TARGETED DELIVERY OF DOXORUBICIN CONJUGATED TO FOLIC ACID AND VITAMIN E D-α-TOCOPHERYL POLYETHYLENE GLYCOL SUCCINATE (TPGS)

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SUMMARY

Targeted prodrug delivery is one of the promising drug delivery systems for cancer treatment. Prodrug may improve the biological distribution and the half-life in the circulation as well as reduce the systemic toxicity and the kidney excretion of the drug. Prodrug is an important strategy to improve the solubility, permeability, stability and provide a means to circumvent the multi-drug resistance (MDR). MDR is caused by the overexpression of MDR transport proteins such as p-glycoproteins (p-gp) in the cell membrane, that efflux the drug by reducing the intracellular drug levels for cancer chemotherapy. Tumors also acquire drug resistance through induction of MDR transport proteins. At present, about 5-7% of the approved drugs worldwide can be classified as prodrugs and approximately 15% of all new drugs approved within 2001 and 2002 were prodrugs. The conjugation of the drug with the polymer is a main strategy to form the polymeric prodrug of the synergistic or additive effect, which occurs with enhanced and simultaneous action of the drug and the polymer in destroying the cancer cells. The rationale for polymer conjugation is to mainly prolong the half-life of therapeutically active agents by increasing their hydrodynamic volume and hence decreasing their excretion rate. Polymer-anticancer drug conjugate has been investigated and some prodrugs have been found successful. Polymers such as N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers, poly(ethylene glycol) and poly(L-glutamic acid) (PGA) have been used often as the carriers for anticancer drugs such as doxorubicin, paclitaxel, camphothecin and gemcitabine. Conjugation of TPGS should be an ideal solution for the drugs that have problems in adsorption, distribution, metabolism and excretion (ADME).

Doxorubicin (DOX) is an effective anticancer agent for cancer treatment, which is hampered by its short plasma half life, low selectivity towards the tumor cells and serious side effects. This

research developed a prodrug strategy to conjugate DOX to d-α-tocopheryl polyethylene glycol succinate (TPGS) and folic acid (FOL) for targeted chemotherapy to enhance the therapeutic effects and reduce the side effects of the drug. We synthesized 2 conjugates, TPGS-DOX and TPGS-DOX-FOL to quantitatively evaluate the advantages of TPGS conjugation and FOL conjugation through passive and active targeting effects. The successful conjugation was confirmed by ^{1}H NMR and FTIR. The DOX content in the conjugates was found to be 13wt% for TPGS-DOX and 6 wt% for TPGS-DOX-FOL. The *in vitro* drug release from the conjugates were found pH dependent, which is in favor of cancer treatment. The *in vitro* cellular uptake and cytotoxicity were evaluated with MCF-7 breast cancer cells. It was found that the cellular uptake of DOX increased 15.2% by TPGS conjugation and further 6.3% by FOL conjugation after 0.5 hour cell culture at 100 μM equivalent DOX concentration at 37°C, The mortality of the MCF-7 cells showed 23.2% increase by TPGS conjugation and further 31.0% increase by targeting effect of FOL after 24 hour cell culture at 100 μM equivalent DOX concentration at 37°C. These advantages were further confirmed by IC50 analysis. Cellular uptake of DOX, TPGS-DOX and TPGS-DOX-FOL conjugates were also visualized by confocal laser scanning microscopy (CLSM). The *in vivo* pharmacokinetics of the conjugates showed prolonged retention time of the DOX in plasma, where they have almost same half-life. The biodistribution data showed that the conjugates lowered the amount of drug accumulated in the heart, thereby reducing the cardiotoxicity, which is said to be the main side effect of the DOX. Also, the gastrointestinal side effect of the drug could be reduced by the TPGS-DOX-FOL conjugate, which has a 6.8- fold and 5.3- fold lesser amount of drug in stomach and intestine respectively.

The TPGS-DOX-FOL prodrug showed greater potential than the TPGS-DOX and DOX for it to become a novel formulation for the delivery of doxorubicin. This can be applied to other drugs as well.

NOMENCLATURE

ACN	Acetonitrile
ADME	Adsorption, Distribution, Metabolism, Excretion
ATP	Adenosine Tri Phosphate
AUC	Area Under the Curve
BD	Biodistribution
CL	Clearance
CLSM	Confocal Laser Scanning Miroscopy
CMC	Critical Micelle Concentration
CyA	Cyclosporine A
DCC	N,N´-dicyclohexylcarbodiimide
DCM	Dichloromethane
DMAP	Dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DOX	Doxorubicin
EPR	Enhanced Permeation and Retention
FBS	Fetal Bovine Serum
FT-IR	Fourier Transform Infrared Spectroscopy
FOL	Folic Acid
FR	Folate Receptor
GI	Gastrointestinal
GPI	Glycosyl phosphatidylinositol
HPLC	High Performance Liquid Chromatography
HPMA	N-(2-hydroxypropyl)-methacrylamide

IC_{50}	Drug concentration at which 50% cells die
MDR	Multi Drug Resistance
MRT	Mean Residence Time
MTD	Maximum Tolerated Dose
NHS	N-hydroxysuccinimide
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
PEI	Poly(ethyleneimine)
PGA	Poly(L-glutamic acid)
P-gp	P-glycoproteins
PHEG	Poly((N-hydroxyethyl)-L-glutamine)
PK	Pharmacokinetics
PLA	Poly(lactic acid)
PLGA	Copoly(lactic acid/glycolic acid)
PVA	Polyvinyl alcohol
PVP	Poly(vinylpyrrolidone)
RME	Receptor Mediated Endocytosis
SA	Succinic Anhydride
SMA	Poly(styrene-co-maleicacid/anhydride)
$t_{1/2}$	Half-life period
TEA	Triethyl amine
THF	Tetrahydrofuran
TPGS	Vitamin E TPGS, d-α-tocopheryl polyethylene glycol 1000 succinate

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CHAPTER 1: INTRODUCTION

1.1 General Background

There has been intensive research on macromolecular 'prodrugs' in the field of drug delivery that refers to modification of the drug's molecular structure such that it makes an inactive form to be administered and then to become active metabolite in the diseased cells. Prodrugs may improve the biological distribution and the half-life in the circulation as well as reduce the systemic toxicity and the kidney excretion of the drug (Cavallaro, Pitarresi et al. 2001; Zhang, Huey Lee et al. 2007). Prodrug is an important strategy to improve the solubility, permeability, stability and provide a means to circumvent the multidrug resistance (MDR). MDR is caused by the overexpression of MDR transport proteins such as p-glycoproteins (p-gp) in the cell membrane, that efflux the drug by reducing the intracellular drug levels for cancer chemotherapy (Schinkel 1997; Stella and Nti-Addae 2007). Tumors also acquire drug resistance through induction of MDR transport proteins (Harris and Hochhauser 1992; Gottesman, Fojo et al. 2002). At present, about 5-7% of the approved drugs worldwide can be classified as prodrugs and approximately 15% of all new drugs approved within 2001 and 2002 were prodrugs (Rautio, Kumpulainen et al. 2008). The conjugation of the drug with the polymer is a main strategy to form the polymeric prodrug of the synergistic or additive effect, which occurs with enhanced and simultaneous action of the drug and the polymer in destroying the cancer cells (Tarek. M. Fahmy 2005). The rationale for polymer conjugation is to mainly prolong the half-life of therapeutically active agents by increasing their hydrodynamic volume and hence decreasing their excretion rate. Polymer-anticancer drug conjugate has been investigated and some prodrugs have been found successful (Kopecek, Kopeckova et al. 2001; Jayant Khandare 2006; Pasut, Canal et al. 2008). Polymers such as N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers, poly(ethylene glycol) and

poly(L-glutamic acid) (PGA) have been used often as the carriers for anticancer drugs such as doxorubicin, paclitaxel, camphothecin and gemcitabine (Greenwald, Choe et al. 2003; Chytil, Etrych et al. 2006; Pasut, Canal et al. 2008). Several polymeric conjugates, for example, PEG conjugation of paclitaxel, camptothecin, methotrexate, PLA-paclitaxel, PEG-Doxorubicin, PLGA-paclitaxel have been developed earlier (Maeda, Seymour et al. 1992; Li, Yu et al. 1996; Riebeseel, Biedermann et al. 2002; Veronese, Schiavon et al. 2005; Pasut 2007).

Most of the anticancer drugs do not differentiate between the cancerous cells and the healthy cells, leading to their systemic toxicity and side effects by affecting the normal cells (Brannon-Peppas and Blanchette 2004). The aim of targeted drug delivery is to decrease the non-specificity to the healthy cells and increase the specificity to the cancer cells by attaching a targeting moiety to the inactive prodrug such that the active drug may then be released in the cancer cells without affecting the healthy cells (de Groot, Damen et al. 2001). The concept of targeting takes its effect when Paul Ehrlich (1854-1915) first postulated the 'magic bullet'. Targeted drug delivery system has been considered as the promising way to increase the therapeutic effects of the antitumor drugs by being specific to tumor cells and by having prolonged duration of drug action (Sudimack and Lee 2000). This leads to reduction in the minimum effective dose of the drug. Though the "passive targeting" is quite effective by the enhanced permeation and retention (EPR) effect, "active targeting" by receptor mediated endocytosis (RME) is found to be more advantageous for most of the anticancer drugs (Tarek. M. Fahmy 2005). Several drug conjugates and drug encapsulated nanoparticles have been reported to actively target the cancer cells to increase the anticancer effects of the drug (Li, Yu et al. 1996; Veronese, Schiavon et al. 2005).

Among the targeting moieties, vitamin folic acid (folate or FOL) has been widely employed as a targeting moiety for various anticancer drugs. It is attracted for its high binding affinity, ease of

modification, small size, stability during storage, and low cost (Lee and Low 1995; Reddy and Low 2000). The high-affinity folate receptor (FR), which is a cell surface-expressed molecule containing folate binding proteins called GPI (glycosyl phosphatidyl inositol) (Lu and Low 2002), is overexpressed in almost all the carcinomas, but has a highly restricted distribution of expression in normal cells. For this reason, folic acid has been covalently conjugated to anticancer drugs for selective targeting against tumor, which can uptake the drug-FOL conjugation by the receptor mediated endocytosis (RME) (Lee and Low 1995). It was reported that folate-targeted liposomal doxorubicin in an MDR cell line can bypass the P-gp efflux effect as compared to the free doxorubicin, showing the effective targeting delivery of doxorubicin by folate (Goren, Horowitz et al. 2000).

A water-soluble derivative of natural vitamin E, D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) or vitamin E TPGS, which is an amphiphilic macromolecule comprising of hydrophilic polar head and a lipophilic alkyl tail, has been used as an effective emulsifier as well as a good solubilizer due to its bulky nature and larger surface area (Fisher 2002). Our group has successfully applied TPGS to prepare nanoparticles of biodegradable copolymers such as PLA-TPGS and PLGA-TPGS for controlled and targeted delivery of paclitaxel, employed as a model anticancer drug (Mu and Feng 2003; Zhang and Feng 2006; Lee, Zhang et al. 2007). TPGS can enhance the solubility and bioavailability of poorly absorbed drugs by acting as a carrier in drug delivery systems, thus providing an effective way to improve the therapeutic efficiency and reduce the side effects of the anticancer drugs (Fisher 2002; Youk, Lee et al. 2005). It also increases the drug permeability across the cell membranes and enhances the absorption of the drug by inhibiting the P-glycoproteins, whereby acting as a vehicle for drug delivery system (Dintaman and Silverman 1999; Mu and Feng 2003). The increased emulsification efficiency and enhanced cellular uptake of nanoparticles by TPGS could result in increased cytotoxicity of the

drug to the cancer cells (Mu and Feng 2003). In recent studies, it is known that TPGS also possesses potent antitumor activity and has effective apoptosis inducing properties (Dintaman and Silverman 1999; Youk, Lee et al. 2005). TPGS should thus be an ideal candidate for polymeric conjugation of the drugs that have problems in pharmacokinetics, i.e. in the process of adsorption, distribution, metabolism and excretion (ADME).

Doxorubicin (DOX), an anthracyclinic drug is a DNA-interacting drug for various cancers especially breast, ovarian, stomach, bladder, brain and lung cancers and is one of the most potent anticancer agents after its discovery in 1969 (Blum and Carter 1974). However, application of doxorubicin in clinical application has been limited because of its short half-life and its extremely high toxicity to the normal cells, especially the heart and gastrointestinal cells, as well (Blum and Carter 1974; Al-Shabanah, El-Kashef et al. 2000). It was indicated that when the cumulative dose of doxorubicin reaches 550 mg/m², the risks of developing cardiac side effects would dramatically increase (Petit 2004). Alternative formulations of doxorubicin have been developed recently, which include folate targeted doxorubicin, DOX-GA3 prodrug, HPMA-doxorubicin conjugate, doxorubicin-PEG-folate conjugate, DOX-PLGA-mPEG-folate micelles (Shiah, Dvorak et al. 2001; Yoo and Park 2004; Yoo and Park 2004; Lee, Na et al. 2005; Veronese, Schiavon et al. 2005).

1.2 Objectives of this Research

The objectives of this research is to develop a novel targeting polymeric prodrug, TPGS-DOX-FOL, that is hoped to combine the advantages of TPGS and FOL applied individually in formulation of prodrugs. The polymer-drug conjugation was confirmed by ^{1}H NMR and FT-IR. The conjugation efficiency, stability and *in vitro* drug release from the conjugate were measured and analyzed. The cellular uptake and *in vitro* cytotoxicity of the TPGS-DOXFOL and TPGS-

DOX conjugates were investigated by using MCF-7 breast cancer cells in close comparison with the pristine drug. Also, the pharmacokinetics and biodistribution were investigated in SD rats for pristine DOX, TPGS-DOX and TPGS-DOX-FOL conjugates.

1.3 Thesis Organization

The thesis includes six chapters. Chapter 1 gives a brief introduction to the research done. It comprises of general background of the project and its objectives as well. Chapter 2 gives a literature review, which was useful in developing novel ideas and concepts in this project and also gives supporting evidences. Chapter 3 gives the materials required and procedures adopted for the preparation of the conjugates. Chapter 4 explains the *in vitro* studies on drug release, cellular uptake and cell viability of the conjugates and the DOX. Chapter 5 gives the *in vivo* pharmacokinetics and biodistribution of the conjugates compared to the free DOX. Finally, the conclusions of the project are drawn based on the results and the interpretations done, followed by few recommendations for future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer: A Deadly Disease

2.1.1 Overview of Cancer

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells that might affect almost any tissue of the body. The spreading of the cancerous cells is called 'metastasis' (http://en.wikipedia.org/wiki/Metastasis). It can result in death, if the spread is not controlled. According to World Health Organization (WHO), cancer causes about 13 % of all the deaths (http://en.wikipedia.org/wiki/Cancer). Cancer is also called malignancy. A cancerous growth or tumor is referred to as a malignant growth or tumor. A non-malignant growth or tumor is referred to as benign. Benign tumors are not cancerous. There are dozens of cancer types such as prostate cancer, lung cancer, colorectal cancer, bladder cancer, cutaneous melanoma, pancreatic cancer, leukemia, breast cancer, endometrial cancer, ovarian cancer, brain cancer, non-Hodgkin lymphoma etc. General classification of cancer includes Carcinoma, Sarcoma, Lymphoma, Leukemia, Germ cell tumor, Blastic tumor etc (http://en.wikipedia.org/wiki/Cancer).

2.1.2 Cancer Prevalence, Causes and Risk Factors

Cancer is one of the leading causes of death with around 10 million people being diagnosed with the disease each year. According to American Cancer Society, 7.6 million people died from cancer all over the world during 2007 and about 1.4 million new cancer cases are expected to be diagnosed in the year 2008 (http://en.wikipedia.org/wiki/Cancer). The 5-year relative survival rate for all cancers diagnosed between 1996 and 2003 is 66 %, up from 50 % 1975 – 1977. The

National Institutes of Health estimate overall costs of cancer in 2007 at $219.2 billion:$89.0 billion for direct medical costs (total of all health expenditures); $18.2 billion for indirect morbidity costs (cost of lost productivity due to illness); $112.0 billion for indirect mortality costs (loss of productivity due to premature death) (http://www.cancer.org/downloads/STT/2008CAFFfinalsecured.pdf). By the year 2050, the global burden is expected to grow to 27 million new cancer cases and 17.5 million cancer deaths simply due to the growth and ageing of the population (http://www.cancer.org/downloads/STT/Global_Cancer_Facts_and_Figures_2007_rev.pdf).

Cancer may affect people at all ages but in most cases the number of cancer patient increases with age. All cancers are almost caused by the abnormalities in the genetic material of the transformed cells. These genetic abnormalities in cancer affect 2 types of genes namely Tumor suppressor genes and Oncogenes. In cancer, the oncogenes are activated and the tumor suppressor genes are inactivated. Here, the oncogenes are responsible for the hyperactive growth and division of the cancer cells, to adjust in different environments and cause programmed cell death. Now the Tumor suppressor genes are responsible for the loss in control over the cell cycle, adhesion with other tissues and interaction with the immune cells. The 2 wide factors that cause the cancerous cells are the external factors and the internal factors. The external factors include

- Tobacco smoking
- Chemicals
- Radiation
- Infections
- Alcohol
- Poor diet
- Lack of physical activity or overweight

The internal factors include

- Inherited mutations
- Hormones
- Growing older
- Immune conditions.

These factors are said to be the most common risk factors for cancer. Many of these risk factors can be avoided and several of these factors may act together to cause normal cells to become cancerous. The chemicals that cause cancer are called carcinogens and those chemicals that cause cancer through mutations in DNA are called mutagens. All mutagens are carcinogens, but all carcinogens are not mutagens. They cause rapid rates of mitosis of the cells and thus inactivate the enzyme that does the DNA repair. One of the most important carcinogens is tobacco. Smoking and its related disease remains the world's most preventable cause of death and so is the cancer also. According to National Cancer Institute (NCI), each year, more than 180,000 Americans die from cancer that is related to tobacco use. Tobacco smoking accounts for at least 30 % of all cancer deaths and 87 % of lung cancer deaths. The risk of developing lung cancer is about 23 times higher in male smokers and 13 times higher in female smokers compared to non-smokers (http://www.cancer.org/downloads/STT/2008CAFFfinalsecured.pdf). Also, quitting smoking substantially decreases the risk of cancer. Prolonged exposure of radiation such as ultra violet radiation from the sun, sun lamps and tanning booths causes early ageing of the skin and skin damage that can lead to skin cancer. Ionizing radiation usually causes cell damage that leads to cancer. This kind of radiation comes from the rays that enter the earth's atmosphere from outer space, radioactive fallout, radon gas, x-rays and other sources. The radioactive fallout can come from accidents at nuclear power plants or from the production, testing or use of atomic weapons. People exposed to fallout may have an increased risk of cancer, especially leukemia and cancer of thyroid, breast, lung and stomach. Radon is a radioactive gas that we cannot see, smell or taste.

People who work in mines may be exposed to radon. People exposed to radon are at increased risk of lung cancer. The risk of cancer from low dose x-rays is very small and that from the radiation therapy is slightly higher. Being infected with certain viruses or bacteria may increase the risk of developing cancer. HPV (*Human papillomavirus*) infection is the main cause of cervical cancer. It also may be a risk factor for other types of cancer. *Hepatitis B and Hepatitis C* viruses can cause liver cancer after many years of infection. Infection with HTLV-1 (*Human T-cell leukemia/lymphoma virus*) increases a person's risk of developing lymphoma and leukemia. HIV (*Human Immunodeficiency Virus*) is the virus that causes AIDS. People who possess HIV have a greater risk of having cancer such as lymphoma and a rare cancer called 'Kaposi's sarcoma'. EBV (*Epstein-Barr Virus*) infection can cause lymphoma. *Human herpesvirus 8* (HHV8) is a risk factor for kaposi's sarcoma. *Helicobacter pylori* bacteria can cause stomach ulcers. It can also cause stomach cancer and lymphoma in stomach lining. The viruses are responsible for about 15% of the cancers worldwide.

The hormonal imbalance causes cancer due to the hormones acting in the same manner as the non-mutagenic carcinogens. Hormones may increase the risk of breast cancer, heart attack, stroke or blood clot. Diethylsilbestrol (DES), a form of estrogen, was given to pregnant woman in the United States between about 1940 and 1971. Woman who took DES during their pregnancy may have a slightly higher risk of developing breast cancer. Their daughters have an increased risk of developing a rare type of cancer of cervix. The effects on their sons are under study. The immune system malfunction also causes cancer to a greater extent and heredity causes cancer as well. Most cancers develop because of changes (*mutations*) in genes. A normal cell may become a cancer cell after a series of gene changes occur. Tobacco use, certain viruses, or other factors in a person's lifestyle or environment can cause such changes in certain types of cells. Some gene changes that increase the risk of cancer are passed from parent to child. These changes are present

at birth in all cells of the body. It is uncommon for cancer to run in a family. However, certain types of cancer do occur more often in some families than in the rest of the population. For example, melanoma and cancers of the breast, ovary, prostate, and colon sometimes run in families. Several cases of the same cancer type in a family may be linked to inherited gene changes, which may increase the chance of developing cancers. However, environmental factors may also be involved. Most of the time, multiple cases of cancer in a family are just a matter of chance. Having more than two drinks each day for many years may increase the chance of developing cancers of the mouth, throat, esophagus, larynx, liver, and breast. The risk increases with the amount of alcohol that a person drinks. For most of these cancers, the risk is higher for a drinker who uses tobacco. People who have a poor diet, do not have enough physical activity, or are overweight may be at increased risk of several types of cancer. For example, studies suggest that people whose diet is high in fat have an increased risk of cancers of the colon, uterus, and prostate. Lack of physical activity and being overweight are risk factors for cancers of the breast, colon, esophagus, kidney, and uterus.

2.1.3 Cancer Treatment

The treatment for cancer varies based on the type of cancer and its stage. The stage of a cancer refers to how much it has grown and whether the tumor has spread from its original location. The goal of the treatment is the complete removal of the cancer without damage to the rest of the body. Cancer can be treated by many methods such as

- Surgery
- Radiation therapy
- Chemotherapy

- Immunotherapy
- Targeted therapy
- Hormonal therapy etc.

Surgery is done by removing the cancer in the respective location by physical operation. It is usually used to remove small cancers and those that are not metastasized. The goal of the surgery can be the removal of either the tumor alone or the entire organ. When the cancer has metastasized to other sites in the body prior to surgery, complete surgical excision is usually impossible. Surgery is also used to control the symptoms like spinal cord compression or bowel obstruction. Radiation therapy is the use of ionizing radiation to kill cancer cells and shrink tumors. It can be administered externally or internally. The effects of radiation therapy are localized and confined to the region being treated. Radiation therapy injures or destroys cells in the area being treated by damaging their genetic material, making it impossible for these cells to continue to grow and divide. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue. Radiation therapy may be used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, stomach, uterus, or soft tissue sarcomas. Radiation is also used to treat leukemia and lymphoma. Chemotherapy is the treatment of cancer with drugs called anticancer drugs, that can destroy cancer cells. In current usage, the term "chemotherapy" usually refers to cytotoxic drugs which affect rapidly dividing cells. Chemotherapy drugs interfere with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target all rapidly dividing cells and are not specific for cancer cells, although some degree of specificity may come from the inability of many cancer cells to repair DNA damage, while normal cells generally can. Hence, chemotherapy has the potential to harm healthy tissue,

especially those tissues like intestinal lining that have a high replacement rate. These cells usually repair themselves after chemotherapy. Because some drugs work better together than alone, two or more drugs are often given at the same time and this is called "combination chemotherapy". Most chemotherapy regimens are given in a combination. Targeted therapy constitutes the use of agents specific for the deregulated proteins of cancer cells. Small molecule targeted therapy drugs like tyrosine kinase inhibitors, are generally inhibitors of enzymatic domains on mutated, overexpressed, or otherwise critical proteins within the cancer cell. Monoclonal antibody therapy is another strategy in which the therapeutic agent is an antibody which specifically binds to a protein on the surface of the cancer cells. The anti-HER2/neu antibody trastuzumab (Herceptin) used in breast cancer, and the anti-CD20 antibody rituximab, used in a variety of B-cell malignancies are some of the antibodies used in targeting and treating cancer cells. Cancer immunotherapy induces the person's own immune system to destroy the tumor. Contemporary methods for generating an immune response against tumours include intravesical BCG immunotherapy for superficial bladder cancer, and use of interferons and other cytokines to induce an immune response in renal cell carcinoma and melanoma patients. Vaccines that are used to generate specific immune responses are the subject of intensive research for various tumors. The growth of some cancers can be inhibited by providing or blocking certain hormones. Common examples of hormone-sensitive tumors include certain types of breast and prostate cancers. Removing or blocking estrogen or testosterone is often an important additional treatment. In certain cancers, administration of hormone agonists, such as progestogens may be therapeutically beneficial. Angiogenesis inhibitors prevent the extensive growth of blood vessels (angiogenesis) that tumors require to survive and thus it can be considered as a treatment for cancer. Some inhibitors, such as bevacizumab, have been approved and are in clinical use. One of the main problems with anti-angiogenesis drugs is that many factors stimulate blood vessel growth, in normal cells and cancer. Anti-angiogenesis drugs only target one factor, so the other

factors continue to stimulate blood vessel growth. Other problems include route of administration, maintenance of stability and activity and targeting at the tumor vasculature.

