design of a DDS which not only prevent the side effects on healthy tissues but also suppress the cellular antiapoptotic defense cancer cells develop over the period of time. The results showed that the DDS consisting of 2 copies of CPT and 2 copies of LHRH and BH3 (suppressor of cellular antiapoptotic defense) was the most effective when compared with the drug alone and the system containing no targeting moiety or just LHRH as targeting moiety.

The final set of experiments were designed to evaluate this DDS mentioned above in the treatment of malignant form of ovarian cancer, thus to confirm the usefulness of the DDS in the treatment of different stages of cancer.

Summarizing the results of experiments in this dissertation, one can conclude that the use of multicomponent polymeric drug delivery system consisting of multiple copies of CPT, LHRH and BH3 substantially limits the side effects on healthy tissues while ensuring cell

death. Therefore, the proposed system has potential in the treatment of all the stages of cancer.

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