2.1.4 Cancer Chemotherapy and its Evolution

Chemotherapy refers to "treatment with drugs or chemicals" to destroy the cancer cells. The drugs destroy the cells by interfering with their life cycle. Cancer cells are more sensitive to chemotherapy than healthy cells because they divide more frequently. Healthy cells can also be affected by chemotherapy, especially the rapidly dividing cells of the skin, the lining of the stomach, the intestines and the bladder. Chemotherapy is often the first choice for treating many cancers. It differs from surgery or radiation in that it is almost always used as a systemic treatment. This means the medicines travel throughout the body to reach cancer cells wherever they may have spread. Treatments like radiation and surgery act in a specific area such as the breast, lung, or colon, and so are considered local treatments. More than 100 drugs are used today for chemotherapy, either alone or in combination with other drugs or treatments. As research continues, more drugs are expected to become available. Chemotherapy drugs can be divided into several groups based on factors such as how they work, their chemical structure, and their relationship to another drug. Some chemotherapy drugs are grouped together because they were derived from the same plant. Because some drugs act in more than one way, they may belong to more than one group. Nanotechnology has been developed in recent times to design more comfortable and effective drug formulations that are patient friendly. The common types of chemotherapeutic drugs are the following.

- Alkylating agents – They directly damage DNA to prevent the cancer cell from reproducing. Alkylating agents are used to treat many different cancers, including acute and chronic

leukemia, lymphoma, Hodgkin disease, multiple myeloma, sarcoma, as well as cancers of the lung, breast, and ovary. Because these drugs damage DNA, they can cause long-term damage to the bone marrow. In a few rare cases, this can eventually lead to acute leukemia. The risk of leukemia from alkylating agents is "dose-dependent," meaning that the risk is small with lower doses, but goes up as the total amount of drug used gets higher. The risk of leukemia after alkylating agents is highest 5-10 years after treatment. The different alkylating agents include *nitrogen mustards* such as mechlorethamine (nitrogen mustard), chlorambucil, cyclophosphamide (Cytoxan®), ifosfamide, and melphalan, *nitrosoureas* which include streptozocin, carmustine (BCNU), and lomustine, *alkyl sulfonates* that include busulfan, *triazines* such as dacarbazine (DTIC), and temozolomide (Temodar®), *ethylenimines* such as thiotepa and altretamine (hexamethylmelamine). The platinum drugs (cisplatin, carboplatin, and oxalaplatin) are sometimes grouped with alkylating agents because they kill cells in a similar way. These drugs are less likely than the alkylating agents to cause leukemia.

- Antimetabolites - Antimetabolites are a class of drugs that interfere with DNA and RNA growth by substituting for the normal building blocks of RNA and DNA. These agents damage cells during the S phase. They are commonly used to treat leukemias, tumors of the breast, ovary, and the intestinal tract, as well as other cancers. Examples of antimetabolites include 5-fluorouracil (5-FU), capecitabine (Xeloda®), 6-mercaptopurine (6-MP), methotrexate, gemcitabine (Gemzar®), cytarabine (Ara-C®), fludarabine, and pemetrexed (Alimta®).
- Anthracyclines - Anthracyclines are anti-tumor antibiotics that interfere with enzymes involved in DNA replication. These agents work in all phases of the cell cycle. Thus, they are widely used for a variety of cancers. A major consideration when giving these drugs is that they can permanently damage the heart if given in high doses. For this reason, lifetime dose

limits are often placed on these drugs. Examples of anthracyclines include daunorubicin, doxorubicin (Adriamycin®), epirubicin, and idarubicin.

- Other anti-tumor antibiotics – They include the drugs actinomycin-D, bleomycin, and mitomycin-C. Mitoxantrone is an anti-tumor antibiotic that is similar to doxorubicin in many ways, including the potential for damaging the heart. This drug also acts as a topoisomerase II inhibitor (see below), and can lead to treatment-related leukemia. Mitoxantrone is used to treat prostate cancer, breast cancer, lymphoma, and leukemia.
- Topoisomerase inhibitors - These drugs interfere with enzymes called topoisomerases, which help separate the strands of DNA so they can be copied. They are used to treat certain leukemias, as well as lung, ovarian, gastrointestinal, and other cancers. Examples of topoisomerase I inhibitors include topotecan and irinotecan (CPT-11). Examples of topoisomerase II inhibitors include etoposide (VP-16) and teniposide. Mitoxantrone also inhibits topoisomerase II.
- Mitotic inhibitors - Mitotic inhibitors are often plant alkaloids and other compounds derived from natural products. They can stop mitosis or inhibit enzymes from making proteins needed for cell reproduction. These work during the M phase of the cell cycle but can damage cells in all phases. They are used to treat many different types of cancer including breast, lung, myelomas, lymphomas, and leukemias. These drugs are known for their potential to cause peripheral nerve damage, which can be a dose-limiting side effect. Examples of mitotic inhibitors include *the taxanes* like paclitaxel (Taxol®), docetaxel (Taxotere®), *epothilones* like ixabepilone (Ixempra®), *the vinca alkaloids* such as vinblastine (Velban®), vincristine (Oncovin®), and vinorelbine (Navelbine®) and *estramustine* like (Emcyt®).

- Corticosteroids - Steroids are natural hormones and hormone-like drugs that are useful in treating some types of cancer (lymphoma, leukemias, and multiple myeloma) as well as other

illnesses. When these drugs are used to kill cancer cells or slow their growth, they are considered chemotherapy drugs. Corticosteroids are also commonly used as *anti-emetics* to help prevent nausea and vomiting caused by chemotherapy. Examples include prednisone, methylprednisolone (Solumedrol), and dexamethasone (Decadron).

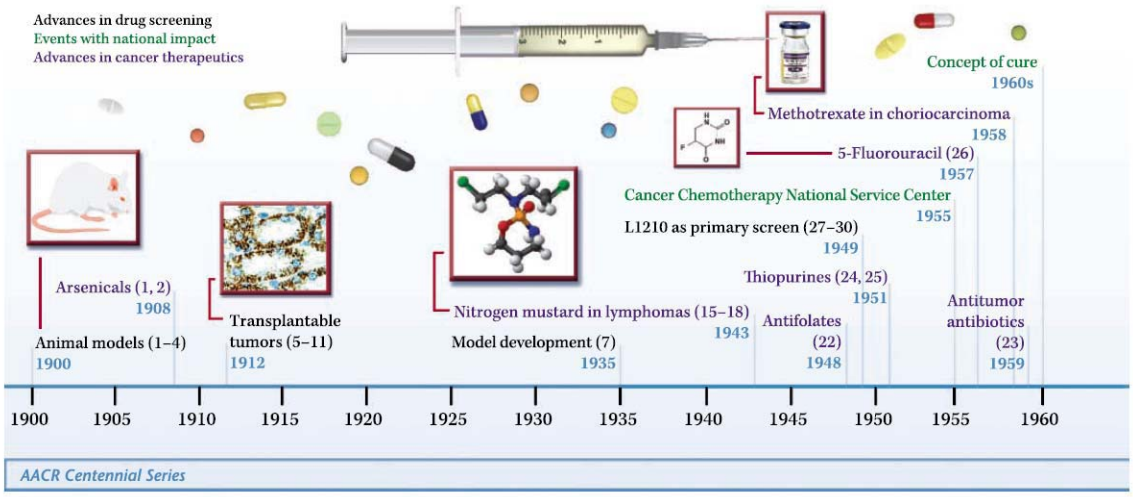


Fig 2-1 Timeline of events in the development of cancer chemotherapy (DeVita and Chu 2008)

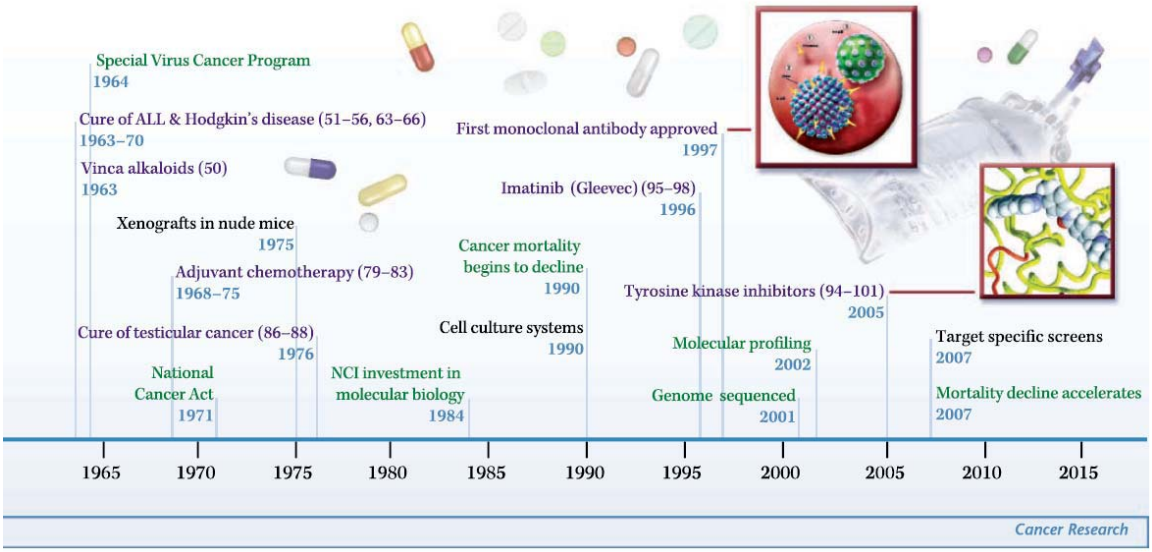


Fig 2-1 Timeline of events in the development of cancer chemotherapy (DeVita and Chu 2008)

(continued)

2.1.5 Barriers encountered in Cancer Chemotherapy

There are four main barriers encountered in cancer chemotherapy which gives rise to increased side effects. They are as follows:

2.1.5.1 Solubility

Solubility has been identified as a critical parameter in cancer chemotherapy. The drug administered either intravenously or orally has to be soluble in the blood or should have a better oral absorption respectively. Since most of the anticancer drugs are hydrophobic, they have a very low solubility, which results in poor therapeutic effect. Research has been carried out to find a method that increases the solubility of these drugs. One such method is the use of polymers to form prodrugs. Prodrugs are polymer-drug conjugates that remain inactive till it reaches the site

of action (Stella and Nti-Addae 2007). Also, they found that polymeric nanoparticles can increase the oral absorption of the drugs in the intestine as well as increase the solubility of drugs in the blood.

2.1.5.2 Macrophages Uptake

Macrophages are white blood cells within tissues, produced by the division of monocytes. Human macrophages are about 21 micrometres in diameter. The important role of macrophages is to find the foreign materials that enter the blood, engulf them and digest them. It is a protective system to prevent the body from attach of pathogens that enter the blood. This is considered to be a barrier for chemotherapy, because the anticancer drugs can be recognized as foreign particles and can be digested by the macrophages, which results in very poor treatment.

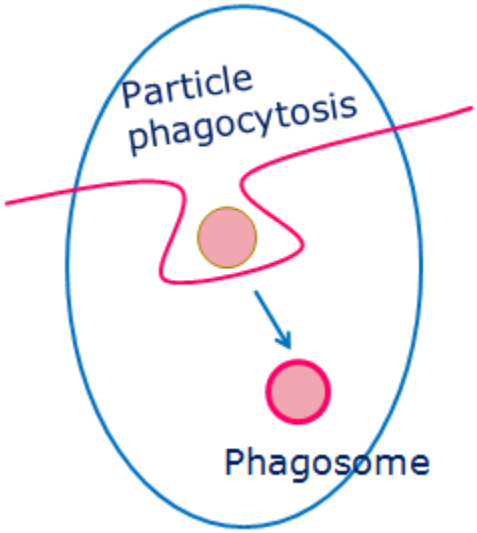


Fig 2-2 Macrophages uptake by phagocytosis

When a macrophage ingests a pathogen, the pathogen becomes trapped in a phagosome, which then fuses with a lysosome. Within the phagolysosome, enzymes and toxic peroxides digest the pathogen. However, some bacteria, such as *Mycobacterium tuberculosis*, have become resistant to these methods of digestion. Macrophages can digest more than 100 bacteria before they finally die due to their own digestive compounds.

2.1.5.3 Multi Drug Resistance (MDR effect)

The MDR is defined as the resistance of tumor cells to the cytostatic or cytotoxic actions of multiple, structurally dissimilar and functionally divergent drugs commonly used in cancer chemotherapy (Gottesman 1993). The most studied mechanism of MDR is that resulting from the overexpression of ABC transporters, localized in the cell membrane, which cause this phenomenon by extruding a variety of chemotherapeutic agents from tumor cells. The ABC transporters are primary-active transporters, driven by energy released from ATP by inherent ATPase activity, and exporting substrates from the cell against a chemical gradient. Three major ABC transporters are involved in MDR, (1) P-glycoproteins (P-gp), (2) ABCG2 protein and the (3) multidrug resistance associated proteins (Perez-Tomas 2006). P-glycoproteins are the most important transporters resulting in decreased anticancer activity of the drugs.

P-glycoproteins were discovered by their ability to confer multidrug resistance (MDR) to cancer cells (Juliano and Ling 1976; Gottesman, Hrycyna et al. 1995). P-gps are large, glycosylated membrane proteins which localize predominantly to the plasma membrane of the cell. They confer drug resistance by active, ATP-dependent extrusion of a range of cytotoxic drugs from the cell.

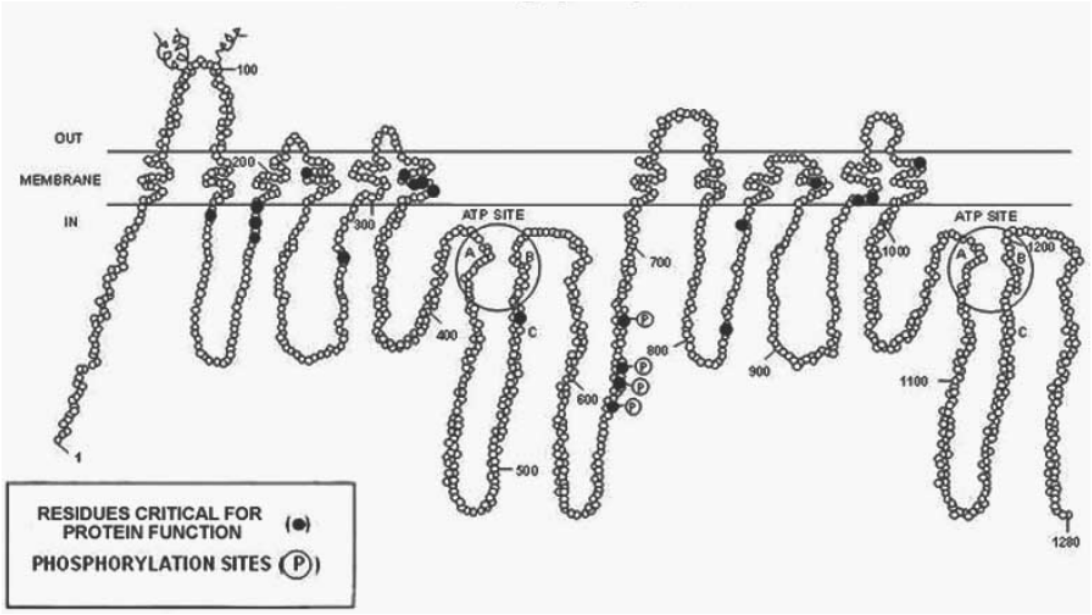


Fig 2-3 Human P-glycoprotein (Perez-Tomas 2006)

The most striking property of the drug transporting P-gps is their ability to transport an incredibly diverse range of compounds, which do not share obvious structural characteristics. Interestingly, many of these compounds are of natural origin (derived from plants, bacteria, fungi, sponges), or minor variants of natural products. The only common structural denominator identified so far is that all transported P-gp substrates are amphipathic in nature. This probably relates to the mechanism of drug translocation by P-gp, which may be dependent on the ability of the drug to insert in one hemileaflet of the membrane lipid bilayer (Higgins and Gottesman 1992) as is also discussed elsewhere in this volume. As a consequence of the promiscuity of the P-gps, they can transport a large number of medically relevant compounds. These include a range of widely used anticancer drugs, such as anthracyclines, *Vinca* alkaloids, epipodophyllotoxins,and taxanes, but many other drugs and pesticides too, such as the immunosuppressive agents cyclosporin A and FK506 (Saeki, Ueda et al. 1993), cardiac glycosides such as digoxin (Tanigawara, Okamura et al.

1992), antibiotics like rifampicin and the anthelmintic pesticide ivermectin (Schinkel, Smit et al. 1994; Schinkel, Wagenaar et al. 1995). The properties of P-gp includes the protection against natural toxins, hormone transport and reproduction, functional role in hematological compartment, role in cell volume regulation, role in lipid transport and other functions. The P-gp plays an important role in the blood-brain barrier (Bradbury 1985; Schinkel 1997). They are also said to limit oral absorption and brain entry through HIV-1 protease inhibitors (Kim, Fromm et al. 1998).

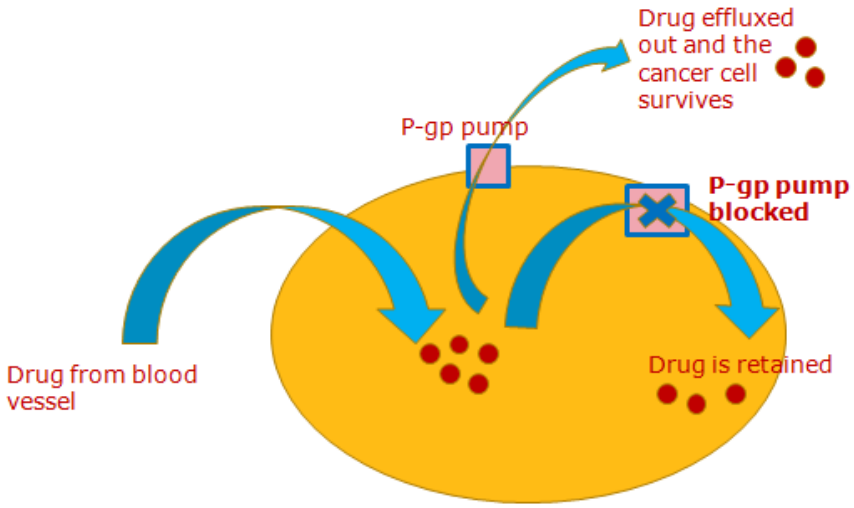


Fig 2-4 Mechanism of P-glycoproteins

2.1.5.4 Stability and Absorption in Small Intestine

The stability and the absorption in small intestine is one of the barriers in delivering the drug to the cancer cells. This is in the case of oral chemotherapy, where absorption in small intestine and crossing the intestinal membrane by diffusion plays an important role. The inner walls of the small intestine have thousands of finger-like outgrowths called villi. The villi increase the surface area for absorption of the digested food. Each villus has a network of thin and small blood vessels

close to its surface. The surface of the villi absorbs the digested food materials. The absorbed substances are transported via the blood vessels to different organs of the body where they are used to build complex substances such as the proteins required by our body. This is called assimilation. If an orally administered drug can harm the stomach lining or decomposes in the acidic environment of the stomach, a tablet or capsule of the drug can be coated with a substance intended to prevent it from dissolving until it reaches the small intestine. These protective coatings are described as enteric, which refers to the small intestine. For the coatings to dissolve, they must come in contact with the less acidic environment of the small intestine or with the digestive enzymes there.

2.1.6 Problems and Side Effects in Chemotherapy

Chemotherapy is a very complicated procedure that gives rise to a high or low risk making it an ineffective or effective therapy respectively. The risk is due to the high toxicity of the chemotherapeutic drug that finally leads to side effects. The side effects of chemotherapy are usually caused by its effects on healthy cells. Chemotherapy interferes with cell duplication. Since cancer cells divide rapidly they are the targets of the treatment. Some of the most common side effects of chemotherapy are listed below.

(1) Blood-Related side effects – One of the most important side effects of chemotherapy is its effect on blood cells namely RBCs (Red Blood Cells), WBCs (White Blood Cells) and Platelets. Normally blood cells are the most rapidly dividing cells in the body, and therefore, the most sensitive to chemotherapy. Chemotherapeutic agents may usually decrease temporarily the levels of these blood components. The time when the blood components are at the lowest level is called as the “nadir”, and usually occurs one to two weeks after the

chemotherapy had begun. When the RBCs decrease significantly, a condition known as "anemia" occurs. When the WBCs decrease significantly, a condition known as "neutropenia" occurs. When the platelets decrease significantly, a condition known as "thrombocytopenia" occurs. Internal bleeding causes anemia. These side effects can be treated with blood transfusions and new medications that speed up the replacement of the lost blood cells.

(2) Hair loss – This is another side effect of chemotherapy and is also called "alopecia". Cells in the hair follicles are responsible for hair growth and maintenance. Because these cells divide rapidly, they are affected by chemotherapeutic drugs. Hair loss may affect the scalp, face and the rest of the body. The rate of hair loss may be rapid. Hair loss is usually temporary.

(3) Nausea and vomiting – Some chemotherapeutic agents can lead to nausea and vomiting. Strong anti-nausea and anti-vomiting medications are available for this purpose. Drinking clear liquids before chemotherapy helps to decrease nausea and vomiting.

(4) Sore throat – The cells lining the inside of the mouth and throat divide rapidly. They are also continuously exposed to infections from the food. Chemotherapy can cause inflammation and infections inside the mouth. This condition is known as "stomatitis" makes swallowing difficult and painful.

(5) Diarrhea – Because the cells lining the intestines and colon divide constantly, they can be affected by chemotherapy. This can cause diarrhea. Increasing fluid intake usually keeps the patient hydrated.

(6) Constipation – It is sometimes caused by chemotherapy. Maintaining a high fiber diet helps to decrease the side effect.

(7) Effect on the skin – because the cells lining the skin divide fairly and rapidly, they are susceptible to chemotherapy. This can cause skin dryness and increased reaction to the sunlight.

(8) Fertility and sexuality – Men wishing to father children may consider sperm banking prior to the start of chemotherapy. Chemotherapy may affect sperm count and viability. Some woman may have changes in their menstrual cycle because of chemotherapy, which could result in total absence of periods. Chemotherapy could also cause dryness of the vagina.

(9) Other possible side effects – Besides the common side effects of the chemotherapy, other side effects can happen, depending on the type of cancer, the type of chemotherapy treatment and the patient's medical condition.

These side effects are due to certain factors such as dosage form of the drug, pharmacokinetics of the drug, toxicity associated with the drug and the drug resistance by the cancer cells. The drug resistance is of three categories namely pharmacokinetic resistance (due to low concentration of drug), kinetic resistance (small fraction of cells in susceptible state) and genetic resistance (due to biochemical resistance). A very important resistance developed by the cancer cells is the Multi Drug Resistance (MDR). This resistance is caused by the membrane proteins, P-glycoproteins that causes the efflux of the drug from the cell and results in low drug accumulation in the cancer cells. It usually acts as the efflux pump to protect the cancer cells (Krishna and Mayer 2000). The dosage form of the anti cancer drug is also a factor for the side effects. Mostly the anti cancer drugs are hydrophobic in nature and that it has to be made hydrophilic in order for it to be soluble in blood and available for the cancer cells. For this purpose, adjuvants are added to the drugs, which cause the side effects. In the case of anti cancer drug Paclitaxel, Cremophor EL has been added as an adjuvant in order to improve its availability to cancer cells and to improve its solubility and this was found to have serious side effects like hypersensitivity, nephrotoxicity,

cardiotoxicity etc. The longer time exposure is believed to have better anti cancer effects and thus sufficient drug concentration for longer time is required to kill cancer cells in a better way. Anti cancer drugs affect healthy cells also. So this might cause toxicity to the normal cells along with the cancerous cells that might cause side effects affecting the liver, heart, kidney etc (Feng SS 2003).

2.1.7 Engineering Aspects of Cancer Chemotherapy

The main engineering aspects of the cancer chemotherapy is to achieve the best efficiency of the anticancer drugs with the least side effects. The chemotherapy involves toxic drugs which are used to treat cancer cells. The problem comes from the anticancer drugs like doxorubicin, paclitaxel, fluorouracil etc itself. The efficiency and the side effects are not interrelated. Sometimes the drug that has maximum efficacy may have higher side effects and vice versa. The side effects of the anticancer drugs not only decrease effective chemotherapy, but also reduce the life of patients. Chemotherapeutic engineering represents a new challenge for chemical engineers. Chemical engineering made important contributions in providing new products and services to meet the needs of modern civilization and improve the quality of life in the past century.

2.2 Polymers as Drug Carriers in Drug Delivery System

Different drug delivery systems have been developed in the last few years to improve pharmacokinetic and pharmacodynamic profile of the drugs (Reddy 2000) . Many polymers have been investigated as candidates for the delivery of natural and synthetic drugs (Brocchini S 1999).

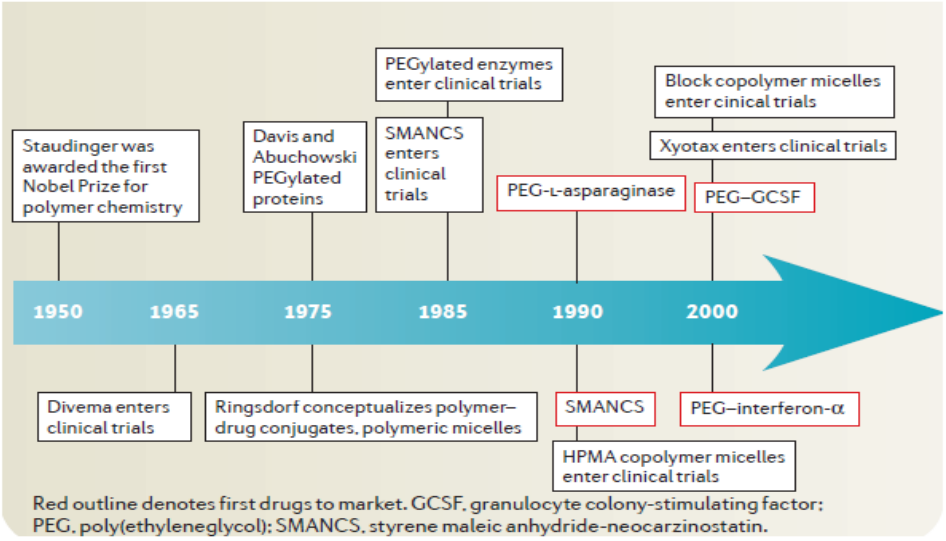


Fig 2-5 Emergence of anticancer polymer therapeutics (Duncan 2006)

In general, an ideal polymer for drug delivery should have characteristics like (1) biodegradability or adequate molecular weight that allows elimination from the body to avoid progressive accumulation *in vivo*, (2) low polydispersity, to ensure an acceptable homogeneity of the final drug formulations and (3) longer residence time either to prolong the drug action or to allow distribution and accumulation in respective body compartments. The polymers used for drug delivery are given as follows:

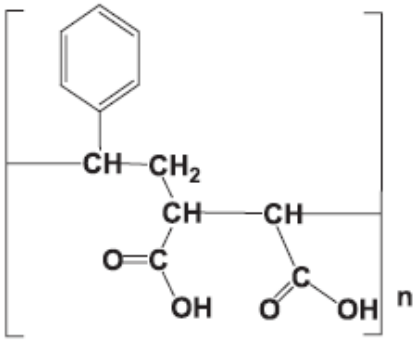
2.2.1 Synthetic Polymers

It includes PEG, N-(2-hydroxypropyl)-methacrylamide copolymers (HPMA), poly(ethyleneimine) (PEI), poly(acroloylmorpholine) (PAcM), poly(vinylpyrrolidone) (PVP), polyamidoamines, divinylethermaleic anhydride/acid copolymer (DIVEMA), poly(styrene-co-maleic acid/anhydride) (SMA), polyvinylalcohol (PVA).

Vinyl polymers are synthesized by radical polymerization of the respective vinyl monomer or by copolymerization of 2 or more different monomers. They can bring about high drug loading due to the reactive pendant groups and thus acts as a polymeric carrier. They are usually non biodegradable and therefore their molecular weight must fall below the renal threshold filtration for these molecules i.e. 40-50 kDa.

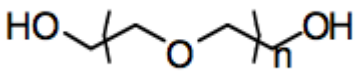
HPMA is one of most widely studied polymers (Kopecek J. 1973; Duncan R. 1983; Lloyd JB 1983). Its derivative with the antitumor drug doxorubicin was the first drug-conjugate design developed. This was developed based on the Ringsdorf model and it entered the clinical trials (Duncan 2001). Doxorubicin was linked via peptidyl spacer to polymer, where the linker is designed to be stable during plasma circulation, but promptly cleaved by lysosomal cathepsin B after cellular endocytosis (Duncan R 1983). HPMA copolymer was studied also in campothecin (Schoemaker, van Kesteren et al. 2002), paclitaxel (Meerum Terwogt, ten Bokkel Huinink et al. 2001) and Pt-malonate conjugation (Gianasi, Buckley et al. 2002; Rademaker-Lakhai, Terret et al. 2004), drugs that suffer from low solubility in water, which can be solved by polymer conjugation.

SMA is a hydrophobic copolymer which is obtained from maleic anhydride and styrene. Neocarcinostatin (NCS)-SMA is a most known conjugate, which exhibits cytotoxicity against mammalian cells. The conjugation was allowed for a half-life increase of 10-20 times with respect to native protein and by the EPR effect, the accumulation in tumor tissue was 30-fold that in muscle (Maeda 1991).



Scheme 2-1 Chemical structure of SMA

PEG is synthesized by the ring opening polymerization of ethylene oxide using methanol or water as initiator to yield methoxy-PEG or diol PEG, respectively. It has unique properties such as (1) lack of immunogenicity, antigenicity and toxicity, (2) high solubility in water and in many organic solvents, (3) high hydration and flexibility of the chain, (4) low polydispersity, (5) prolonged pharmacokinetic properties of drugs and (6) approval by FDA for human use (Pasut 2007). PEG is considered a non-biodegradable polymer. But slow degradation by alcohol dehydrogenase (Kawai 2002), aldehyde dehydrogenase (Mehvar 2000) and cytochrome P-450 (Beranova, Wasserbauer et al. 1990) has been reported for PEG oligomer. Therefore, its body clearance depends upon its molecular weight. The main limitation of PEG as drug carrier is the presence of only two reactive groups which leads to an intrinsically low drug payload. To overcome this limitation, the construction of Dendron structure at the PEG's end chain has been afforded, leading to enhanced drug loading (Choe, Conover et al. 2002; Schiavon, Pasut et al. 2004). Some of the conjugates prepared with PEG are PEG-camptothecin, PEG-Doxorubicin etc.



Scheme 2-2 Chemical structure of PEG

2.2.2 Natural Polymers

It includes dextran, pullulan, mannan, dextrin, chitosans, hyaluronic acid, proteins. Polysaccharides have been widely studied in drug delivery. Their pharmacokinetic is largely

influenced by molecular weight, electric charge, chemical modifications, and degree of polydispersity and/or branching. Their applications range from delivery of small drugs to preparation of protein conjugates (Mehvar 2003). Dextran is the most widely used polymer of this class (Brocchini S 1999). Dextran-Doxorubicin conjugate entered the phase I clinical trials, but displayed a toxicity attributed to uptake of dextran by the liver reticuloendothelial cells (Danhauser-Riedl, Hausmann et al. 1993).

2.2.3 Pseudosynthetic Polymers

It includes PGA, poly(L-lysine), poly(malic acid), poly(aspartamides), poly((N-hydroxyethyl)-L-glutamine) (PHEG). PGA, poly(L-lysine), poly(aspartamides), PHEG are easily synthesized and are biodegradable. The drug loading is high because any monomer possess a side reactive group for coupling. In this, PGA-Paclitaxel conjugate has reached the most advanced clinical stage. Here PGA with a 17,000 Da molecular weight was conjugated to Paclitaxel through an ester bond reaching the better high loading of 37% (Singer, Baker et al. 2003). The final conjugate had a molecular weight of 49,000 Da.

2.3 Drug Targeting to Cancer Cells

Targeted drug delivery to cancer cells is generally categorized as either passive or active targeting in the case of the presence or absence of site-directing ligands, respectively (Allen TM 1996; Willis and Forssen 1998). Targeted drug delivery systems promise to expand the therapeutic effects of drugs by increasing delivery to the target tissue as well as the target – non-target tissue ratio, which leads to a reduction in minimum effective dose and toxicity of the drug, and an improvement in therapeutic efficacy at equivalent plasma concentrations.

2.3.1 Active Targeting

Active targeting requires site-directed ligands to bind and interact with surfaces at the target site. Various targeting moieties or ligands against tumor-cell-specific receptors have been immobilized on the surface of drug carriers to deliver them within cells via receptor mediated endocytosis. Targeting ligands attached to the surface of nanoparticles may act as 'homing devices', improving the selective delivery of drug to specific tissue and cells. This is especially true for targets that are readily accessible from the vasculature.

Ligand	Drug	System	Target cells	Evaluation
Nucleic acids				
Aptamers[108]		PLA	Prostate epithelial cells	*In vitro*
ECM proteins				
Integrin[109]	Raf genes	Liposomes	Melanoma cells	*In vivo*
RGD peptides[110]	siRNA	Poly(ethylene imine)	Tumor vasculature	*In vivo*
Fibrinogen[111]	Radioisotopes	Albumin	Tumor vasculature	*In vivo*
Von Willebrand Factor[75,76] (Rexin-G®)	Cyclin gene	Viral particles	Pancreatic cancers	*In vivo*
Lipids				
MP Lipid A[87]		PLGA	Dendritic cells	*In vitro*
Carbohydrates				
Galactose[112]	Retinoic acid	PLA	Hepatocytes	*In vitro*
Hyaluronic acid[113]	Doxorubicin	Liposomes	CD44+ melanoma cells	*In vitro*
Peptidomimetics[114]	Various	mPEG/PLGA	Brain cells	Various
Antibodies to:				
HER2 receptor[115]		Gelatin/HAS	HER2 cells	*In vitro*
HER2 receptor[26]	Doxorubicin	Liposomes	HER2 cells	*In vivo*
CD19[29]	Doxorubicin	Liposomes	B cell lymphoma	*In vivo*
Vitamins				
Folate[116]	Doxorubicin	Liposomes	Leukemia cells	*In vivo*
Other				
Albumin[72,73,74] (Abraxane®)	Paclitaxel	Albumin-drug conjuate	Breast cancers	*In vivo*

Fig 2-6 List of ligand targeted nanoparticulate systems evaluated for *in vitro* and *in vivo* therapeutics delivery (Tarek. M. Fahmy 2005)

When tumor cells were administered intravenously in mice, active targeting was found to increase the therapeutic index of the drug when tumors were just growing (Ahmad, Longenecker et al. 1993; Moase, Qi et al. 2001).

2.3.1.1 Concept of “Magic Bullets”

Fig 2-7 Dr. Paul Ehrlich (Father of Chemotherapy)

The concept of targeted therapy was first postulated by Paul Ehrlich by introducing ‘magic bullet’ in the year 1906.

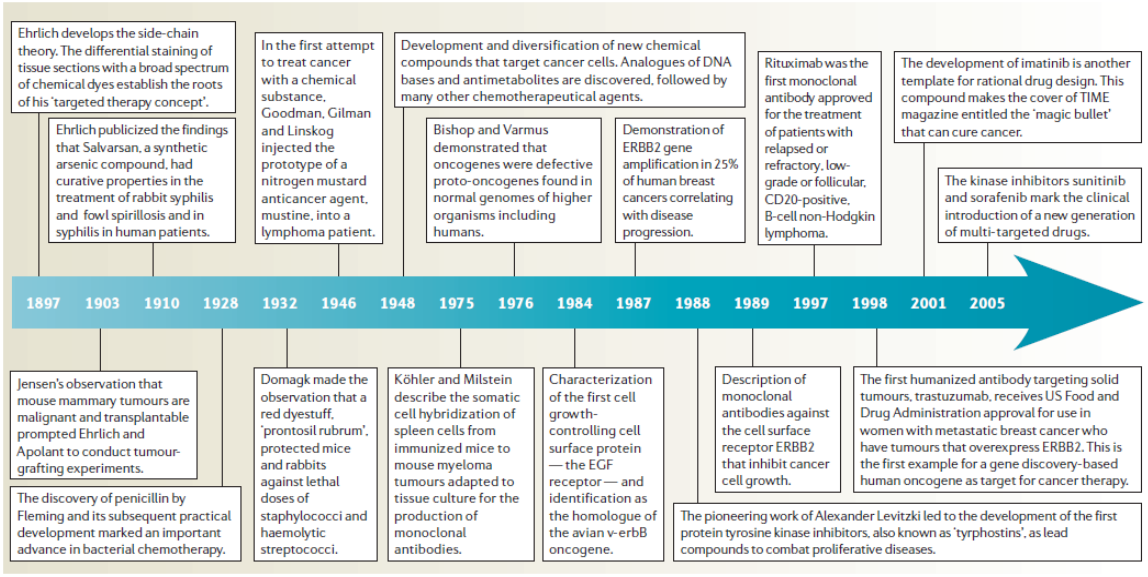


Fig 2-8 Cancer Therapy Progress since Ehrlich’s finding (Strebhardt and Ullrich 2008)

Since then, magic bullet started finding the usage in clinical trials based on (1) finding the proper target for a particular disease state, (2) finding a drug that effectively treats the disease and (3) finding a means of carrying the drug in a stable form to specific sites while avoiding non specific interactions that clears any foreign particles from the body.

2.3.1.2 Folic Acid

Folic Acid is one of the most extensively studied small molecule targeting moieties for drug delivery, which is used to avoid non-specific attacks of the anticancer drug on normal tissues as well as to increase their cellular uptake within the target cells as studied in several previous studies (Lu JY 1999; Reddy and Low 2000; Lu and Low 2002). Folate targeted drug delivery has emerged as an alternative therapy for the treatment and imaging of many cancers and inflammatory diseases. It was said that the administration of folic acid accelerated the progression of leukemia (Farber, Cutler et al. 1947; Kim 2008). Folates are low molecular weight pterin-based vitamins required by eukaryotic cells for one-carbon metabolism and *de novo* nucleotide synthesis. Folate was often covalently attached to a wide variety of drug delivery carriers such as liposomes, polymer conjugates, and nano-particulates (Gabizon, Horowitz et al. 1999; Goren, Horowitz et al. 2000; Reddy and Low 2000). The high affinity vitamin is a commonly used ligand for cancer targeting because folate receptors (FRs) are frequently over-expressed in a range of tumor cells (Antony 1992). Folate specifically binds to FRs with a high affinity ($K_D = \sim 10^{-9}$ M), enabling a variety of folate derivatives and conjugates to deliver molecular complexes to cancer cells without causing harm to normal cells. The FR is a tumor-associated protein, and it can actively internalize bound folates and folate-drug conjugates via the natural process of endocytosis (Kamen 1986; Leamon 1991).

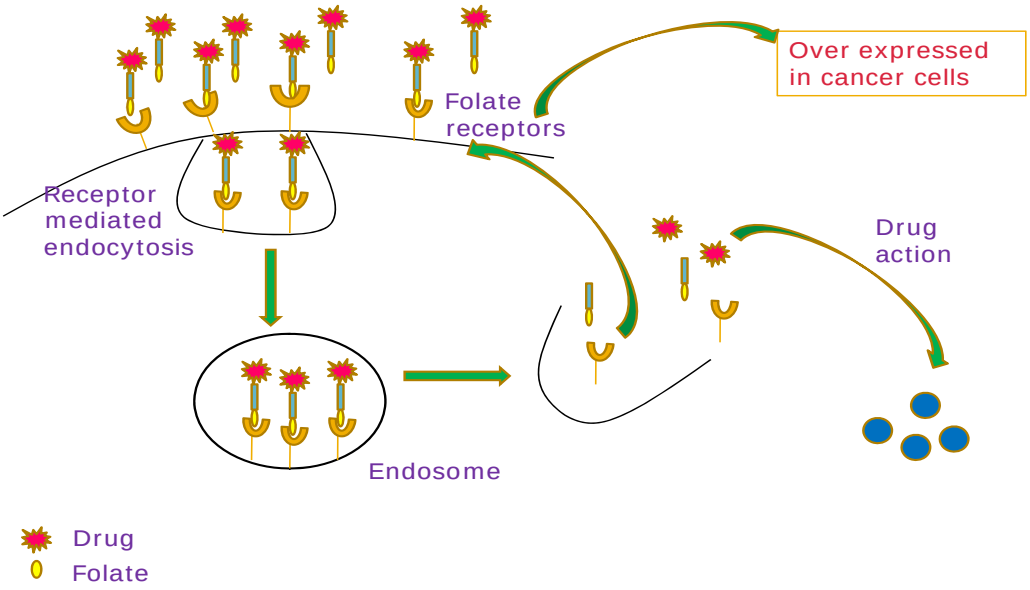


Fig 2-9 Folate mediated targeting

It has been used as a targeting moiety combined with a wide array of drug delivery vehicles including liposomes, protein toxins, polymeric NPs, linear polymers, and dendrimers to deliver drugs selectively into cancer cells using FR-mediated endocytosis (Benns, Mahato et al. 2002; Quintana, Raczka et al. 2002). The attractiveness of folate has been further enhanced by its high binding affinity, low immunogenicity, ease of modification, small size, stability during storage, compatibility with a variety of organic and aqueous solvents, low cost, and ready availability (Reddy and Low 1998).

2.3.1.3 Monoclonal Antibody (Herceptin)

The discovery of antigens that are particularly overexpressed on the surface of cancer cells suggests that by using certain monoclonal antibodies (mAbs) to selectively mark tumor cells, malignant tissues could be distinguished from normal tissues (Liu, K. M.; Derr et al. 1996). These mAbs could be used as vehicles to deliver cytotoxic drugs selectively to tumor cells (Chari, Jackel et al. 1995; Chari 1998). The mAb moiety then binds to the antigens on cancer cells and the conjugate is internalized via receptor-mediated endocytosis followed by the release of parent drug to restore its original activity.

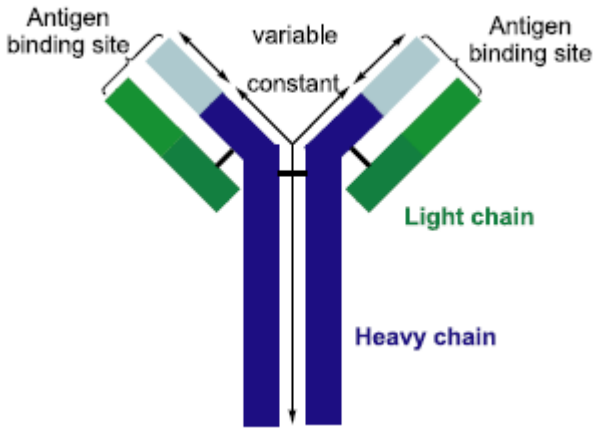


Fig 2-10 Antibody structure

Monoclonal antibodies are monospecific antibodies that are identical because they are produced by one type of immune cell that are all clones of a single parent cell. One of the main applications of the monoclonal antibody is in cancer treatment which involves the antibodies to bind only to cancer cell-specific antigens and induce an immunological response against the target cancer cell. These can also be modified for delivery of active conjugates etc.

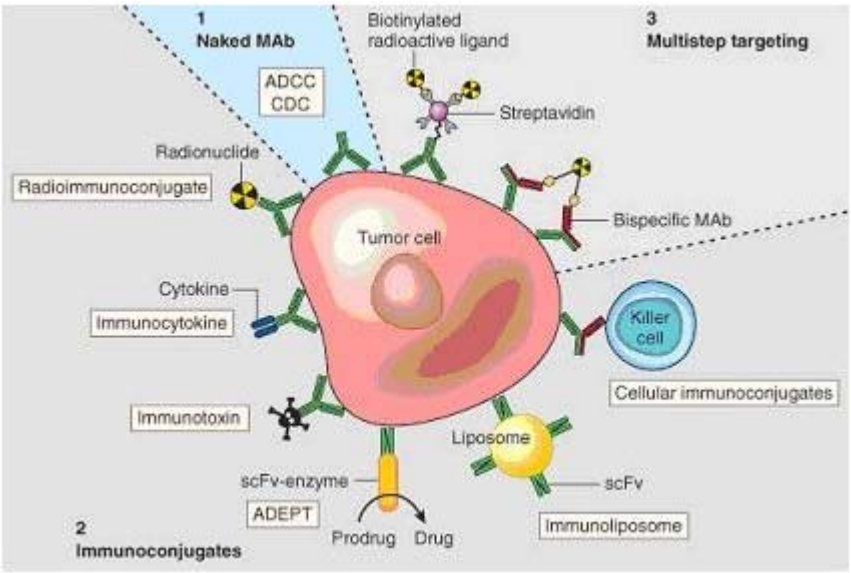


Fig 2-11 Monoclonal antibodies for cancer

(http://www.edinformatics.com/biotechnology/MonoclonalAb.jpg)

It is also possible to design bispecific antibodies that can bind with their Fab (Antigen binding fragment) regions both to target antigen and to a conjugate or effector cell. Monoclonal antibodies have been generated and approved to treat diseases like cancer, cardiovascular disease, inflammatory disease, multiple sclerosis, viral infection etc. It was reported by the Pharmaceutical Research and Manufacturers of America, that in 2006, U.S. companies had 160 different monoclonal antibodies in clinical trials or awaiting approval by Food and Drug Administration (FDA).

Herceptin is one of a new group of cancer drugs called monoclonal antibodies. Herceptin, also called trastuzumab, is a monoclonal antibody that interferes with the HER2/neu receptor. It is designed to target HER positive cancer cells. They are thought to stop the cancer cells from

growing. The HER2 receptors are proteins that are embedded in the cell membrane and communicate molecular signals from outside the cell to inside the cell, and turn genes on and off. In some cancers, including breast cancers, the HER2 receptor is defective and stuck in the "on" position, and causes breast cells to reproduce uncontrollably, causing breast cancer (Hudis 2007). Antibodies are molecules from the immune system that bind selectively to different proteins.

Type	Application	Mechanism	Mode
infliximab	▪ rheumatoid arthritis ▪ Crohn's disease	inhibits TNF-α	chimeric
basiliximab	▪ Acute rejection of kidney transplants	inhibits IL-2 on activated T cells	chimeric
abciximab	▪ Prevent coagulation in coronary angioplasty	inhibits the receptor GpIIb/IIIa on platelets	chimeric
daclizumab	▪ Acute rejection of kidney transplants	inhibits IL-2 on activated T cells	humanized
gemtuzumab	▪ relapsed acute myeloid leukaemia	targets an antigen on leukemia cells	humanized
alemtuzumab	▪ B cell leukemia	targets an antigen CD52 on T- and B-lymphocytes	humanized
rituximab	▪ non-Hodgkin's lymphoma	targets phosphoprotein CD20 on B lymphocytes	chimeric
palivizumab	▪ RSV infections in children	inhibits an RSV protein	humanized
trastuzumab	▪ anti-cancer therapy for a specific kind of breast cancer	targets the HER2/neu (erbB2) receptor	humanized
etanercept	▪ rheumatoid arthritis	contains TNF receptor	fusion protein
adalimumab	▪ rheumatoid arthritis ▪ Crohn's disease	inhibits TNF-α	human
Nimotuzumab	▪ Approved in SCCHN, Glioma ▪ Clinical trials for other indications underway	EGFR inhibitor	Humanized

Fig 2-12 Monoclonal antibodies for various applications

Trastuzumab is an antibody that binds selectively to the HER2 protein. When it binds to defective HER2 proteins, the HER2 protein no longer causes the breast cells to reproduce uncontrollably. This increases the survival of people with cancer. However, cancers usually develop resistance to trastuzumab. The combination of Trastuzumab with chemotherapy has been shown to increase both survival and response rate, in comparison to Trastuzumab alone (Nahta and Esteva 2003).

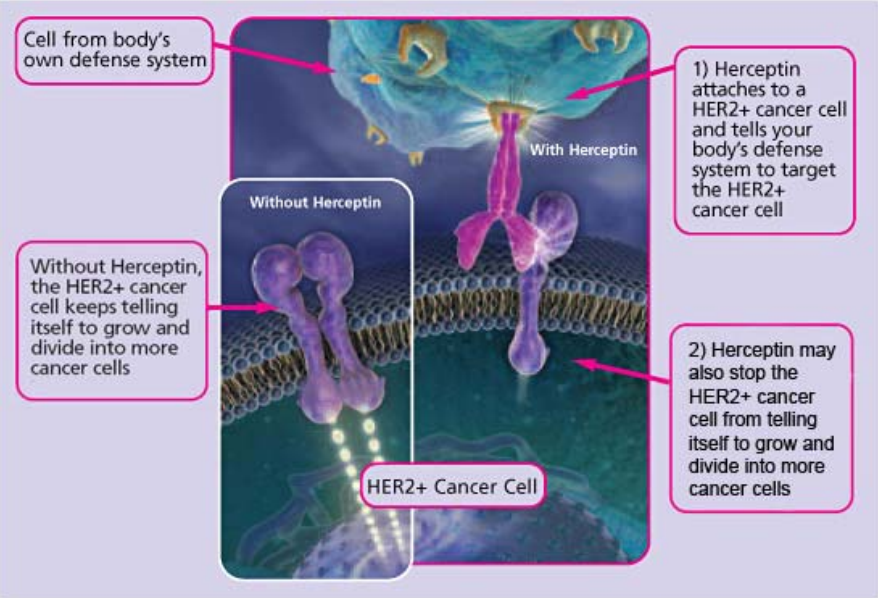


Fig 2-13 Herceptin action with breast cancer cells (http://www.herceptin.com/metastatic/what-is/how-does-it-work.jsp)

Cells treated with trastuzumab undergo arrest during the G1 phase of the cell cycle, so there is reduced proliferation. Also, trastuzumab suppresses angiogenesis by both induction of anti-angiogenic factors and repression of pro-angiogenic factors. It is thought that a contribution to the unregulated growth observed in cancer could be due to proteolytic cleavage of HER2/neu that results in the release of the extracellular domain. Trastuzumab has been shown to inhibit HER2/neu ectodomain cleavage in breast cancer cells (Albanell, Codony et al. 2003). One of the significant complications of trastuzumab is its effect on the heart. Trastuzumab is associated with cardiac dysfunction in 2-7% of cases (Seidman, Hudis et al. 2002). Approximately 10% of patients are unable to tolerate this drug because of pre-existing heart problems; physicians are balancing the risk of recurrent cancer against the higher risk of death due to cardiac disease in this population. The risk of cardiomyopathy is increased when trastuzumab is combined with

anthracycline chemotherapy (which itself is associated with cardiac toxicity). The other side effects are tumor pain, diarrhea, flu-like symptoms, headaches, allergic reactions etc.

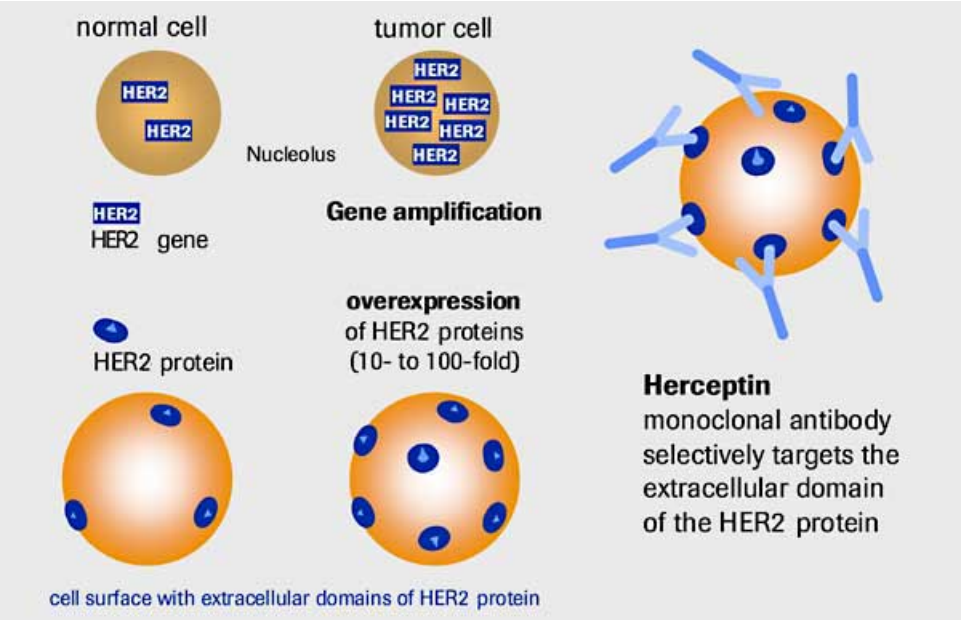


Fig 2-14 Mechanism of action of Herceptin (http://www.roche.com/pages/facets/9/herc2.jpg)

2.3.1.4 Polyunsaturated Fatty Acids

The polyunsaturated fatty acids (PUFAs) function to target the tumors. Essential fatty acids are polyunsaturated fatty acids (PUFAs) that can be obtained only from the diet. There are several known PUFAs having 18, 20, and 22 carbons, and 2–6 unconjugated cis-double bonds separated by one methylene. Vegetable oils are the source of alinolenic acid (LNA), linoleic acid (LA), and arachidonic acid (AA), while cold-water fish is the supply for eicosapentaenoic acid (EPA) and

docosahexaenoic acid (DHA). AA can be obtained also from meat (Hardman 2002; Tapiero 2002).

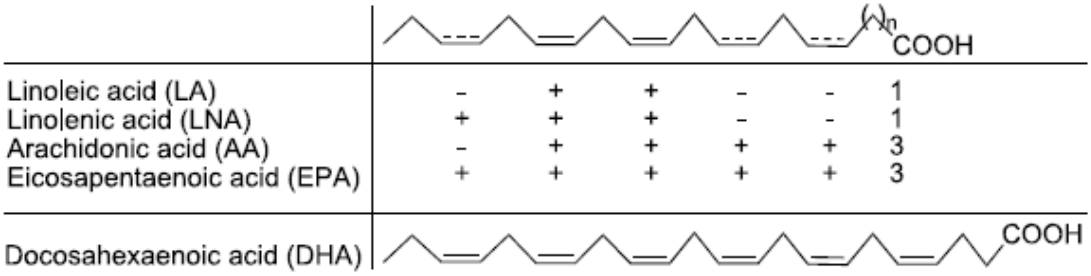
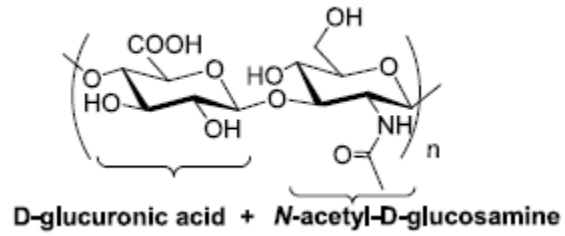


Fig 2-15 PUFAs

PUFAs have exhibited anticancer activity against CFPAC, PANC-1, and Mia-Pa-Ca-2 pancreatic and HL-60 leukemia cell lines, and their antitumor activities have been evaluated in preclinical and clinical studies (Wigmore, Ross et al. 1996; Hawkins, Sangster et al. 1998). Moreover, it has been shown that PUFAs are taken up greedily by tumor cells, presumably for use as biochemical precursors and energy sources (Sauer, Nagel et al. 1986; Sauer and Dauchy 1992). In addition, PUFAs are readily incorporated into the lipid bilayer of cells, which results in disruption of membrane structure and fluidity (Takahashi, Przetakiewicz et al. 1992; Grammatikos, Subbaiah et al. 1994). This has been suggested to influence the chemosensitivity of tumor cells (Diomede and J.; Salmona 1993). These findings strongly suggest the benefits in the use of PUFAs for tumor-targeting drug delivery. For example PUFA–taxoid conjugates have a high potential to become efficacious tumor-targeting chemotherapeutic agents in cancer therapy.

2.3.1.5 Hyaluronic Acid



Scheme 2-3 Hyaluronic Acid

Hyaluronic acid (or hyaluronan) (HA) is a linear, negatively charged polysaccharide, containing two alternating units of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) with molecular weight of 105–107. HA is responsible for various functions within the extracellular matrix such as cell growth, differentiation, and migration. A wide range of activities can be explained by a large number of HA-binding receptors such as cell surface glycoprotein CD44, receptor for hyaluronic acid-mediated motility (RHAMM), and several other receptors possessing HA-binding motifs, for example, transmembrane protein layilin, hyaluronic acid receptor for endocytosis (HARE), lymphatic vessel endocytic receptor (LYVE-1), and also intracellular HA-binding proteins including CDC37, RHAMM/IHABP, P-32, and IHABP4 (Huang, Grammatikakis et al. 2000; Ponta, Sherman et al. 2003). It has been shown that the HA level is elevated in various cancer cells (Toole, Wight et al. 2002). The higher concentration of HA in cancer cells is believed to form a less dense matrix, thus enhancing the cells motility as well as invasive ability into other tissues (Yang, Zhang et al. 1993) and also providing an immunoprotective coat to cancer cells (McBride and Bard 1979). It is well known that various tumors, for example, epithelial, ovarian, colon, stomach, and acute leukemia, overexpress HA-

binding receptors CD44 (Day and Prestwich 2002) and RHAMM (Turley, Belch et al. 1993). Consequently, these tumor cells show enhanced binding and internalization of HA (Hua, Knudson et al. 1993). HA can be coupled with an active cytotoxic agent directly to form a non-toxic prodrug. Alternatively, a suitable polymer with covalently attached HA and drug can be used as a carrier. Direct conjugations of a low molecular weight HA to cytotoxic drugs such as butyric acid, paclitaxel, and doxorubicin have been reported. It has been shown that these bioconjugates are internalized into cancer cells through receptor-mediated endocytosis, followed by intracellular release of active drugs, thus restoring their original cytotoxicity.

2.3.1.6 Peptides

Peptide-based targeting of tumor-associated receptors is an attractive approach in tumor-specific drug delivery because high-affinity sequences can be discovered through screening of combinatorial libraries. Recently, numbers of peptides and their conjugates with cytotoxic agents that target different cancer cell receptors have emerged as potential tumor-specific chemotherapeutic agents. Gastrointestinal (GI) peptides have many physiological functions as hormones, neurotransmitters, and growth factors. Each of these peptides usually targets more than one receptor. Thus, these peptides and their truncated analogs, possessing appropriate recognition properties, could serve as tumor-targeting molecules in combination with cytotoxic agents. Somatostatin (SST) is a hormonal neuropeptide existing in two active forms, that is, SST-14 and SST-28 with 14 and 28 amino acid residues, respectively. SST-14 and SST-28 interact with cells through a minimum of five membrane receptor subtypes (SSTR1–5) inhibiting the secretion of various hormones including the growth hormone (GH) also known as somatotropin (Schally 1988). The SSTR1–5 membrane receptors are expressed at significantly elevated levels in tumor cells and possess high binding affinity to somatostatin (Weckbecker, Raulf et al. 1993; Orlando,

Raggi et al. 2004). Thus, somatostatin is a good candidate for delivery of cytotoxic agents specifically to GI tumor cells. Bombesin (BBN) and the bombesin-like peptide, gastrin-releasing peptide (GRP), consist of 14 and 27 amino acid residues, respectively, and have several physiological functions as gastrointestinal hormones and neurotransmitters (Schally, Comaru-Schally et al. 2001). Moreover, these peptides also function as growth factors and modulate tumor proliferation (Cuttitta, Carney et al. 1985).

2.3.2 Passive Targeting and EPR Effect

Strategies on delivering various drug formulations to cancerous cells make use of the passive targeting. Aggressive tumors inherently develop leaky vasculature with 100-800 nm pores due to rapid formation of vessels that must serve the fast-growing tumor. This defect in vasculature coupled with poor lymphatic drainage serves to enhance the permeation and retention of drug formulations within the tumor region. This is often called EPR (Enhanced Permeation and Retention) effect (Teicher 2000; Sledge and Miller 2003). Thus the passive targeting uses the unique properties of the tumor microenvironment, (1) leaky tumor vasculature, which is highly permeable to macromolecules relative to normal tissue and (2) a dysfunctional lymphatic drainage system which results in enhanced fluid retention in the tumor interstitial space (Matsumura and Maeda 1986; Maeda and Matsumura 1989).

The EPR effect, related to the transport of macromolecular drugs composed of liposomes, micelles, proteinaceous or polymer-conjugated macromolecules, lipid particles, and nanoparticles into the tumor, is the hallmark of solid tumor vasculature. These macromolecular species are therefore ideal for selective delivery to tumor. The EPR effect has facilitated the development of macromolecular drugs consisting of various polymer-drug conjugates (pendant type), polymeric micelles, and liposomes that exhibit far better therapeutic efficacy and far fewer side effects than the parent low-molecular-weight compounds. Normal tissues contain capillaries with tight

junctions that are less permeable to nanosized particles. Passive targeting can therefore result in increase in drug concentrations in solid tumors of several-fold relative to those obtained with free drugs (Moghimi, Hunter et al. 2001).

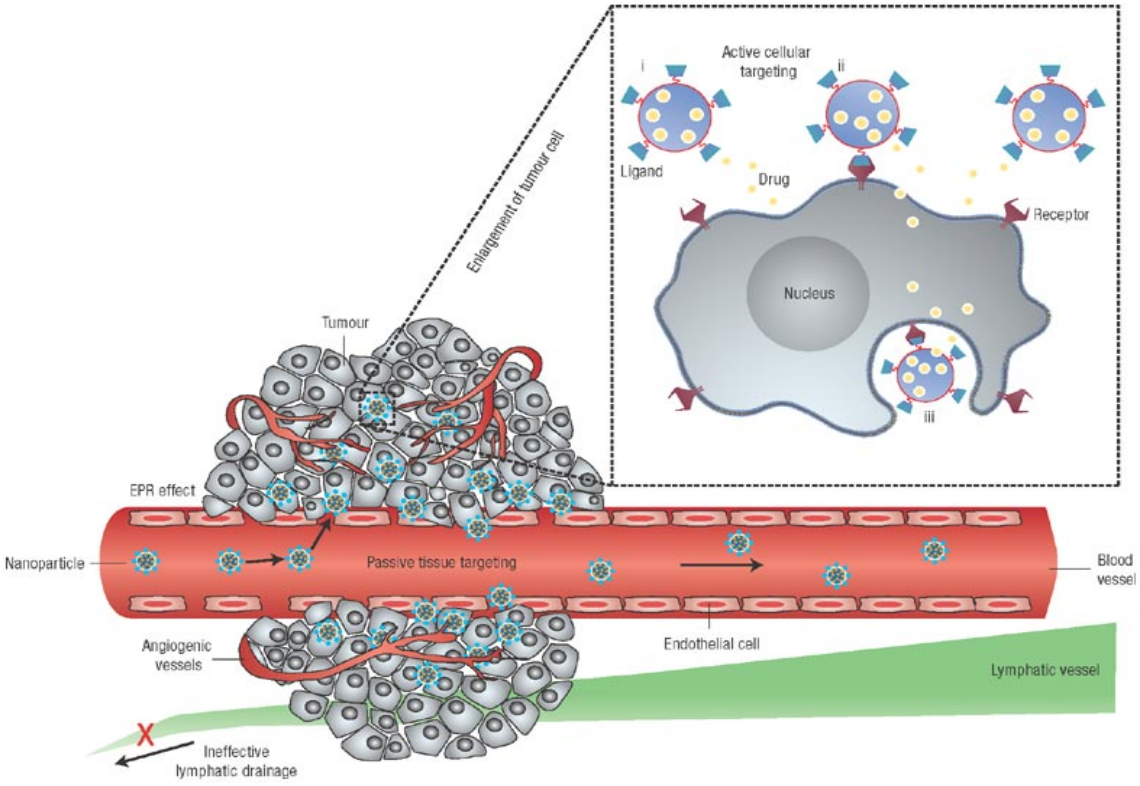


Fig 2-16 Representation of EPR effect and active targeting for drug delivery to tumors (http://www.nature.com/nnano/journal/v2/n12/images/nnano.2007.387-f1.jpg)

The key mechanism for the EPR effect for macromolecules in solid tumors was found to be retention, whereas low-molecular weight substances were not retained but were returned to circulating blood by diffusion (Noguchi, Wu et al. 1998). It was found that macromolecules remain at high levels in the blood circulation; this phenomenon applies to most plasma proteins

and biocompatible synthetic polymers or their conjugates. Here, macromolecules are defined as larger than 40 KDa.

Factors involved in enhanced vascular permeability in solid tumors (Maeda, Wu et al. 2000)

Architectural defectiveness	
Angiogenesis: High vascular density	
Impaired lymphatic drainage	
Generation of permeability-enhancing factors	
(1) Bradykinin/^{3}hydroxyprolyl bradykinin	(6) Matrix metalloproteinases
(2) Nitric oxide ($NO^{\cdot}$)	(7) Other proteases (e.g. kallikrein)
(3) Peroxynitrite ($ONOO^-$)	(8) Other cytokines (e.g. tumor necrosis factor)
(4) Prostaglandins	
(5) VPF (VEGF)	

2.4 Drug Delivery Strategies for Cancer Chemotherapy

2.4.1 Liposomes

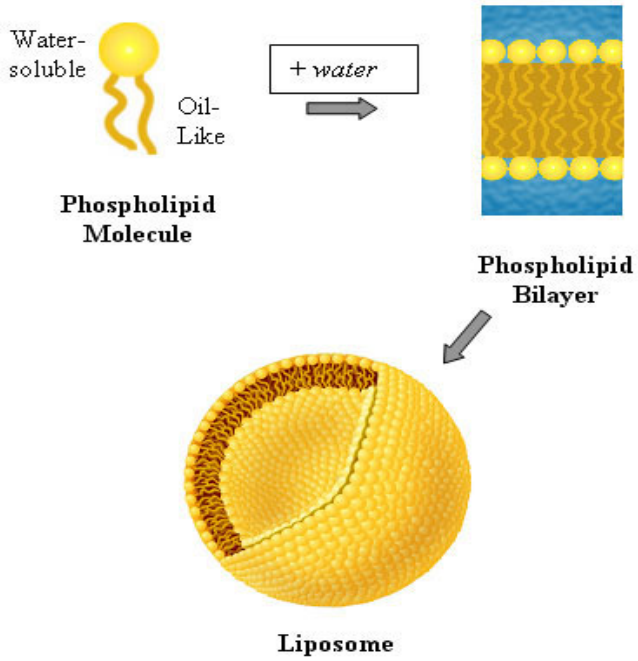


Fig 2-17 Liposome formation (http://www.nanolifenutra.com/images/image_liposome_01.jpg)

Liposomes are drug delivery vehicles which were first proposed by Gregoriadis and are composition of amphiphilic phospholipids and cholesterol that self-associate into bilayers encapsulating an aqueous interior. These may be formulated into small structures (80-100 nm in size) that encapsulate either hydrophilic drugs in the aqueous interior or hydrophobic drugs within the bilayer.

Encapsulation of drugs is achieved using a variety of loading methods, most notably the pH gradient method used for loading vincristine (Waterhouse, Madden et al. 2005) or the ammonium sulfate method for loading doxorubicin (Haran, Cohen et al. 1993). Additionally, the liposome surface can be engineered to improve its properties (Allen, Sapra et al. 2002; Sapra and Allen 2003). So far, the most noteworthy surface modification is the incorporation of polyethylene glycol (PEG) which serves as a barrier, preventing interactions with plasma proteins and thus retarding recognition by the reticuloendothelial system (RES) (Gabizon, Shmeeda et al. 2003) and enhancing the liposome circulation lifetime. However, despite this versatility, there have been major drawbacks to the use of liposomes for targeted drug delivery, most notably, poor control over release of the drug from the liposome (i.e. the potential for leakage of the drug into the blood), coupled with low encapsulation efficiency, manufacturability at the industrial scale and poor stability during storage (Soppimath, Aminabhavi et al. 2001; Hans 2002).

2.4.2 Nanoparticles

In recent decades, there has been increased interest in the use of nanoparticles for drug delivery applications. Nanoparticles are colloidal-sized particles, possessing diameters ranging between 1 and 1000 nm, and drugs may be encapsulated, adsorbed or dispersed in them. A wide variety of nanoparticles composed of a range of materials including lipids, polymers and inorganic materials have been developed, resulting in delivery systems that vary in their physicochemical properties and thus their applications (Liggins and Burt 2002; Gabizon, Shmeeda et al. 2003; Klumpp,

Kostarelos et al. 2006). Nanoparticles are used to deliver hydrophilic drugs, hydrophobic drugs, proteins, vaccines etc. They can be synthesized by dispersion of polymers and polymerization of monomers, which involves solvent extraction/evaporation method, salting out method, dialysis method, supercritical fluid spray technique and nanoprecipitation method (Feng SS 2003). Among these, the solvent extraction method is the most commonly used one that uses single emulsion method and double emulsion method for hydrophobic drugs and hydrophilic drugs respectively.

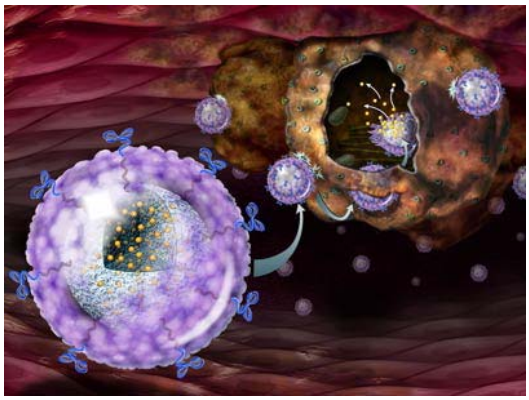


Fig 2-18 drug delivery by targeted nanoparticles
(http://web.mit.edu/newsoffice/2008/nanoparticles.jpg)

The solid biodegradable polymeric nanoparticles have certain advantages that makes is an attractive area of drug delivery. First, by varying the polymer composition of the particle and morphology, one can effectively tune in a variety of controlled release characteristics, allowing moderate constant doses over prolonged periods of time (Shive and Anderson 1997). There has been variety of materials used to engineer solid nanoparticles both with and without surface functionality (Brigger, Dubernet et al. 2002). Perhaps the most widely used are the aliphatic polyesters, specifically the hydrophobic poly(lactic acid) (PLA), the more hydrophilic poly(glycolic acid) (PGA), and their copolymers, poly(lactide-co-glycolide) (PLGA). The

degradation rate of these polymers, and often the corresponding drug release rate, can vary from days (PGA) to months (PLA) and is easily manipulated by varying the ratio of PLA to PGA. Second, physiologic compatibility of PLGA and its homopolymers PGA and PLA have been established for safe use in humans. These materials have a history of over 30 years in various human clinical applications, including drug delivery systems (Langer and Folkman 1976; Visscher, Robison et al. 1985). Thus, PLGA nanoparticles can be formulated in a variety of ways that improve drug pharmacokinetics and biodistribution to target tissue by either passive or active targeting. Also the advantages include targeting drugs to tumors, size availability for intravenous injection, reduction in uptake of drugs to RES, improving biodistribution of drugs in the body (Kim, Lee et al. 2003).

The synthesized nanoparticles can be characterized for size and size distribution, surface and bulk morphology, surface chemistry, surface charge, physical and chemical status of the drug and drug encapsulation efficiency (Feng SS 2003). Recently, nanoparticles using the innovative PLA-TPGS co-polymer has been used to achieved to deliver anticancer drugs like Paclitaxel, Docetaxel, Doxorubicin etc (Mu and Feng 2002; Mu and Feng 2003; Feng, Mu et al. 2004; Win and Feng 2005; Zhang and Feng 2006; Zhang, Huey Lee et al. 2007; Pan and Feng 2008; Pan, Wang et al. 2008).

2.4.3 Micelles

Polymeric micelles are formed from spontaneous association of amphiphilic copolymers in an aqueous phase. They are characterized by a diameter not exceeding 100 nm. The attractive force leading to micellization is based on an interaction between the hydrophobic and electrostatically neutral parts of copolymers. Self-assembly starts when the copolymer concentration reaches a threshold value known as the critical micelle concentration (CMC). Usually, the CMC of amphiphilic copolymers is 1000-fold weaker than that of low molecular weight surfactants (10^{-6} –

10^{-7} M) (La, Okano et al. 1996). The micelle shape depends on the length of the lipophilic chains (Zhang, Yu et al. 1996). The formation of micelles effectively removes the hydrophobic portion of the amphiphile from the solution minimizing unfavourable interactions between the surrounding water molecules and the hydrophobic groups of the amphiphile. If the amphiphile concentration in solution remains above the CMC, micelles are thermodynamically stabilized against disassembly. Upon dilution below CMC, micelles will disassemble with the rate of disassembly being largely dependent on the structure of amphiphiles and interactions between the chains (C. Allen 1999).

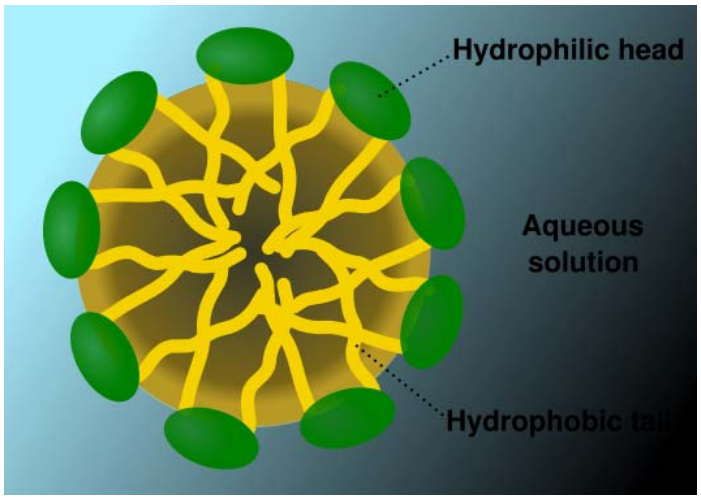


Fig 2-19 Structure of Micelle (http://politicook.net/wp-content/uploads/2008/05/532px-micelle_scheme-ensvg.png)

Currently, polymeric micelles are popular pharmaceutical nanocarriers for the delivery of poorly water soluble drugs, which can be solubilized within the hydrophobic inner core of the micelle (Bader H 1984; Jones and Leroux 1999). As a result, micelles can substantially improve solubility and bioavailability of various hydrophobic drugs (Lukyanov and Torchilin 2004). The small size (10-100 nm) of micelles allows for the micelle efficient accumulation in pathological tissues with the permeabilized vasculature, such as tumors and infarcts, via enhanced permeation and

retention (EPR) effect (Wu, Da et al. 1993; Maeda, Wu et al. 2000; Torchilin 2001). The hydrophilic blocks commonly used in drug delivery are polyethers like poly(propylene oxide) and PEG (Vakil and Kwon 2005) with a molecular mass comprised between 1 and 15 KDa (Torchilin 2004). Other hydrophilic polymers may be used (Torchilin, Trubetskoy et al. 1995). Polymeric nanocarriers must have a size larger than 42-50 KDa in order to prevent their elimination by the glomerular excretion of kidneys (Seymour, Duncan et al. 1987). Various hydrophobic anticancer agents including paclitaxel (Nakayama M 2006) and docetaxel (Le Garree D. Gori S 2005) were incorporated into the hydrophobic core of polymeric micelles. These drugs can be chemically conjugated to macromolecules.

2.4.4 Microspheres

Microspheres are prepared by commonly used methods such as solvent evaporation and spray drying. It is also used for microencapsulation methods (Vasir, Tambwekar et al. 2003). Mostly the polymeric microspheres are synthesized. In the solvent evaporation method, spherical droplets can be formed by dispersing hydrophobic monomers in aqueous solution or hydrophilic monomers in an organic phase. Usually, double emulsion method is used. In this, the first water in oil emulsion, in which drug is dispersed in water, is dispersed in another aqueous medium to get the final oil in water emulsion. Microspheres can protect the drug molecules against degradation, control their release after administration and facilitate their passage across biological barriers. Using a double emulsion solvent evaporation method, some researchers have achieved a constant release of drug from the micelles after initial burst (Yang YY 2000). Recently, polymeric microspheres are synthesized using PMMA polymer, which are useful in tattoo making. Since PMMA is completely hypoallergenic, it can be used in various applications.

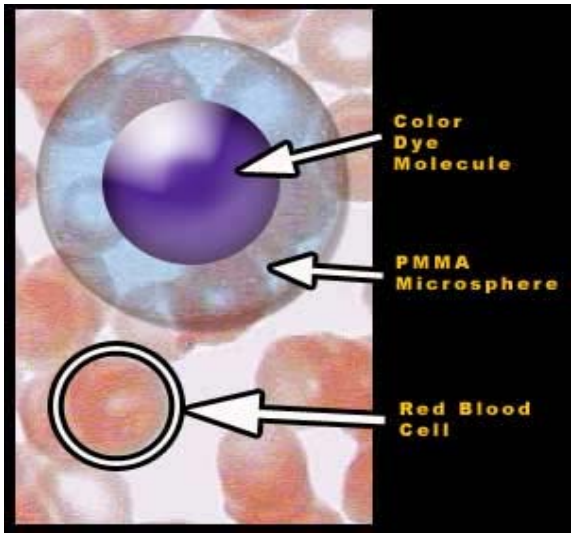


Fig 2-20 Microspheres (http://www.crazychameleonbodyartsupply.com/images/PMMA-microsphere.jpg)

2.4.5 Paste

Polymer paste in chemotherapeutic are used to maximize local drug level in tumor environment but minimize systemic exposure to normal tissues during local administration or direct injection of chemotherapeutic agents. The polymer paste is prepared by loading the chemotherapeutic agents like paclitaxel. The base component used is PCL that has low melting point 50-60° C and biodegradation life time of 6-9 months *in vivo* (Pitt, Gratzl et al. 1981). The paste is also said to suppress tumor growth by intra tumoral injection of the paste and due to its slow release (Jackson, Gleave et al. 2000).

2.5 Prodrugs

2.5.1 Concept of Prodrug

A Prodrug is a form of a drug that remains inactive during its delivery to the site of action and is activated by the specific conditions in the targeted site as illustrated in Fig 2-21. The conjugation of a drug with a polymer is called ‘polymeric prodrug’. Albert and his coworkers were the first

ones to suggest the concept of prodrug approach for increasing the efficiency of drugs in 1950. The prodrug approach has been one of the most promising means of site-specific drug delivery (Takakura and Hashida 1995). Currently, 5-7% of the drugs approved worldwide can be classified as prodrugs and approximately 15% of all new drugs approved in 2001 and 2002 were prodrugs (Rautio, Kumpulainen et al. 2008). Here, antitumor drug-macromolecular conjugates are called as 'macromolecular prodrugs'.

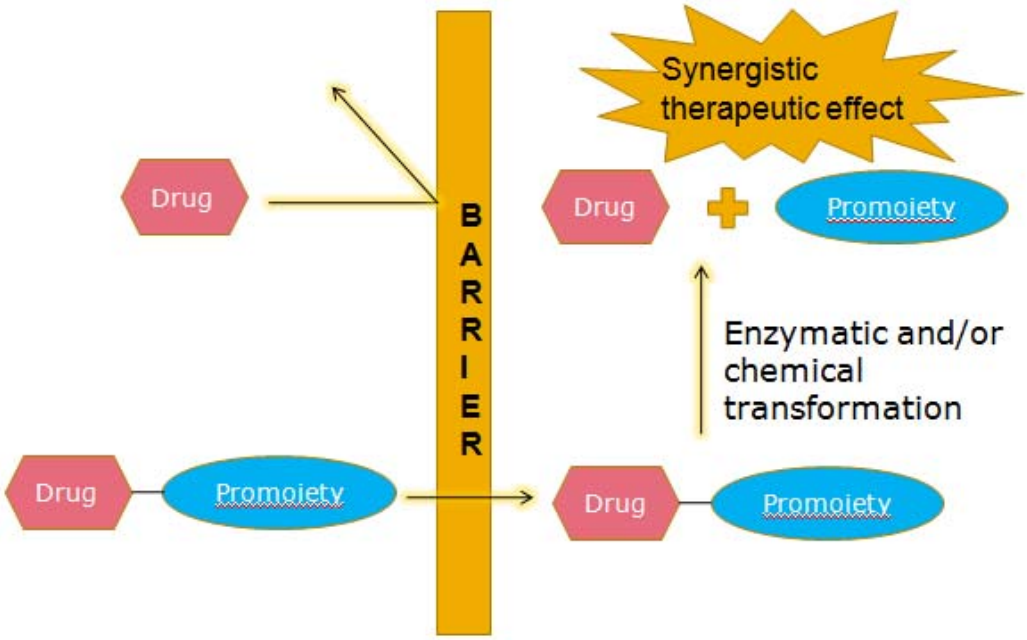


Fig 2-21 An illustration of the Concept of Prodrug (Stella and Nti-Addae 2007)

2.5.2 Why Prodrugs?

The prodrugs are developed mainly to overcome the drawbacks of the drugs such as site specificity, permeability, resistance and hydrophobicity. The use of prodrugs can be reasoned due to its advantages that include (1) an increase in water solubility of low soluble or insoluble drugs, and thus enhancement of drug bioavailability, (2) protection of drug from deactivation and

preservation of its activity during circulation, transport to targeted organ or tissue and intracellular trafficking, (3) an improvement in pharmacokinetics, (4) a reduction in antigenic activity of the drug leading to a less pronounced immunological body response, (5) the ability to provide passive or active targeting of the drug specifically to the site of its action, (6) the possibility to form an advanced complex drug delivery system, which in addition to drug and polymer carrier, includes several other active components that enhance the specific activity of the main drug.

2.5.3 Classification of Prodrugs

Prodrugs are classified mainly into 2 types

(1) Carrier-linked prodrugs

The Carrier-linked prodrugs are drugs that are attached through a metabolically labile chemical linkage to another molecule designated as the 'promoiety'. The promoiety alters the physical properties of the drug to increase water or fat solubility or provide site directed delivery. The Carrier-linked prodrugs are further divided into (1) bipartate, (2) tripartate and (3) mutual prodrugs. The bipartate prodrug is composed of one carrier group attached to the drug (eg. Prednisolone, Benzocaine etc) and the tripartate prodrug is composed of carrier group attached to the drug via linker (eg. Bacampicillin, Pivampicillin etc). The mutual prodrugs are composed of 2 drugs linked together (eg. Sultamacillin) (D. Bhosle 2006). The advantages of the carrier-linked prodrugs are increased absorption, injection site pain relief, elimination of unpleasant taste, decreased toxicity, decreased metabolic inactivation, increased chemical stability and prolonged or shortened action.

(2) Bioprecursor prodrugs

Bioprecursors are those metabolized into new compound that may itself be active or further metabolized into an active metabolite (eg. Amine to aldehyde to carboxylic acid). They rely on

oxidative and reductive activation reactions unlike the hydrolytic activation of carrier-linked prodrugs. The oxidative activation reactions are N- and O- Dealkylation (Phenacetin), Oxidative Deamination (Cyclophosphamide), N-Oxidation (Pralidoxime chloride) and Epoxidation (Carbamazepine). The reductive activation reactions are Azo Reduction (Sulfasalazine), Sulfoxide Reduction (Suldinac), Disulfide Reduction (Thiamin), Bioreductive Alkylation (Mitomycin C) and Nitro Reduction.

2.5.4 Polymer-Drug Conjugation

The rationale for polymer conjugation is the possibility to prolong the half-life of therapeutically active agents by increasing their hydrodynamic volume and hence decreasing their excretion rate. Futher more, polymer chains can prevent the approach of antibodies, proteolytic enzymes or cells on conjugated molecules, an effect obtained by the steric hindrance of polymer strands. Immunogenicity is likely to be one of the most serious problems, especially when dealing with heterologous proteins that commonly cause adverse response when recognized as non-self by the body immune system. The prevention of immunogenicity can be attributed to the shielding effect of polymeric chains surrounding the protein. This steric hindrance prevents interaction of antibodies or degrading enzymes with the protein. In general, the conjugation of hydrophilic polymers deeply changes the behavior of the parent (free) compound both *in vitro* and *in vivo*. This change happens with both proteins and low molecular weight agents. Some advantages are (1) increased water solubility, (2) enhanced bioavailability and prolonged plasma half-life, (3) protection towards degrading enzymes, (4) prevention or reduction of aggregation, immunogenicity and antigenicity and (5) specific accumulation in organs, tissues and cells, by active or passive targeting (Maeda, Wu et al. 2000).

The difficulties encountered in the development of successful conjugates of low molecular weight drugs can be attributed to the vast number of chemical and biological factors that has to be taken into consideration namely,

- Conjugate features – eg. Size, polydispersity, solubility, hydrophilic/lypophilic balance, stability, biodegradability, drug loading, free drug amount as impurity, mechanism of drug release
- *In vivo* behavior – eg. Biodistribution, pharmacokinetics, interaction with the blood components and cells, intracellular trafficking, specific targets, metabolism.

Compound	Name	Status	Indication
Polyglutamate–paclitaxel	CT-2103; Xyotax	Phase III	Various cancers, particularly non-small-cell lung cancer; ovarian cancer as a single agent or in combination therapy
Polyglutamate–camptothecin	CT-2106	Phase I	Various cancers
HPMA copolymer–doxorubicin	PK1; FCE28068	Phase II	Various cancers, particularly lung and breast cancer
HPMA copolymer–doxorubicin–galactosamine	PK2; FCE28069	Phase I/II	Particularly hepatocellular carcinoma
HPMA copolymer–paclitaxel	PNU166945	Phase I	Various cancers
HPMA copolymer–camptothecin	MAG-CPT	Phase I	Various cancers
HPMA copolymer–carboplatin platinate	AP5280	Phase I/II	Various cancers
HPMA copolymer–DACH-platinate	AP5346; ProLindac	Phase I/II	Various cancers
Dextran–doxorubicin	AD-70, DOX-OXD	Phase I	Various cancers
Modified dextran–camptothecin	DE-310	Phase I	Various cancers
PEG–camptothecin	Prothecan	Phase II	Various cancers

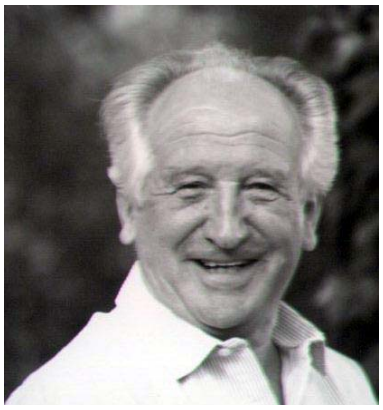
DACH, diaminocyclohexane; HPMA, *N*-(2-hydroxypropyl)methacrylamide; PEG, poly(ethyleneglycol).

Fig 2-22 Polymer-drug conjugates (Duncan 2006)

Usually, a covalent and strategically positioned linkage with the polymer prevents the activity of small drugs. To ensure drug release, several methods have been developed primarily based on

either hydrolytically unstable bond or enzymatically labile spacers between the drug and the polymer. To maximize the outcomes and better tailor the polymer conjugation, a number of different polymers and chemical approaches were also developed, yielding a selection of new structures like dendrimers (Tomalia DA 1985), dendronized polymers, graft polymers, block copolymers (Pechar, Ulbrich et al. 2000), branched polymers (Stiriba, Kautz et al. 2002), multivalent polymers, stars and hybrid glycol and peptide derivatives.

2.5.5 Ringsdorf model



Helmut Ringsdorf

The Ringsdorf model proposed in 1975 by Helmut Ringsdorf, describes the ideal polymeric prodrug model for the polymer-low molecular weight drug conjugates. The spacer should assist mild drug fixation. The spacers are classified as permanent and temporary spacer. The permanent spacers are those that interfere in the biological activity of the drug and temporary spacers are those that do not interfere in the biological activity of the drug.

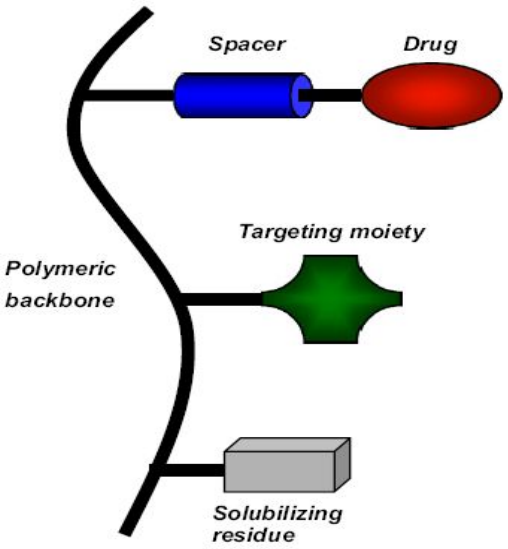


Fig 2-23 Ideal polymeric prodrug model (Pasut 2007)

The targeting moiety of the Ringsdorf model is used for specific resorption at the biological target cells. The solubilizing residue of the model functions in adding non-toxic, non-immunogenic and soluble character to polymer chain.

2.5.6 Design of Polymeric Prodrugs

Design of polymeric prodrugs is one of the approach developed for improved use of drugs for therapeutic applications. A prodrug is a chemical entity of an active parent drug with altered physico-chemical properties (Hoste, De Winne et al. 2004). The most complete realization of the prodrug approach is possible by the use of an advanced type of prodrug- the drug delivery system. This system can be constructed to target a desired organ, its cells or organelles as well as to release a specific amount of the drug at desired times. The polymer prodrug conjugate can also increase aqueous solubility, enhance biodistribution and retain the inherent pharmacological properties of the drug intact (Oliyai R 1993).

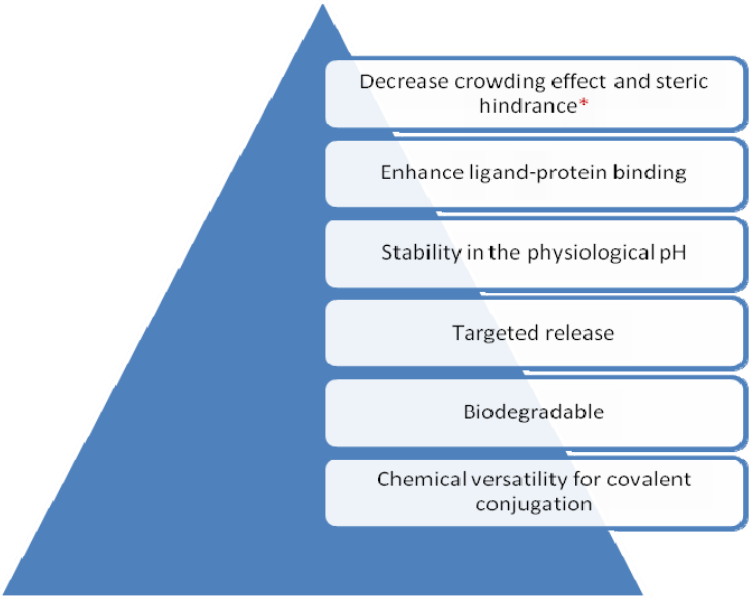


Fig 2-24 Incorporation of spacers in prodrug conjugation

There are 3 major types of polymeric prodrugs currently used (David, Kopeckova et al. 2004). The first type of prodrug are broken down inside cells to form active substance or substances. The second type of prodrug is usually the combination of two or more substances. Under specific intracellular conditions, these substances react forming an active drug. The third type of prodrug, targeted drug delivery systems, usually includes three components, a targeting moiety, a carrier and one or more active components. The targeting ability of the delivery system depends on the several variables including receptor expression, ligand internalization, choice of antibody, antibody fragments or no-antibody ligands and binding affinity of the ligand (Allen 2002). Therefore, the selection of suitable polymer and a targeting moiety is vital to the effectiveness of prodrugs.

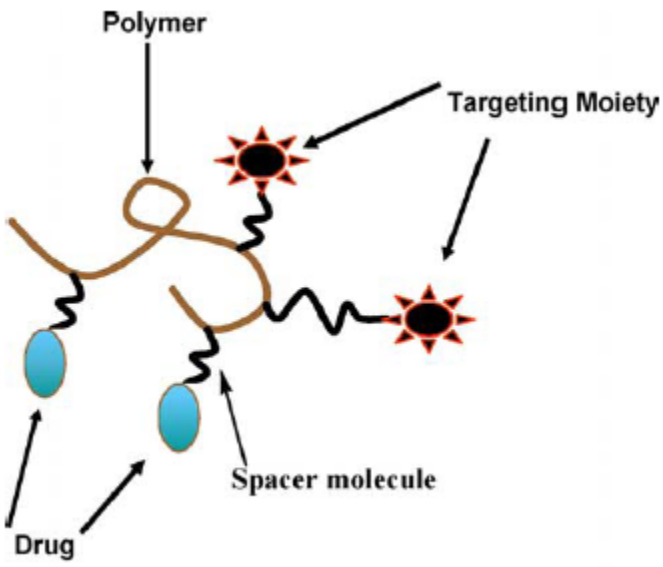


Fig 2-25 Polymeric prodrug with targeting agent (Jayant Khandare 2006)

2.5.7 Critical Aspects of Polymer Conjugation

The critical aspects of polymer conjugation includes the structure-activity relationship (SAR) of conjugation, steric hindrance, enhanced reactivity of polymers by incorporation of spacers and targeting of polymeric drugs that include active and passive targeting. The SAR means the effect of a drug, in its conjugated form, on an animal, plant or the environment as it relates to its molecular structure. Very few reports suggest the differences in SAR due to variations of the conjugated sites of a drug with the polymer. Such studies are possible if a drug candidate has different sites for conjugation and their activity mechanisms are established. The drug, Methotrexate (MTX), is an ideal candidate for these studies, as it has two –COOH groups available for the covalent linkage with the polymeric carrier. The drug delivery is relatively maintained when the gamma-carboxyl is chemically modified, whereas the alpha-carboxyl has much less bulk tolerance (Rosowsky, Forsch et al. 1981). Recently, design and synthesis of dextran-peptide-MTX conjugates for tumor-targeted delivery of chemotherapeutics via the

mediation of matrix metalloproteinase II and matrix metalloproteinase IX was reported (Chau, Tan et al. 2004). Steric hindrance describes how molecular groups interfere with other groups in the structure or other molecules during chemical conjugation. This effect is due to the interaction of the molecules as dictated by their shape and/or spatial relationships. The macroscale architecture of polymers causes steric hindrance for covalent conjugation with drugs in general, and large peptide molecules in particular. Steric hindrance drives chemical conformations and may affect the chemical conjugation with bulkier unstable molecules. Therefore, a conjugation reaction involving polymers, peptides and unstable molecules requires methodologies to reduce this effect. The most preferred method to decrease steric hindrance has been to alter the synthesis approach either by incorporating a spacer arm or by increasing the reactivity of the polymer or biomolecules (Khandare, Kolhe et al. 2005). During bioconjugation, high molecular weight biomolecules and polymers exhibit steric hindrance for the reactions. This is especially true for the linear polymers, in general, and dendrimers in particular. Therefore, the hindrance must be reduced either by incorporation of the spacer molecule or by increasing the reactivity of the bioconjugating moiety. Instead of conjugating two large molecules directly, one may be reacted first with small, reactive spacer arm moiety to increase the final reactivity. Further, the resultant conjugate can be coupled with the second molecule (Khandare, Kolhe et al. 2005). Crowding of functional groups and steric hindrance may lead to lower conjugate ratios with unreacted polymers. The reactivity of functional polymers to couple with other biomolecules, which may be low, could be enhanced by first conjugating the polymer with reactive bis functional molecules. The resulting polymer–spacer conjugate moiety often enhances the reactivity and decreases steric hindrance for further coupling with drugs or biomolecules (Khandare, Kolhe et al. 2005). Commonly used as spacers for conjugating polymers with drugs and other biomolecules include a-amino acids such as glycine, alanine, and serine. Polymer carriers used for conjugation with anticancer drugs are often linked by polypeptides (Li 2002). Most of the conjugation methods

involve the use of spacers, which provide chemical flexibility for coupling biological compounds to the polymers. Conjugation of low molecular weight drugs to high molecular weight carriers results in high molecular weight prodrugs, which substantially changes the mechanisms of cellular drug entrance. While small molecular weight drugs enter cells primarily by diffusion, high molecular weight drugs are internalized mainly by endocytosis.

2.5.8 Characteristics of Prodrugs

In recent years, numerous prodrugs have been designed and developed to overcome barriers to drug utilization, such as low oral absorption properties, lack of site specificity, chemical instability, toxicity, bad taste, odour, pain at application site, etc. It has been suggested that the following characteristics of a prodrug must be improved for site-specific drug delivery.

(1) The prodrug must be readily transported to the site of action

(2) The prodrug must be selectively cleaved to the active drug utilizing special enzymatic profile of the site

(3) Once the prodrug is selectively generated at the site of action, the tissue must retain the active drug without further degradation.

2.5.9 Mechanism of Action

Macromolecules normally cannot enter cells by passive diffusion across the plasma membrane. The general mechanism whereby they pass the cell membrane is endocytosis. A macromolecule, when dissolved in the extracellular fluid can enter a cell at a relatively slow rate. This process is called ‘fluid-phase endocytosis’. Macromolecular prodrugs using carriers without any special affinity to tumor cells are considered to be endocytosed by this mechanism. In ‘adsorptive endocytosis’, macromolecules bound to the plasma membrane are internalized at rates usually faster than those by fluid-phase endocytosis. Tumor cells may endocytose cationic

macromolecular prodrugs, following adsorption on the plasma membrane by electrostatic force by this process. Actively targeted macromolecular prodrugs with carriers of glycoproteins, hormones, lectins, etc. are rapidly and effectively internalized by adsorptive or receptormediated endocytosis, which occurs via coated pits. In conjunction with drug release problems, the rate and extent of endocytosis of macromolecular prodrugs are of particular importance to their pharmacological efficacy. The pharmacological activity of macromolecular prodrugs requires the release of free drugs by chemical and/or enzymatic reactions from the conjugate. In terms of drug release, the stability of the linkage between the carrier and the drug, and the site of regeneration of the free drug from the conjugate are important factors. Since the site of action of most antitumor drugs, such as nuclei, is located in the intracellular space of tumor cells, the therapeutic efficacy of a macromolecular prodrug greatly depends on where the free drugs are released. The most well-known concept for the mechanism of action of macromolecular prodrugs is the principle of a 'lysosomotropic' delivery which was advocated more than two decades ago by Trouet et al. (Trouet A 1972) for a DNA-daunorubicin complex. Another mechanism for the intra lysosomal drug release involves the low pH in the lysosomal milieu. In this approach, free active drugs are generated from the conjugates by a chemical reaction under the acidic condition. In order to elucidate the mechanism of action of macromolecular produrgs cellular interactions and *in vitro* antitumor activities of mitomycin C-dextran conjugates have been studied in a cell culture system (Matsumoto, Yamamoto et al. 1986). Macromolecular prodrugs endocytosed by the tumor cells also may have exhibited cytotoxicity, but contribution of this mechanism seem to be minimal because drug release is slower and mitomycin C is unstable (Beijnen JH 1985) at a low pH in endosomes and lysosomes, in addition to slow internalization rate.

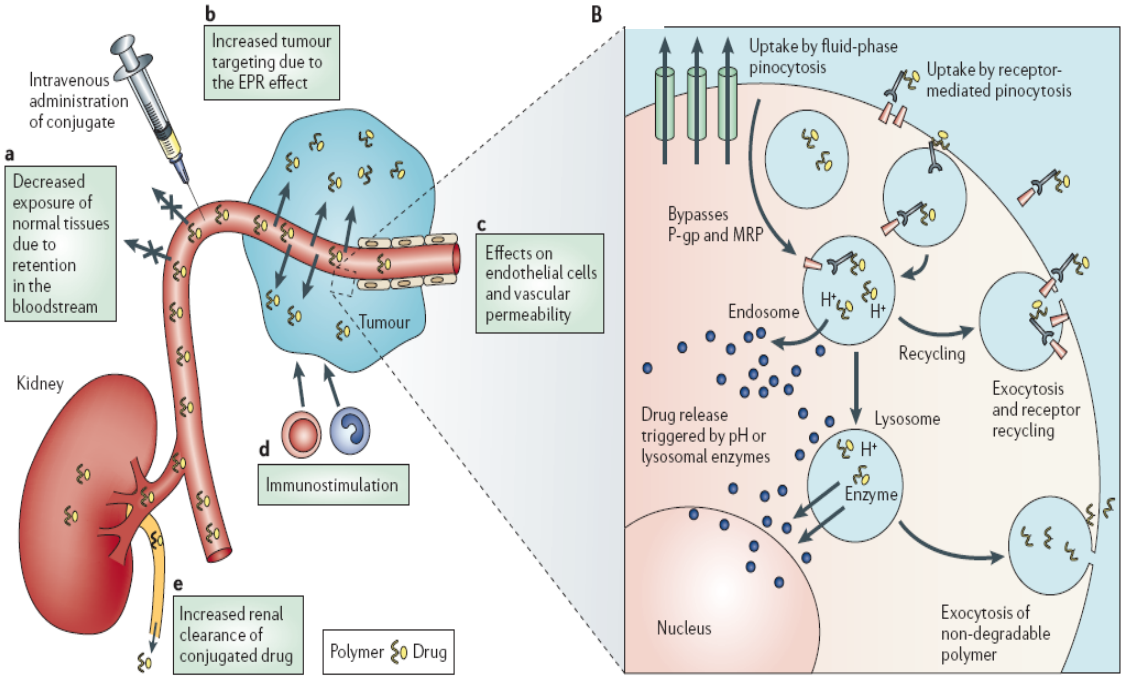


Fig 2-26 Mechanism of action of polymer drug conjugate (Duncan 2006)

Hydrophilic polymer–drug conjugates administered intravenously can be designed to remain in the circulation. The clearance rate of the conjugates depends on conjugate molecular weight, which governs the rate of renal elimination. Drug that is covalently bound to the polymer by a linker, that is stable in the circulation, is largely prevented from accessing normal tissues (including sites of potential toxicity), and biodistribution is initially limited to the blood pool. The blood concentration of drug conjugate drives tumor targeting due to the increased permeability of angiogenic tumor vasculature (compared with normal vessels), providing the opportunity for passive targeting due to the enhanced permeability and retention effect (EPR effect). Through the incorporation of cell-specific recognition ligands it is possible to bring about the added benefit of receptor-mediated targeting of tumor cells. It has also been suggested that circulating low levels of conjugate (slow drug release) might additionally lead to immunostimulation. On arrival in the tumor interstitium, polymer-conjugated drug is internalized by tumor cells through either fluid-

phase pinocytosis (in solution), receptor-mediated pinocytosis following non-specific membrane binding (due to hydrophobic or charge interactions) or ligand–receptor docking. Depending on the linkers used, the drug will usually be released intracellularly on exposure to lysosomal enzymes. The active or passive transport of drugs such as doxorubicin and paciltaxel out of these vesicular compartments ensures exposure to their pharmacological targets. Intracellular delivery can bypass mechanisms of resistance associated with membrane efflux pumps such as p-glycoprotein. Non-biodegradable polymeric platforms must eventually be eliminated from the cell by exocytosis. Rapid exocytic elimination of the conjugated drug before release would be detrimental and prevent access to the therapeutic target.

2.5.10 Bioconversion of Prodrugs

Conversion of the prodrug to the parent drug at the target site is critical for the prodrug approach to be successful. Typically, activation involves metabolism by enzymes that are distributed throughout the body (Williams 1985; Rooseboom, Commandeur et al. 2004). Many prodrugs contain an ester bond, which is formed by derivatizing a phenolic, hydroxyl, or carboxyl group present in the drug molecule. When the ester bond of the prodrug is cleaved, the active drug is released. The cleavage of the ester bond typically occurs through hydrolysis or oxidation. The most important esterases that catalyze hydrolyses of prodrugs include carboxylesterase, acetylcholinesterase, butyrylcholinesterase, paraoxonase, and arylesterase. Oxidation cleavage of ester-based prodrugs is catalyzed by cytochrome P450s. Since esterases in particular are widely distributed throughout the body and therefore the ester bond is quite labile *in vivo*, many ester-based prodrugs have been developed (Beaumont, Webster et al. 2003). In many cases, these prodrugs were designed to improve the oral bioavailability of drugs (Wang, Jiang et al. 1999; Beaumont, Webster et al. 2003).

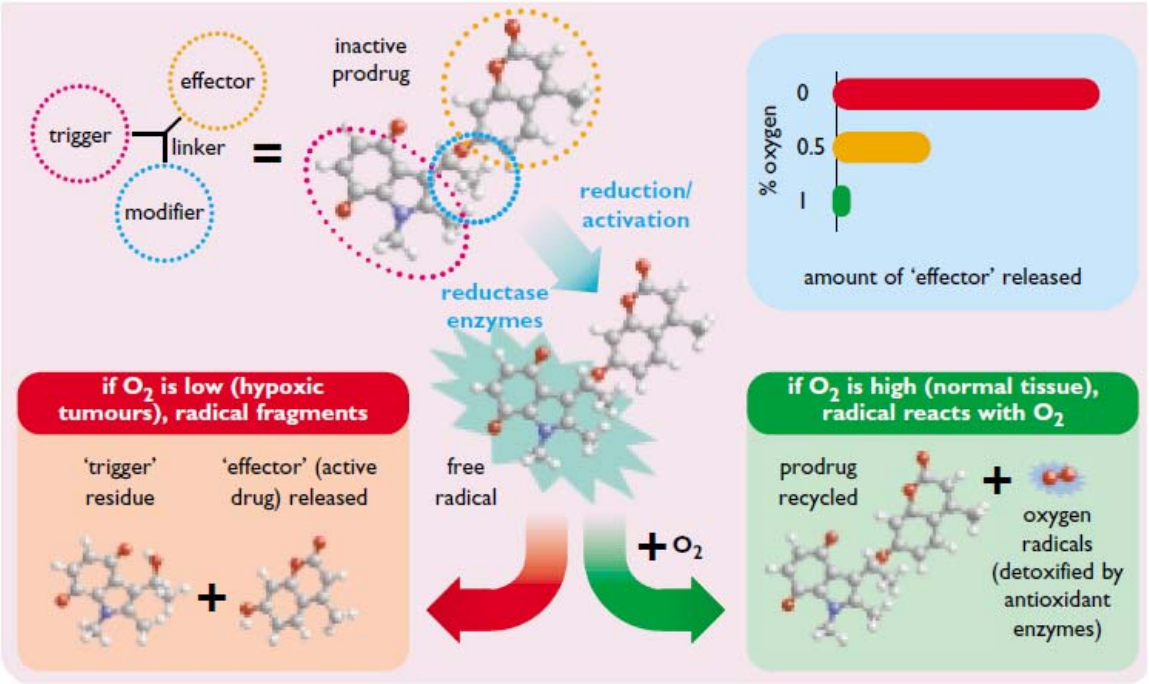


Fig 2-27 Selective release of active drugs in regions of low oxygen concentration in tumors (Scientific Yearbook 2001-02; Pg 36)

There is a problem with the ester prodrugs is the difficulty in predicting their rates of bioconversion and, thus, their pharmacological or toxicological effects. This is particularly a problem when one is trying to use animal data to predict the prodrug's bioconversion in human. Species differences can generally result from the existence of different types of esterases in biological media and differences in their respective substrate specificities (Liederer and Borchardt 2005). Even within one species, the rate of hydrolysis is not always predictable for the same reasons (Hosokawa, Endo et al. 1995). Additionally, bioconversion can be affected by various factors such as age, gender and disease. The enzymes involved in the bioconversion of ester prodrugs are Esterases, which is classified into Esterases A, which includes paraoxonase, and Esterases B, that includes carboxylesterase, acetylcholinesterase, cholinesterase and human

valacyclovirase. The factors affecting the bioconversion of ester prodrugs are species differences, interindividual variation, stereochemistry and structural effects.

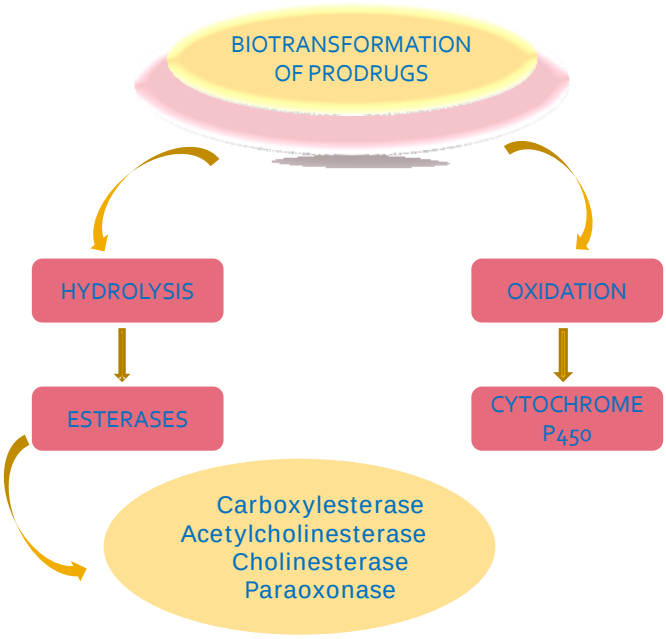
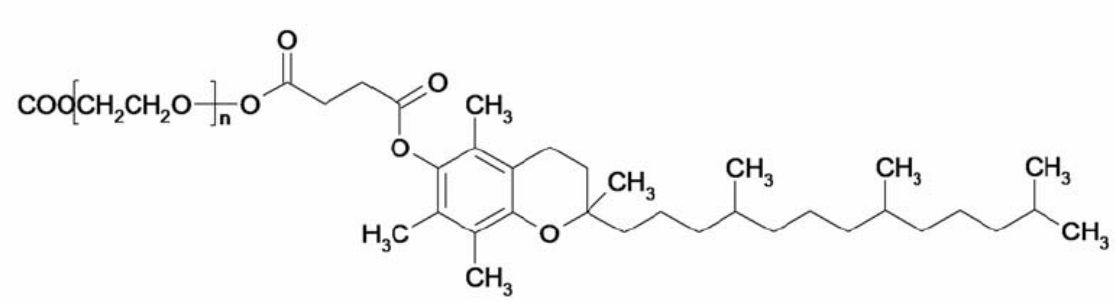


Fig 2-28 Enzymes involved in biotransformation of prodrugs

2.6 Vitamin E TPGS, an amphiphilic polymer

2.6.1 Structure and Properties



Scheme 2-4 Chemical structure of Vitamin E TPGS

D-α-tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS or TPGS) is a water soluble derivative of natural vitamin E and prepared by esterification of d-α-tocopheryl acid succinate with polyethylene glycol 1000. It is an amphiphilic macromolecule comprising of hydrophilic polar head and a lypophilic alkyl tail. Its molecular weight is approximately 1542 Da. The hydrophile/lipophile balance (HLB) of TPGS is ~13. It is basically a waxy solid appearing white to light brown in color with a melting point approximately 37-41°C. It is stable in air as well. It is used an effective emulsifier as well as a good solubilizer due to its bulky nature and larger surface area (Fisher 2002). TPGS has found wide utility in pharmaceutical formulations as follows.

- Improving drug bioavailability
    - Surfactant properties enhance solubilization of poorly water soluble drugs
    - Stabilization of the amorphous drug form
    - Enhances drug permeation by P-glycoprotein efflux inhibition
- Emulsion vehicle
- Functional ingredient in self-emulsifying formulations
- Thermal binder in melt granulation/extrusion processing
- Reducing drug sensitivity on skin or tissues
- Carrier for wound care and treatment
- Water-soluble source of vitamin E

2.6.2 Absorption/Bioavailability Enhancer

TPGS has received increased attention in the literature for its ability to enhance the absorption of several drugs that have otherwise poor bioavailability. Sokol et al. in 1991 clinically demonstrated that TPGS can enhance absorption of the highly lipophilic drug cyclosporin, which

is used for immunosuppressive therapy to manage rejection of transplanted organs. This is a crucial finding for organ transplant recipients. Due to the impaired absorption of cyclosporine, massive doses are required to achieve therapeutic blood plasma concentrations. The study showed that TPGS provides a substantial improvement of cyclosporine absorption and a significant reduction of the high cost of immunosuppressive therapy. While Sokol originally suggested that the increased bioavailability was due to micelle formation enhancing the solubility, others have since provided evidence supporting enhanced permeability due to P-glycoprotein (P-gp) inhibition (Croockewit, Koopmans et al. 1996; Dintaman and Silverman 1999). While many of the examples of TPGS use are poorly water soluble drugs there are also examples of using TPGS with poorly permeable drugs that are water soluble (Prasad, Puthli et al. 2003). Many studies have been conducted to evaluate the mechanism by which TPGS affects bioavailability. Its action is attributable to its ability to improve solubility through micelle formation and through enhancing permeability across cell membranes by inhibition of multi-drug efflux pump P-gp. For oral delivery, TPGS enhances drug efficacy by improving the solubilization or emulsification of the drug in the finished dosage form and through formation of a self-emulsifying drug delivery system in the stomach which may be due to TPGS, which improves the permeability of a drug across cell membranes by inhibiting P-glycoprotein and thus enhance absorption of a drug through intestinal wall and into the bloodstream. It can act as a reversal agent of P-gp mediated multidrug resistance and inhibit P-gp substrate drugs transport (Dintaman and Silverman 1999). TPGS is more effective P-gp inhibitor than many related excipients with surfactant properties such as cremophor EL, Tween 80, Pluronic P85 and PEG 300. However, it is significantly less potent than other clinically tested pharmacologically active compounds such as cyclosporine, tariquidar and zosuquidar (Dantzig, Law et al. 2001; Mistry, Stewart et al. 2001).

2.6.3 Solubilization of Poorly Water Soluble Compounds

It is estimated that over 40% of new drug entities are poorly water soluble. Some drug delivery systems that can utilize the solubilizing ability of TPGS are solid dispersions, self-emulsifying drug delivery systems, self-microemulsifying drug delivery systems, spray drying and others. Much work has been done to investigate the effect of TPGS on the aqueous solubility of poorly water soluble drugs. It was shown that TPGS can enhance the solubility and bioavailability of poorly absorbed drugs by acting as a carrier in drug delivery systems, thus providing an effective way to improve the therapeutic efficiency and reduce the side effects of the anticancer drugs (Fisher 2002; Youk, Lee et al. 2005). One of the early discoveries on the solubilizing potential of TPGS is attributable to the work of Ismailos et al. who followed up on the discovery that TPGS can be co-administered with cyclosporine A resulting in dramatic decrease in the dosage required of this costly drug (Ismailos 1994). Also, it was found that TPGS improves the solubility of the poorly water soluble drug amprenavir (Yu, Bridgers et al. 1999). Below the critical micelle concentration, there is no increase in solution. A more recent application involves taxoids which, while important for their chemotherapeutic action, are poorly water soluble and difficult to administer in oral formulation. TPGS is one of the best excipients in which taxoids are soluble. It shows excellent solubilization properties for oral formulation containing paclitaxel and TPGS (Varma and Panchagnula 2005).

2.6.4 Controlled Delivery Applications

TPGS can be a good emulsifier or surfactant in fabricating nano/microparticles. TPGS emulsified PLGA nanoparticles fabricated by a modified solvent extraction/evaporation method have narrow polydispersity range from 0.005-0.045 and size around 300-800 nm. TPGS can also achieve emulsification efficiency as the amount of TPGS needed in the fabrication process was only 0.015% (w/w), which was far less than 1% for PVA needed in similar process (Mu and Feng

2002; Mu and Feng 2003). Mu and Feng also found that TPGS could be a good component of the polymeric matrix material in fabrication of PLGA nanoparticles. Feng et al found that nanoparticles coated with TPGS eliminated the side effects caused by human intestinal epithelia cells and cancer cell mortality (Feng, Mu et al. 2004). The polymeric nanoparticles, in which active agent is dissolved, entrapped, encapsulated, adsorbed, attached or chemically coupled, are an exciting new area of research. Here the co-polymerization of TPGS with a polymer such as PLZ, PCL or PLGA can improve the emulsification efficiency, drug encapsulation efficiency and enhance the cellular uptake of the nanoparticles, thereby increasing the therapeutic effect. These have been demonstrated on microencapsulated paclitaxel (Mu and Feng 2003; Zhang and Feng 2006). TPGS is also said to be a more effective and safer emulsifier than PVA with easier usage in fabrication and characterization of polymeric nanospheres for drug delivery (Mu and Feng 2002).

2.6.5 Non-Oral Delivery Applications

2.6.5.1 Nasal/Pulmonary Delivery

Use in nasal/pulmonary delivery formulations show that TPGS increases the immune response toward diphtheria toxoid loaded poly(caprolactone) microparticles (Somavarapu, Pandit et al. 2005). TPGS has also recently been found to be an adjuvant for nasally applied anti-tetanus toxoid, anti-diphtheria toxoid in mice (Alpar, Eyles et al. 2001). TPGS, which has good physiological compatibility with the mucous membrane surface, serves as wetting agent to smooth the membrane surfaces, which in turn delimit the flow channels. In this formulation, TPGS plays an important role not only as a surface-active agent but also as an emulsifier [US patent 4, 668, 513 (1987)].

2.6.5.2 Ophthalmic Delivery

It describes many drug delivery systems in which TPGS is compatible and has shown utility. These drug delivery systems include: bioadhesive hydrogels, liposomes, nanoparticles, and the use of excipients with solubility enhancing properties (Bourlais, Acar et al. 1998).

2.6.5.3 Parental Delivery

Here, TPGS has been used in clinical trials and has been the subject of a pharmacokinetic study (Lissianskaya 2004; Hanauske 2005). TPGS is included in the formulations of taxane analogues to improve their solubility. It may also have some therapeutic value against cancer cells as it has been found to induce apoptosis and inhibit the growth of human lung carcinoma cells implanted in nude mice (Youk, Lee et al. 2005).

2.6.5.4 Dermal Delivery

Dermal applications can use TPGS's surface active properties to improve the surface wetting of films with skin. Incorporating TPGS in hot-melt extruded hydroxypropylcellulose and polyethyleneoxide films resulted in nearly doubling the adhesive strength of the films (Repka and McGinity 2001). This result may indicate that TPGS could be an important additive in transdermal/transmucosal or wound care systems. It may also serve as a human skin penetration enhancer was shown for radiolabeled hydrocortisone. It also has good bioadhesive properties.

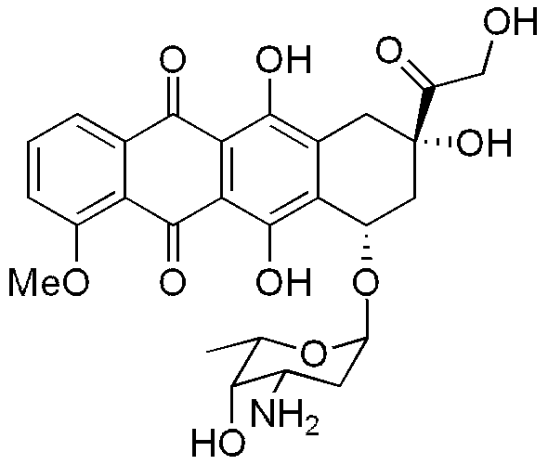
2.6.6 Anti-cancer Activity

TPGS is PEG 1000- conjugates to derivative of α-tocopheryl succinate (TOS) while TOS is a succinyl derivative of vitamin E and has been found to have anticancer properties against leukemia, melanomas, breast, colorectal, malignant brain, lung and prostate cancers (Neuzil, Weber et al. 2001; Yu, Liao et al. 2001). TOS differs from other vitamin E derivatives in that

TOS itself does not act as an antioxidant (Neuzil 2002). In xenograft experiments, TOS suppressed tumor growth, both alone and in combination with other anticancer agents (Barnett, Fokum et al. 2002; Weber, Lu et al. 2002). The anticancer activity of TOS is mediated by its unique apoptosis-inducing properties which appear to be mediated through diverse mechanisms involving the generation of reactive oxygen species (ROS) (Wang, Witting et al. 2005). ROS can damage DNA, proteins and fatty acids in cells resulting in apoptotic cell death depending on the strength and duration of ROS generation. It has poor water solubility but its conjugation to PEG makes it water soluble.

2.7 Doxorubicin, an anti-cancer drug

2.7.1 Structure and Properties



Scheme 2-5 Structure of Doxorubicin

Doxorubicin (trade name Adriamycin; also known as hydroxydaunorubicin) is a drug used in cancer chemotherapy. It is an anthracycline antibiotic, closely related to the natural product

daunomycin, and like all anthracyclines it intercalates DNA. It is commonly used in the treatment of a wide range of cancers, including hematological malignancies, many types of carcinoma, and soft tissue sarcomas. The drug is administered in the form of hydrochloride salt intravenously. It is photosensitive and it is often covered by an aluminum bag to prevent light from affecting it. Its IUPAC name is (8*S*,10*S*)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2*H*-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione. It has a molecular mass of 543.52 g/mol with 5% oral bioavailability and 12-18.5 hrs half-life. Doxorubicin (DXR) is a 14-hydroxylated version of daunorubicin, the immediate precursor of DXR in its biosynthetic pathway. Daunorubicin is more abundantly found as a natural product because it is produced by a number of different wild type strains of *streptomyces*. In contrast, only one known non-wild type species, *streptomyces peucetius* subspecies *cesius* ATCC 27952, was initially found to be capable of producing the more widely used doxorubicin (Lomovskaya, Otten et al. 1999). This strain was created by Arcamone et. al in 1969 by mutating a strain producing daunorubicin, but not DXR, at least in detectable quantities (Arcamone, Cassinelli et al. 1969).

2.7.2 Mechanism of Action

The action mechanism of doxorubicin is complex and still somewhat unclear, though it is thought to interact with DNA by intercalation (Fornari, Randolph et al. 1994). Doxorubicin is known to interact with DNA by intercalation and inhibition of macromolecular biosynthesis (Momparler, Karon et al. 1976). 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. The planar aromatic chromophore portion of the molecule intercalates between two base pairs of the DNA, while the six-membered daunosamine sugar sits in the minor groove and interacts with flanking base pairs immediately adjacent to the intercalation site, as evidenced by several crystal structures (Pigram, Fuller et al. 1972; Frederick, Williams et al. 1990).

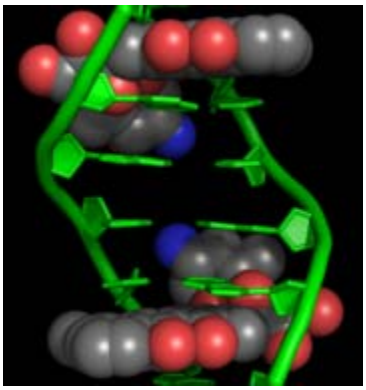


Fig 2-29 Doxorubicin intercalating DNA
(http://en.wikipedia.org/wiki/File:Doxorubicin%E2%80%93DNA_complex_1D12.png)

2.7.3 Limitations and Side Effects

Although doxorubicin is one of the most effective chemotherapeutic agents with most frequently usage, its clinical use is limited due to the acute side-effects of doxorubicin that includes nausea, vomiting, and heart arrhythmias. It can also cause neutropenia (a decrease in white blood cells), as well as complete alopecia (hair loss). When the cumulative dose of doxorubicin reaches 550 mg/m², the risks of developing cardiac side effects, including congestive heart failure, dilated cardiomyopathy, and death, dramatically increase (Petit 2004). Doxorubicin cardiotoxicity is characterized by a dose-dependent decline in mitochondrial oxidative phosphorylation. Reactive oxygen species, generated by the interaction of doxorubicin with iron, can then damage the

myocytes (heart cells), causing myofibrillar loss and cytoplasmic vacuolization. Additionally, some patients may develop Palmar plantar erythrodysesthesia, or, "Hand-Foot Syndrome," characterized by skin eruptions on the palms of the hand or soles of the feet, characterized by swelling, pain and erythema. Due to these side effects and its red color, doxorubicin has earned the nickname "red devil" or "red death". Doxorubucin can also cause reactivation of Hepatitis B. Besides these side effects, it has another limitation namely the multi-drug resistance (MDR). Multi-drug resistance in the cancer treatment by overexpression of MDR transporter proteins such as P-gp and multidrug resistance associated protein (MRP). These are expressed in many tumor cells like liver, kidney and colon cells, as well as malignant cells. Doxorubicin is a substrate of P-gp, that results in short half-life in circulation and low therapeutic efficiency (Krishna and Mayer 2000).

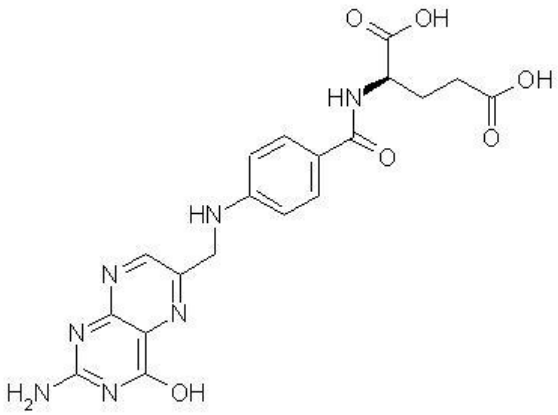
2.7.4 Systems for Delivery of Doxorubicin

Various researchers have studied ways to target doxorubicin delivery to cancer tissues or to diminish the side effects. To overcome the limitations and side effects of doxorubicin, different formulations have been developed successfully. The doxorubicin can be delivered to the cancer cells into the body by the drug delivery systems that include nanoparticles, prodrugs, micelles, liposomes etc. The nanoparticles, especially polymeric nanoparticles, are said to have better delivery of doxorubicin to the cancer cells due to its smaller size and encapsulation of drug by the polymer, which result in sustained release (Zhang, Huey Lee et al. 2007). Over the past decade, polymeric micelles have received much attention to deliver anticancer drugs. Micelles are used for improving the delivery of doxorubicin due to its size, which is less than 100 nm, and escape from renal exclusion and reticulo-endothelial system giving them enhanced vascular

permeability. For DOX, biodegradable polymeric micelles were extensively utilized for passive targeting to solid tumors (Yokoyama, Kwon et al. 1992; Yoo and Park 2001; Yoo, Lee et al. 2002) and active targeting as well (Yoo and Park 2004). Prodrugs are developed to deliver the drug with reduced side effects by increasing the half-life of the drug. The prodrugs developed include DOX-GA3 prodrug (Houba, Boven et al. 2001), HPMA-doxorubicin conjugate (Shiah, Dvorak et al. 2001), doxorubicin-PEG-folate conjugate, doxorubicin-cephalosporin prodrug (Veinberg, Shestakova et al. 2004), N-(phenylacetyl) doxorubicin (Zhang, Xiang et al. 2006), PEG-doxorubicin conjugates (Rodrigues, Beyer et al. 1999; Veronese, Schiavon et al. 2005). Doxorubicin loaded liposomes have enhanced efficiency in some solid tumors compared with free doxorubicin, because they passively target solid tumors through the enhanced permeability and retention effect, resulting in increased drug payloads delivered to tumors (Gaber MH 1995; Laginha, Verwoert et al. 2005).

2.8 Folic Acid

2.8.1 Structure and Properties of Folic Acid



Scheme 2-6 Structure of Folic Acid

Folic acid is also known as Vitamin B9 or Folacin. Folate is said to be the naturally occurring form of folic acid. Here, folic acid and folate are forms of the water-soluble Vitamin B9. Vitamin B9 (Folic acid and Folate inclusive) is essential to numerous bodily functions ranging from nucleotide synthesis to the remethylation of homocysteine. It is especially important during periods of rapid cell division and growth, such as in infancy and pregnancy. Both children and adults require folic acid to produce healthy red blood cells and prevent anemia. It is a yellow orange crystalline powder that has a molar mass of 441.4 g/mol and melting point of 250°C.

2.8.2 Structure and Functions of Folate Receptors

The Folate Receptor (FR) is a folate binding protein known as glycosylphosphatidylinositol anchored protein, that can actively internalize bound folates and folate conjugated compounds via receptor-mediated endocytosis (Kamen 1986; Leamon 1991). It has been found that FR is up-regulated in more than 90% of non-mucinous ovarian carcinomas. It is also found at high to moderate levels in kidney, brain, lung, and breast carcinomas while it occurs at very low levels in most normal tissues (Kamen and Smith 2004). The FR density also appears to increase as the stage of the cancer increases (Elnakat and Ratnam 2004). It is thus hypothesized that folate conjugation to anti-cancer drugs will improve drug selectivity and decrease negative side effects. The family of human FR (M_r ~ 38 kDa) consists of three well-characterized isoforms (FR-α, -β, and γ) that are ~70–80% identical in amino acid sequence, but distinct in their expression patterns (Shen, Ross et al. 1994). FR-α and FR-β are both membrane-associated proteins as a consequence of their attachment to a glycosylphosphatidylinositol (GPI) membrane anchor. FR-α, however, can be distinguished from FR-β by its higher affinity for the circulating folate coenzyme, (6*S*)-5-methyltetrahydrofolate (5- CH_3 H_4 folate), and by its opposite stereospecificity for reduced folate coenzymes (Wang, Shen et al. 1992). FR-α also binds folic acid and physiologic folates with

slightly higher affinity(K_D ~ 0.1 nM) (Kamen and Caston 1986) than FR-β (K_D ~ 1 nM) (da Costa and Rothenberg 1996). FR-γ and a truncated form of the protein, FR-γ′, lack the GPI anchor and are constitutively secreted in barely detectable amounts as soluble forms of the human FR. The binding affinity of the secreted FR-γ for folic acid is reportedly to be ~ 0.4 nM (Shen, Wu et al. 1995). The role of FR in cellular folate transport is not well understood, although a 'potocytosis' model has been proposed (Anderson, Kamen et al. 1992). FRs were found to be clustered in non-coated membrane regions called caveolae. Localization of FRs in caveolae and receptor internalization can be induced by receptor crosslinking and is regulated by cholesterol (Smart, Mineo et al. 1996).

2.8.3 Biological Mechanism

The FR functions to concentrate exogenous folates and various derivatives into the cell cytosol by endocytosis (Kamen 1986). The term endocytosis refers to the process whereby the plasma membrane invaginates and eventually forms a distinct intracellular compartment. The endocytic vesicles (endosomes) that contain the FR–folate complex rapidly become acidified to ~pH 5 and thereby allow the FR to release the folate molecule (Lee, Wang et al. 1996). At this point, cytosolic entry of the vitamin can occur by: (1) direct membrane translocation of the protonated vitamin species; (2) anion exchange-assisted transport of the vitamin out of the endosome (Anderson, Kamen et al. 1992); and (3) simple leakage of the folate during imperfect membrane fusion events (Turek, Leamon et al. 1993). It has been known for nearly a decade that simple covalent attachment of folic acid to virtually any macromolecule produces a conjugate that can be internalized into FR-bearing cells in an identical fashion to that of free folic acid (Leamon 1991).

2.8.4 Drug Delivery by Receptor Mediated Endocytosis

Receptor-mediated endocytosis (RME), also called clathrin-dependent endocytosis, is a process by which cells internalize molecules (endocytosis) by the inward budding of plasma membrane vesicles containing proteins with receptor sites specific to the molecules being internalized. After the binding of a ligand to plasma membrane spanning receptors, a signal is sent through the membrane, leading to membrane coating, and formation of a membrane invagination. The receptor, its ligand, and anything nearby are then internalized in sub-micrometre sized clathrin-coated vesicles. Once internalized, the clathrin-coated vesicle uncoats (a pre-requisite for the vesicle to fuse with other membranes) and individual vesicles fuse to form the early endosome. Since the receptor is internalized with the ligand, the system is saturable and uptake will decline until receptors are recycled to the surface.

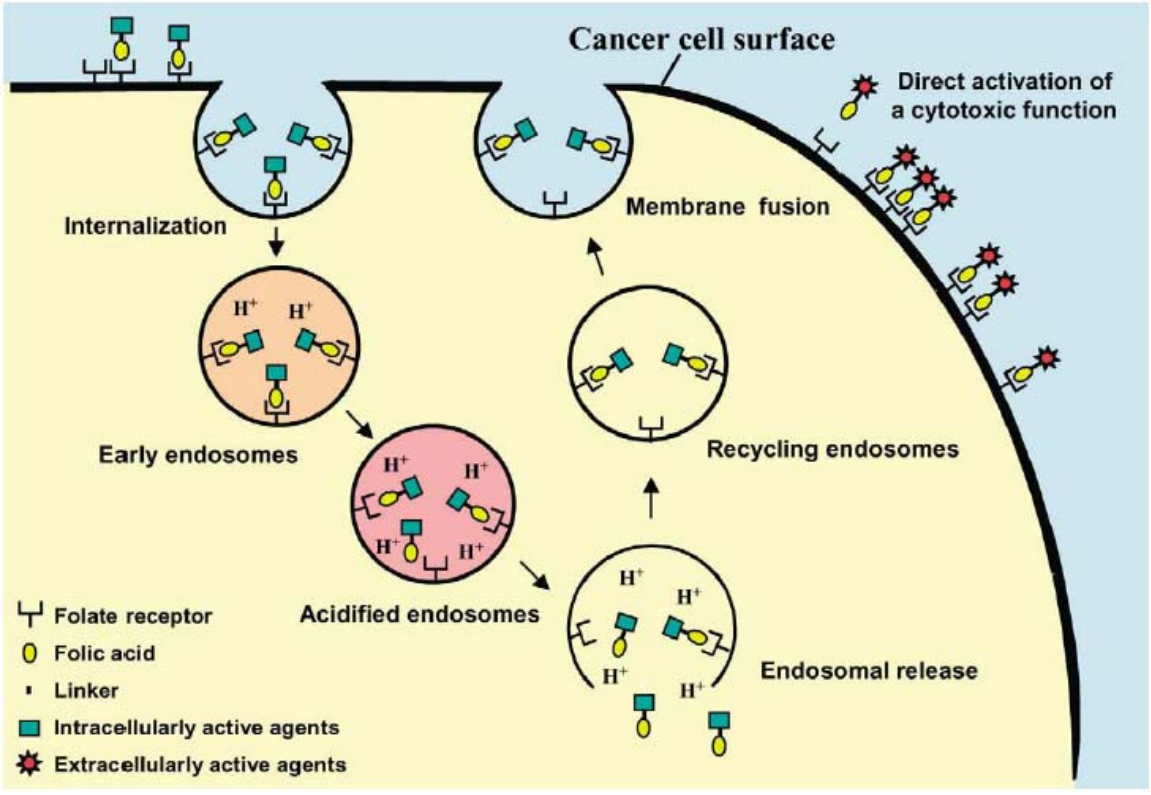


Fig 2-30 Receptor mediated endocytosis (Lu and Low 2002)

2.8.5 Applications

The prevalence of FR overexpression among human tumors makes it a good marker for targeted drug delivery to these tumors. Two strategies have been developed for FR-specific drug targeting: (1) coupling to monoclonal antibodies (e.g., MOv18) against the FR; and (2) coupling to folic acid, in which folic acid functions as the targeting ligand. High affinity FR binding is retained when folate is covalently linked via its g-carboxyl group to a foreign molecule.Among the targeting moieties, vitamin folic acid (folate or FOL) has been widely employed as a targeting moiety for various anticancer drugs. It is attracted for its high binding affinity, ease of modification, small size, stability during storage, and low cost (Lee and Low 1995; Guo, Hinkle et al. 1999; Reddy and Low 2000). The high-affinity folate receptor (FR), which is a cell surface-expressed molecule containing folate binding proteins called GPI (glycosyl phosphatidyl inositol) (Lu and Low 2002), is overexpressed in almost all the carcinomas, but has a highly restricted distribution of expression in normal cells. For this reason, folic acid has been covalently conjugated to anticancer drugs for selective targeting against tumor, which can uptake the drug-FOL conjugation by the receptor mediated endocytosis (RME) (Lee and Low 1995). Folate targeted drug delivery has emerged as an alternative therapy for the treatment and imaging of many cancers and inflammatory diseases. Due to its small molecular size and high binding affinity for cell surface folate receptors (FR), folate conjugates have the ability to deliver a variety of molecular complexes to pathologic cells without causing harm to normal tissues. Complexes that have been successfully delivered to FR expressing cells, to date, include protein toxins, immune stimulants, chemotherapeutic agents, liposomes, nanoparticles, and imaging agents.

CHAPTER 3: SYNTHESIS AND CHARACTERIZATION OF TPGS-DOX-FOL CONJUGATE

3.1 Introduction

TPGS has been synthesized by conjugating PEG 1000 to α-tocopheryl succinate (TOS) and thus TPGS-DOX and TPGS-DOX-FOL conjugates were also synthesized in a similar way. Firstly, the terminal hydroxyl group of the TPGS was reacted with succinic anhydride by the ring opening polymerization mechanism in the presence of DMAP to form TPGS-SA. Secondly, the carboxyl group of the TPGS is activated by NHS using DCC as the catalyst. Now, the amine group of DOX interacts with the activated carboxyl group in TPGS-SA to form TPGS-DOX conjugate. Thirdly, the NHS ester of folic acid (NHS-FOL), formed by activating folic acid using NHS and DCC as catalyst, is allowed to interact with hydrazine hydrate to form Folate-Hydrazide (Guo, Hinkle et al. 1999). Finally, the TPGS-DOX conjugate and the Folate-Hydrazide was reacted in the presence of acetic acid to form the TPGS-DOX-FOL conjugate. The mechanism for the reaction was shown in scheme 3-1, scheme 3-2, scheme 3-3 and scheme 3-4. The synthesized conjugates were characterized by Fourier Transform Infrared Spectroscopy (FT-IR) and Nuclear Magnetic Resonance (NMR) for the molecular structure. It was further characterized using microplate reader for drug loading efficiency and the stability of the conjugate was also studied in PBS.

3.2 Materials

TPGS was purchased from Eastman Chemical Company (TN, USA). Doxorubicin hydrochloride, phosphate buffered saline (PBS), N,N'-Dicyclohexylcarbodiimide (DCC), Dimethylaminopyridine (DMAP), N-hydroxysuccinimide (NHS), Succinic anhydride (SA),

Triethylamine (TEA), diethyl ether, tetrahydrofuran (THF), Hydrazine hydrate and Folic acid were obtained from Sigma-Aldrich (St. Louris, MO, USA). All solvents used are HPLC grade, which include Dichloromethane (DCM), Acetone and Dimethyl sulfoxide (DMSO) from Sigma-Aldrich and ethyl acetate from Merck. All reagent water used in the laboratory was preheated with Milli-Q Plus System (Millipore Corporation, Bredford, USA).

3.3 Methods

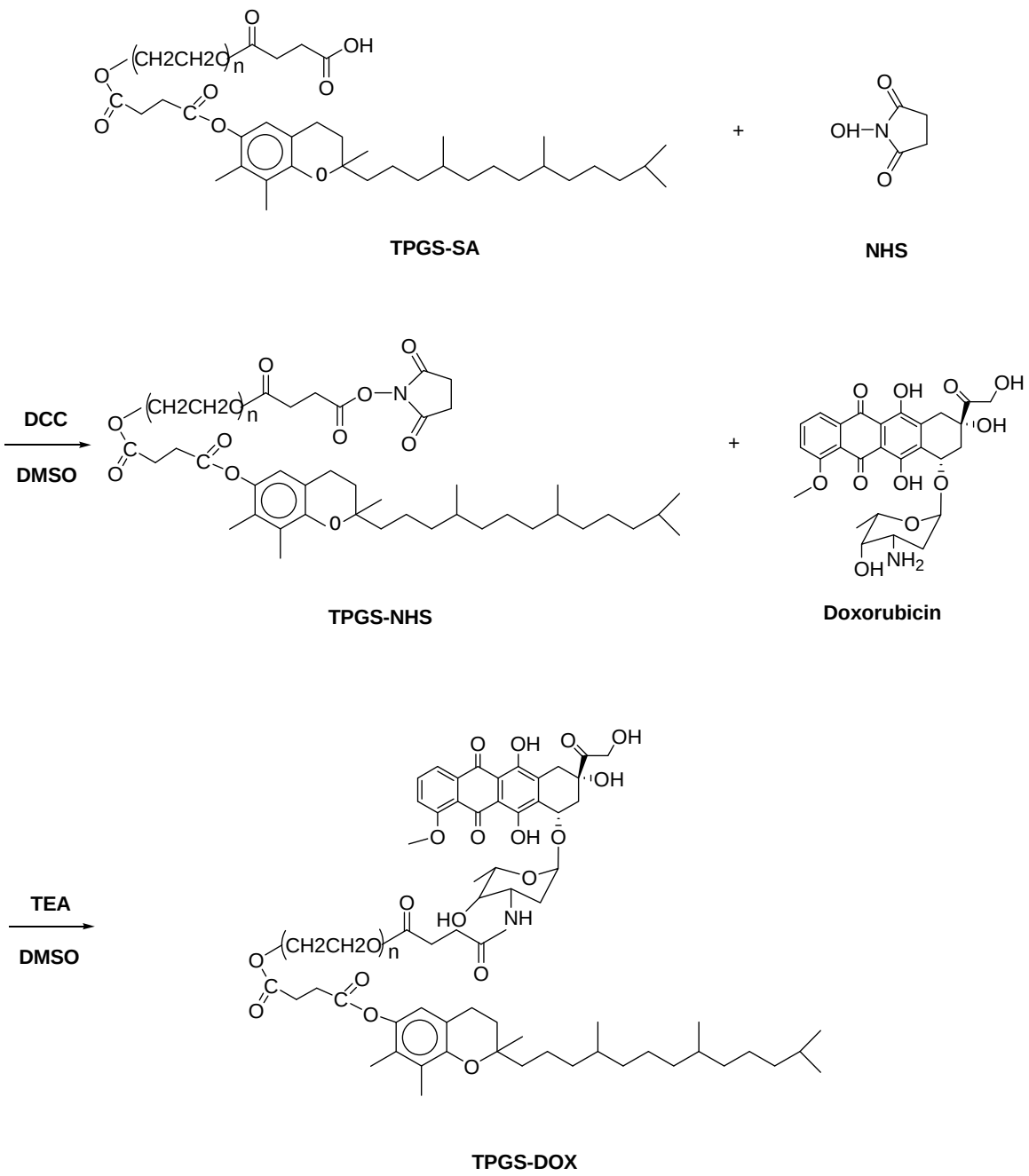
The TPGS-DOX and the TPGS-DOX-FOL conjugates were synthesized and characterized by the methods described below.

3.3.1 Synthesis of TPGS-DOX

3.3.1.1 Succinoylation of TPGS

Succinoylated TPGS was synthesized by the ring-opening polymerization mechanism in the presence of DMAP where the hydroxyl group of TPGS reacts with Succinic anhydride. In brief, TPGS (0.77 g), succinic anhydride (0.10 g) and DMAP (0.12 g) were mixed and allowed to react at 100° C under nitrogen atmosphere for 24 hrs (Cao and Feng 2008). The mixture was cooled to room temperature and taken up in 5.0 mL cold DCM. It is then filtered to remove excessive succinic anhydride and precipitated in 100 mL diethyl ether at -10°C overnight. The white precipitate was filtered and dried in vacuum to obtain succinoylated TPGS. However, complete succinoylation is necessary to avoid the polymer cross-linking during the Doxorubicin conjugation with TPGS-SA (Tomlinson, Heller et al. 2003).

3.3.1.2 TPGS-DOX Conjugation



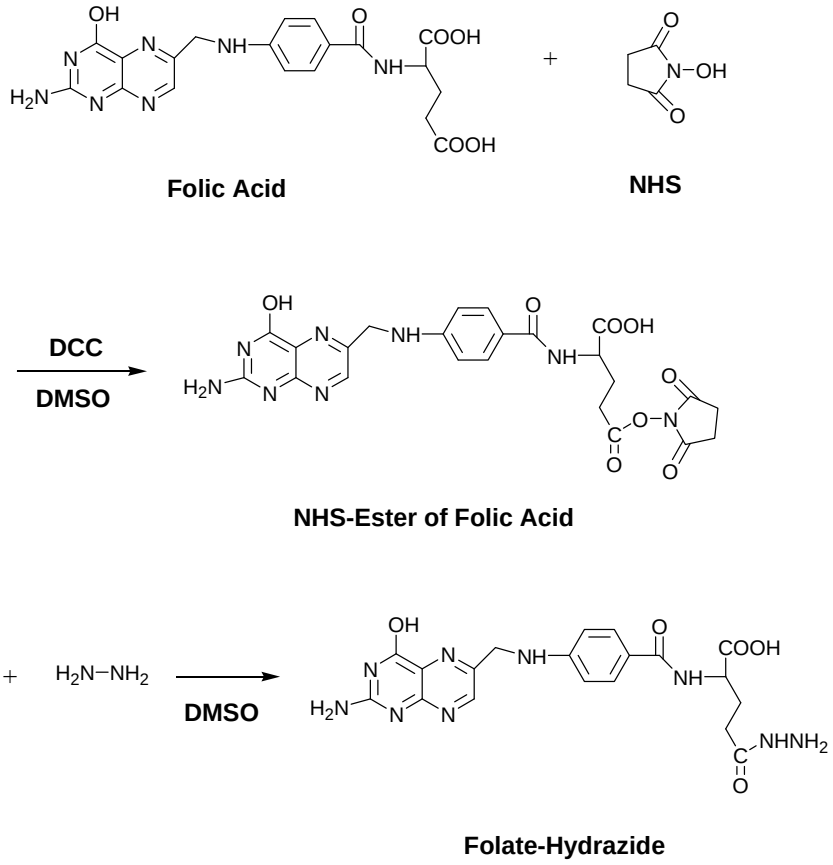
Scheme 3-1 Scheme of TPGS-DOX Conjugation

The succinoylated TPGS (191.3 mg) was reacted with DOX.HCl (102.5 mg) in the presence of DCC (74.2 mg), NHS (41.4 mg) and TEA (50 μL) in DMSO at room temperature under nitrogen atmosphere for 24 hrs. The obtained product was filtered to remove N,N-dicyclohexylurea (DCU) and then dialyzed using MWCO 1,000 membrane in DMSO for 24 hrs to remove excess reagents and the unconjugated DOX. It was further dialyzed against Millipore water for 24 hrs to remove DMSO. The resultant solution was freeze-dried to get the red powder of TPGS-DOX conjugate. The conjugate scheme is shown in Scheme 3-1.

3.3.2 Synthesis of TPGS-DOX-FOL

3.3.2.1 Folate-Hydrazide Synthesis

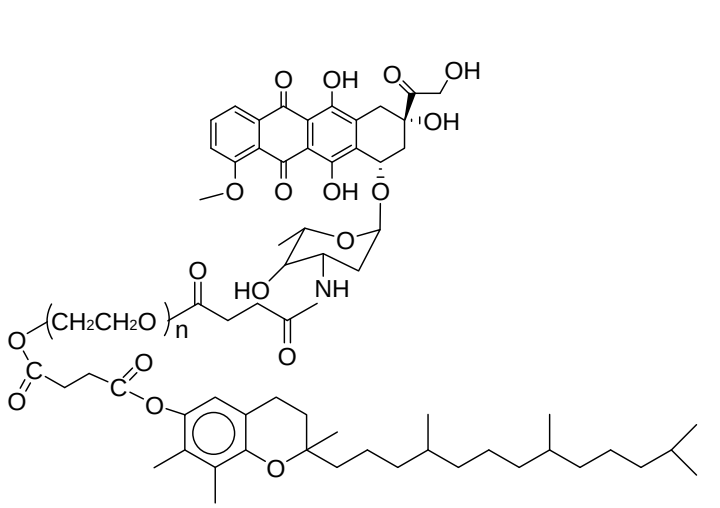
As to interact with hydrazine hydrate, NHS ester of folate (FOL) is required. Three grams of folic acid were dissolved in 60 mL DMSO. 1.1- molar excess of NHS and DCC were then added and reacted for 24 hrs at room temperature under stirring and N2 atmosphere, shielded from light. The by-product DCU was then removed by filtration and the DMSO solution of NHS-FOL was stored at -20° C until use. In the synthesis of folate-hydrazide, 60 mL of the above NHS-folate solution were added to 1.7 mL hydrazine hydrate with constant stirring at room temperature under nitrogen atmosphere for about 6 hrs. The product folate-hydrazide was converted to a hydrochloride salt with the addition of 17 mL 0.5 N HCl and then precipitated with four volumes of acetonitrile/dithylether (1:1) overnight. The precipitate was pelleted by centrifugation, redissolved in a small volume of water and then reprecipitaed with 10 volumes of ethanol in freezer overnight. The pellet was then washed sequentially using ethanol and diethylether and then dried under vaccum to obtain a yellow powder (Guo, Hinkle et al. 1999).



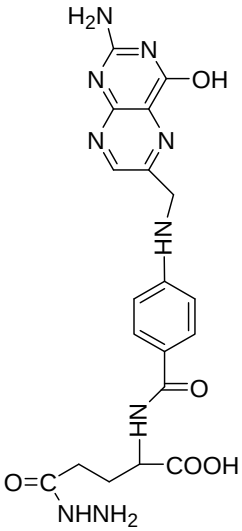
Scheme 3-2 Scheme of FOL-Hydrazide formation

3.3.2.2 TPGS-DOX-FOL Conjugation

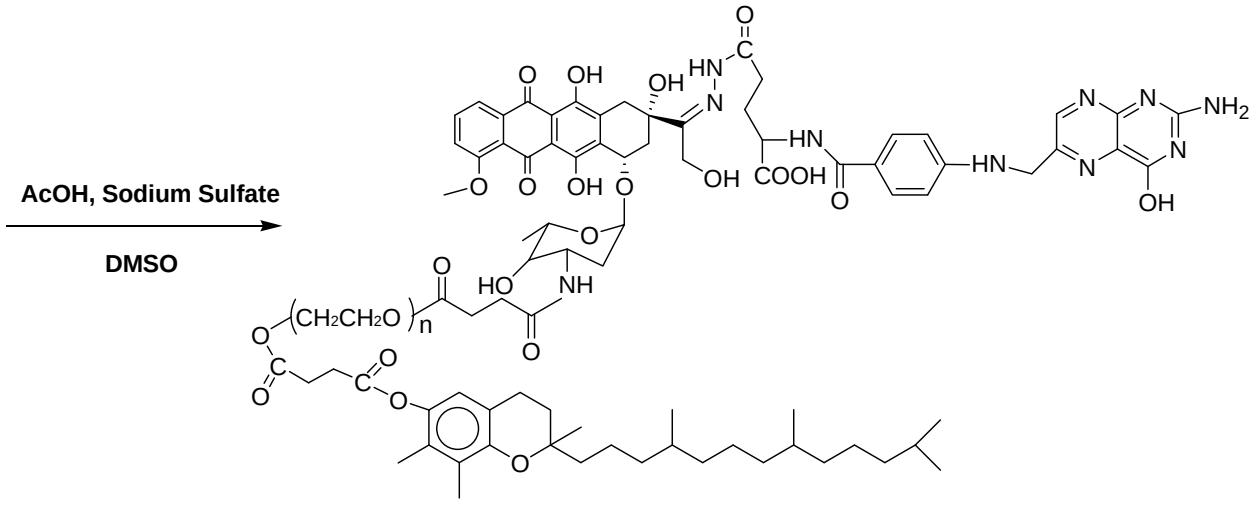
The TPGS-DOX-FOL conjugate synthesis involves the following steps. The TPGS-DOX conjugate (230 mg, 0.10 mmol) and FOL-hydrazide (220 mg, 0.46 mmol) were dissolved in anhydrous DMSO (20 mL), and AcOH (30 μL, 0.48 mmol). The reaction was performed for 24 hrs with stirring in the dark under nitrogen atmosphere at room temperature. Then, the mixture was filtered and dialyzed against DMSO for 24 hrs, followed by DI water for 48 hrs. The product was obtained after freeze-dry.



TPGS-DOX



Folate-Hydrazide



TPGS-DOX-FOL

Scheme 3-3 Scheme of TPGS-DOX-FOL Conjugation

3.3.3 Characterization of TPGS-DOX and TPGS-DOX-FOL Conjugates

3.3.3.1 FT-IR

The chemical structure of TPGS-DOX and TPGS-DOX-FOL were studied by the Fourier Transform Infrared Spectroscopy (FT-IR) (Shimadzu, Japan). For the sample preparation, we used 99% KBr with 1% TPGS-DOX or TPGS-DOX-FOL conjugate and mix them. The mixture is then pressed using high pressure into a transparent tablet.

3.3.3.2 ^{1}H-NMR

The molecular structure of TPGS-DOX, FOL-NH-NH2 and TPGS-DOX-FOL were confirmed by ^{1}H-Nuclear Magnetic Resonance (NMR) in DMSO-d6 graded solvent at 300MHz (Bruker ACF300, Germany).

3.3.3.3 Drug Conjugation Efficiency

The amount of DOX conjugated to TPGS in the TPGS-DOX conjugate and in the TPGS-DOX-FOL conjugate were measured individually using a microplate reader (GENios, Tean, Switzerland) in DMSO with fluorescence detection at Excitaion wavelength, λ_{ex} = 480 nm and Emission wavelength, λ_{em} = 560 nm. A standard curve was obtained using pristine DOX at a concentration range of 100 ng/mL – 500 μg/mL in DMSO.

3.4 Results and Discussion

3.4.1 FT-IR Spectra

Fig 3-1 shows the FT-IR spectra of FOL, TPGS-DOX and TPGS-DOX-FOL, from which we can see that the 3000-1500 1/cm region is known as the functional group region of FOL and the other important region, 900-700 1/cm region, is characteristic of the bending of the functional groups (Cummings and McArdle 1986). The absorption in 2960-2820 1/cm region stands for the C-H stretches both symmetric as well as asymmetric. The region of 1720-1560 1/cm showed the presence of C=O group. The region of 3600-3300 1/cm corresponds to -OH, -NH- and NH2 group. From the spectrum of TPGSDOX conjugate, we can find that the broad band in 3500-3300 1/cm attributed to the overlapping of O-H and N-H stretching frequency. The peak at 1450 1/cm indicated the N-H deformation in secondary amine structure, which was attributed to the linkage between –COOH group in TPGS and –NH2 group in doxorubicin.

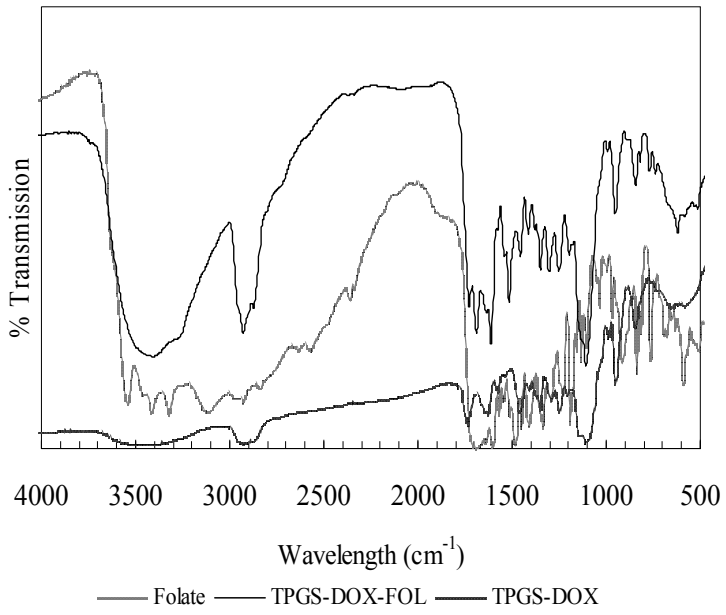


Fig 3-1 FT-IR Spectra of FOL, TPGS-DOX and TPGS-DOX-FOL

In the spectrum of TPGS-DOX-FOL, the region of 3500-3300 cm-1 was reduced. The peaks in 1720-1560 cm-1 was shifted to 1620-1460 due to the presence of C=N by the interaction between DOX and FOL, revealing the successful conjugate of TPGS-DOX and FOL.

3.4.2 ^{1}H-NMR Spectra

The typical ^{1}H NMR spectra of TPGS-DOX, FOL, FOL-NH-NH2 and TPGS-DOX-FOL are shown in Fig 3-2a, 3-2b, 3-2c and 3-2d respectively. The spectrum of TPGS-DOX (Fig 3-2a) contained signals from DOX and TPGS exhibiting typical peaks of DOX between 5-6 ppm, which are characteristic of phenolic protons of DOX, and peaks of TPGS at 3.6 ppm, which is the characteristic of methylene protons of poly ethylene oxide (PEO) part in TPGS. The peak at around 8 ppm is characteristic of amide protons, which indicates that the DOX has been conjugated with the TPGS by forming an amide bond. The spectrum of FOL (Fig 3-2b) exhibited typical peaks of FOL at 1.85-2.10 ppm (β-CH_2 of glutamic acid), 2.30 ppm (γ-CH2 of glutamic acid), 6.62 and 7.61 ppm (aromatic protons), 8.1 ppm (aliphatic amide proton) and 8.6 ppm (pteridine proton). The peak at 11.4 ppm is also characteristic of carboxylic groups. The spectrum of FOL-NH-NH_2 (Fig 3-2c) was similar to that of FOL. However, the exhibited peak at 8.1 ppm has split into multiple peaks from one sharp peak. This suggested the presence of additional amide groups. The peak at 11.4 ppm has been replaced by a smaller peak at around 11 ppm, which indicated that one of the carboxylic groups of FOL has reacted. The spectrum of TPGS-DOX-FOL (Fig 3-2d) retained the characteristic large peak of TPGS-DOX at 3.6 ppm and the characteristic peaks of FOL- NH-NH_2 from 6-9 ppm are also present. This confirmed the conjugation of TPGS-DOX-FOL. In addition, the peak at 11.4 ppm suggested the presence of carboxylic group, which further implies successful conjugation, as this group is originally present in the FOL- NH-NH_2.

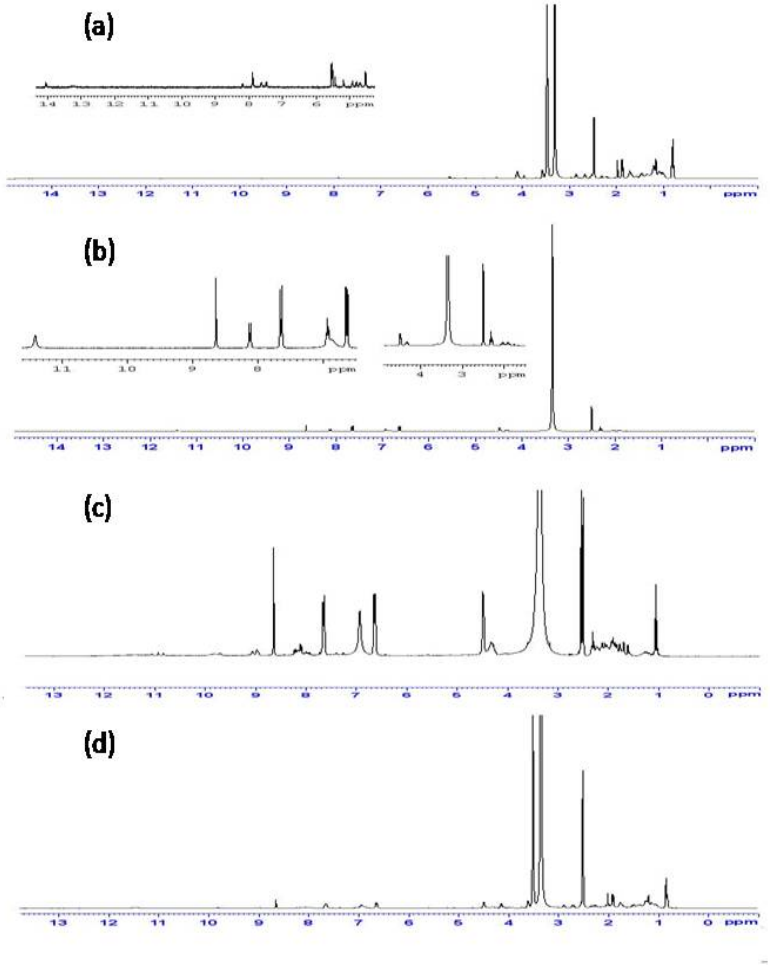


Fig 3-2 ^{1}H-NMR spectra of (a) TPGS-DOX with the insert for a higher magnification of the region between 6 and 14 ppm, (b) FOL with the insert for a magnification of the region between 8 and 11 ppm and 3 and 4 ppm, (c) FOL-NH-NH_2, (d) TPGS-DOX-FOL

3.4.3 Drug Loading Efficiency

The DOX content in the TPGS-DOX conjugate was determined using the microplate reader with fluorescence detection at 480 nm and was found to be 6.0 wt% and the DOX content in TPGS-DOX-FOL conjugate was determined to be 13.0 wt%. This drug loading capacity of the TPGS-DOX conjugate seems comparable to other polymer-DOX conjugates such as PEG-DOX

conjugates, which has a drug loading of 2.7 – 8.0 wt% varying due to branching of polymer and the nature of the linker (Veronese, Schiavon et al. 2005), HPMA copolymer-DOX conjugate which has a drug loading of 8.5 wt% (Vasey, Kaye et al. 1999), another PEG-DOX conjugate with 2.5-5 wt% drug loading (Rodrigues, Beyer et al. 1999) and PGA-DOX conjugate having a drug loading of 5-16% (Hoes, C. J. T., J. Grootoonk, et al. 1993). It is also found to be better than the other polymer-drug conjugates like PEG-Gemcitabine conjugate, wherein the Gemcitabine drug is loaded at 0.98-1.95 wt% (Pasut, Canal et al. 2008). This facilitates the possibility for the conjugate to go through the clinical trials. Now, the drug loading capacity of TPGS-DOX-FOL conjugate is also found to be a better value comparing other polymer-drug conjugates like FOL-PEG-Gemcitabine which has a drug loading of 2.11 wt% (Pasut, Canal et al. 2008) and can be further improved by altering parameters like the type of branching and using a linker or a spacer.

3.4.4 Conclusions

The TPGS-DOX-FOL conjugate was synthesized via the reaction between the TPGS-DOX, which is prepared by the interaction of the succinoylated TPGS and amine group of DOX, and FOL-NH-NH_2, which is formed by the interaction of NHS ester of folate and hydrazine hydrate. The conjugates TPGS-DOX and TPGS-DOX-FOL were characterized by FT-IR and ^{1}H-NMR to study the molecular structure and to confirm the conjugation. This shows successful synthesis of the conjugates. The drug loading in case of both the TPGS-DOX and the TPGS-DOX-FOL conjugate is found to be satisfactory when compared to the drug loading of other polymer-drug conjugates.

CHAPTER 4: *IN VITRO* STUDIES ON DRUG RELEASE KINETICS, CELLULAR UPTAKE AND CELL CYTOTOXICITY OF TPGS-DOX AND TPGS-DOX-FOL CONJUGATES

4.1 Introduction

The drug release from the conjugate is an important factor to be considered for its therapeutic efficiency. Knowing that mostly, the drug release is mediated by simple hydrolysis, we have studied the release kinetics of the drug at 37°C with different pH values. Also, the cellular uptake of the conjugates has been studied by calculating the percentage uptake of the conjugate *in vitro* using the breast cancer cells, MCF-7. The cellular uptake of the conjugates are then visualized by Confocal Laser Scanning Microscopy (CLSM). TPGS is said to enhance the cellular uptake in the human intestinal Caco-2 cell line (Traber, Thellman et al. 1988) and in the human colon carcinoma cells (Win and Feng 2006) by inhibiting the action of P-glycoprotein. Further, it was demonstrated that the folate can target the cancer cells and increase the cellular uptake of the conjugates (Zhang, Xiang et al. 2006; Zhang, Huey Lee et al. 2007). The *in vitro* cell viability study was done using CCK-8 assay in MCF-7 breast cancer cells. All the experiments were done for the TPGS-DOX-FOL conjugate in comparison with the conjugate TPGS-DOX and the pristine DOX.

4.2 Materials and Methods

4.2.1 Materials

Phosphate buffered saline (PBS), Dulbecco's Modified Eagel Medium (DMEM), penicillin-streptomycin solution, Trypsin-EDTA, Triton-X 100 and Tris buffer were obtained from Sigma-

Aldrich (St. Louris, MO, USA). Cell Counting Kit – 8 (CCK-8) was obtained from Dojindo Laboratories, Tokyo. Fetal bovine serum (FBS) and RPMI medium without folate were received from Gibco (Life Technologies, AG, Switzerland).

4.2.2 *In vitro* Drug Release

In vitro DOX release from the conjugate was performed in triplicates in 1X PBS at pH 3.0, 5.0 and 7.0 at 37°C, respectively. The solution of the conjugates TPGS-DOX and TPGS-DOX-FOL of 200 μg/mL equivalent DOX concentrations was placed in a dialysis bag (MW cutoff 1,000) and incubated in 20 mL of the PBS solution with gentle shaking in the water bath shaker. The PBS solution outside the dialysis solution was collected at designated time intervals and equal volume of fresh medium was compensated. The released DOX was determined by fluorescence detection at 480 nm using the microplate reader (GENios, Tecan) with the excitation wavelength at 480 nm and the emission wavelength at 580 nm with the help of a calibration curve of DOX in PBS, range from 0 to 1 μg/mL with R^2=0.9992.

4.2.3 Cell Culture

MCF-7 breast adenocarcinoma cells (American Type Culture Collection, VA) were used as the *in vitro* model to study the cellular uptake and the cell viability. The cells were cultured in the RPMI 1640 medium without folate or DMEM, both supplemented with 10% PBS, 1% penicillin-streptomycin solution, and incubated in SANYO CO2 incubator at 37°C in humidified environment of 5% CO2. The medium was replenished every day until confluence was achieved. The cells were then washed with PBS and harvested with 0.125% Trypsin-EDTA solution.

4.2.4 *In vitro* Cellular Uptake

MCF-7 breast adenocarcinoma cells were seeded in 96-well black plates (Costar, IL, USA) at a density of $3x10^5$cells/well. After the cells reached about 70-80% confluence, they were incubated with 100μL of TPGS-DOX-FOL or TPGS-DOX or free DOX solution in medium at 1 μg/mL drug concentration for 0.5, 1.5, 4, 6 hrs, respectively. For each sample, we seeded six wells for positive control and six wells for sample wells. At the designated time interval, the sample wells were washed three times with 50 μL cold PBS and then added 100 μL culture medium. After that, all the cells were lysed by 50 μL 0.5% Triton in 0.2M NaOH. The fluorescence intensity of each sample was detected by the microplate reader (Tecan, Mannedorf, Switzerland, λ_{ex} = 480 nm, λ_{em} = 580 nm) calibrated with standard solutions of DOX in similar condition. Cellular uptake efficiency was expressed as the percentage of the fluorescence associated with the cell *vs.* that presented in the positive control (Mo and Lim 2005).

4.2.5 Confocal Laser Scanning Microscopy (CLSM)

MCF-7 breast adenocarcinoma cells were incubated with TPGS-DOX-FOL conjugate or TPGS-DOX conjugate or free DOX medium solution at 1 μg/mL DOX concentration at 37°C for 4 hrs. The cells were then rinsed with cold PBS three times, fixed by 75% ethanol for 20 mins, and then washed twice by PBS. The cells were finally mounted by the mounting medium (DAKO® Fluorescent Mounting Medium) and observed under confocal laser scanning microscopy (Zeiss LSM 510, Germany)**.** Fluorescein Isothiocyanate (FITC) dye was conjugated to TPGS in the presence of DCC (Kolhe, Khandare et al. 2004) to observe the TPGS uptake in the cells. The fluorescence was observed at λ_{ex} = 495 nm, λ_{em} = 520 nm. The uptake of pristine DOX, TPGS-DOX, TPGS-DOX-FOL was observed at λ_{ex} = 480 nm, λ_{em} = 580 nm. Also, folic acid uptake by the cells was observed at λ_{ex} = 543 nm, λ_{em} = 590 nm.

4.2.6 *In vitro* Cytotoxicity

In vitro cytotoxicity study of free DOX, TPGS-DOX and TPGS-DOX-FOL conjugates were quantitatively measured by employing on MCF-7 breast adenocarcinoma cells. MCF-7 cells were cultivated in RPMI 1640 medium without folate, supplemented with 10% FBS and 1% antibiotics at 37°C in humidified environment of 5% carbon dioxide. The cells were seeded at a density of $5x10^3$ cells/well in 96-well plates (Costar, IL, USA) incubated for 24 hrs and the medium was then replaced by the free DOX , TPGS-DOX conjugate or TPGS-DOX-FOL conjugate respectively at various equivalent drug concentrations from 0.002 to 100 μM in the medium. The cell viability was determined by the CCK-8 assay. At the designated time intervals 24, 48, 72 hrs, the medium was removed and the wells were washed twice with PBS. 100μl of the CCK-8 solution is added to each well of the plate and incubated for about 3 hrs in the incubator. Each well was analyzed by the microplate reader with absorbance detection at 570 nm. The cell viability was calculated using the formula,

$$\textit{Cell viability (\%)} = (Abs_s / Abs_c) \times 100$$

Where Abs_s is the fluorescent absorbance of the wells containing the drug samples and Abs_c is the fluorescent absorbance of the wells containing the culture medium used as a positive control.

4.2.7 Statistics

Statistical analysis was conducted by using the Student's t-test with a significance of $p<0.05$.

4.3 Results and Discussion

4.3.1 *In vitro* Drug Release

Fig 4-1 shows the *in vitro* release profiles of DOX from the conjugates TPGS-DOX and TPGS-DOX-FOL at various pH conditions such as 3.0, 5.0 and 7.0. It can be seen that the release of

DOX from the conjugates was in a slow and sustained manner, which is pH dependent and increases as the pH decreases. The cumulative release of the drug from TPGS-DOX-FOL and TPGS-DOX at pH 3.0 for the first 15 days was found to be 30.23±2.16% and 98.34±1.1% respectively. This is understandable. For TPGS-DOX, the TPGS and DOX are linked through the degradable amide bond that can be broken easily under acidic conditions by hydrolysis. Also DOX was stable under the pH range <6.5. Since the proton concentration inside the lysosomes is higher than that outside the cells, gradual hydrolysis occurs inside the cells at lower pH which help in the degradation of the bond to release the DOX. For TPGS-DOX-FOL, instead, there is an additional bond, which has to be broken for the DOX to get released, which makes the release relatively slower than that from the TPGS-DOX. At pH 5.0, the release percentage of DOX from TPGS-DOX and TPGS-DOX-FOL after 15 days was found to be 53.26±3.6% and 5.24±0.78% respectively and at pH 7.0, it is 39.2±2.15% and 3.87±1.61% respectively. The release profile confirmed that the linkage is stable under alkaline conditions (pH >7) outside the cell and degraded in acidic conditions which attributes positive effects of the prodrug strategy. It should be pointed out that such a pH dependent drug release feature has advantages for cancer treatment, which is equivalent a passive targeting effect since tumor has been found in an acidic condition in the body.

The pharmacological activity of macromolecular prodrug requires the release of drugs from the conjugate by chemical or enzymatic reactions and this plays an important role in cancer chemotherapy. In terms of drug release, the stability of the linkage between the carrier and the drug and the site of regeneration of the free drug from the conjugate are important factors. In most cases, the anticancer drug is released from the polymer carrier through simple hydrolysis (Takakura and Hashida 1995). The free DOX is known to be stable in the pH range 3.0-6.5 (Vigeveni, A. and M. J. Williamson. “Doxorubicin”. New York, Academic Press. 1980). A well-known concept for the mechanism of action of macromolecular prodrugs is the principle of

'lysosomotropic' delivery which was advocated more than two decades ago by Trouet et al. (Trouet A 1972). Thus the release profile confirmed that the biodegradable bonds between the drug and the carrier are stable under alkaline conditions and that are broken under acidic conditions in the lysosomes.

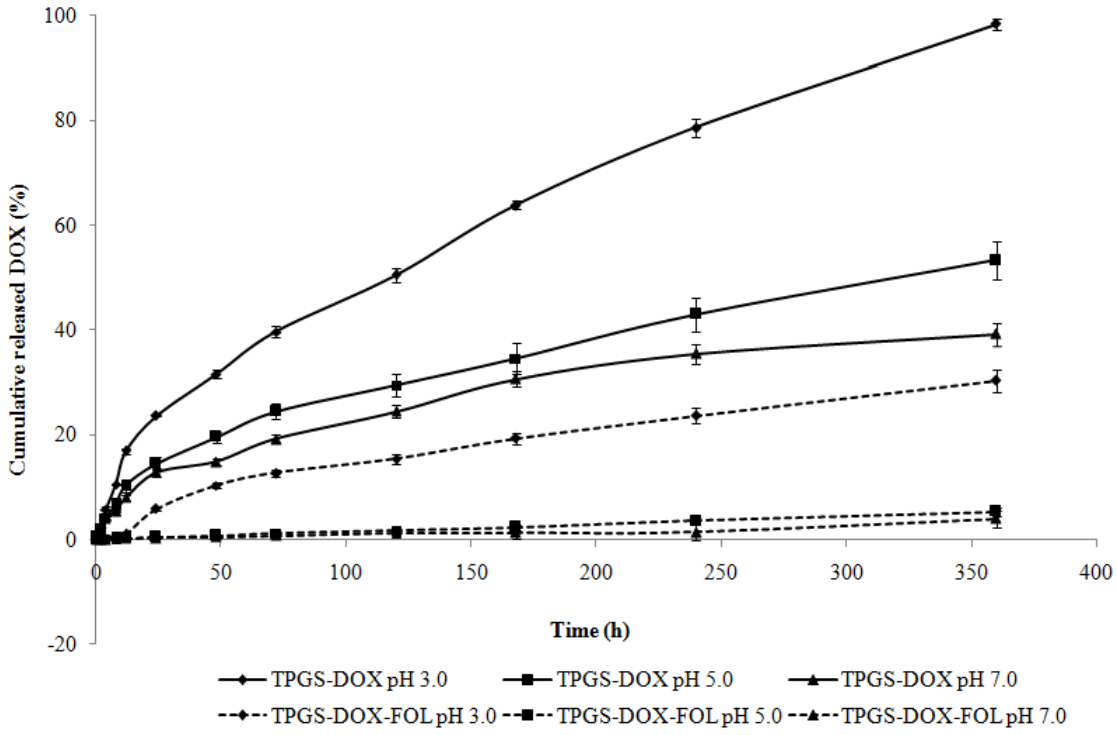


Fig 4-1 *In vitro* release of DOX from TPGS-DOX and TPGS-DOX-FOL conjugates incubated in phosphate buffer at 37°C at 3 different pH (Mean±SD and n=3)

4.3.2 *In vitro* Cellular Uptake

The targeting effect of the TPGS-DOX-FOL conjugate was evaluated by investigating the cellular uptake of the free DOX, and the conjugates of TPGS-DOX and TPGS-DOX-FOL by using the

MCF-7 breast cancer cells as an *in vitro* model. Fig 4-2 shows the cellular uptake efficiency of the DOX, TPGS-DOX and TPGS-DOX-FOL after 0.5, 1.5, 4 and 6 h incubation with MCF cells at 1 μg/mL DOX equivalent concentration at 37°C. It can be seen from the figure that after 0.5 hour culture, the cellular uptake of DOX, TPGS-DOX and TPGS-DOX-FOL is 70.2, 80.9 and 86.0% respectively, which shows 15.2% increase by TPGS conjugation and further 6.3% increase by targeting effect of FOL. After 1.5 hour culture, the cellular uptake of DOX, TPGS-DOX and TPGS-DOX-FOL is 77.4, 81.3 and 87.0% respectively, which shows 5.04% increase by TPGS conjugation and further 7.01% increase by targeting effect of FOL. After 4 hour culture, the cellular uptake of DOX, TPGS-DOX and TPGS-DOX-FOL is 79.2, 84.2 and 92.8% respectively, which shows 6.31% increase by TPGS conjugation and further 10.2% increase by targeting effect of FOL. After 6 hour culture, the cellular uptake of DOX, TPGS-DOX and TPGS-DOX-FOL is 83.1, 87.0 and 95.5% respectively, which shows 4.69% increase by TPGS conjugation and further 9.77% increase by targeting effect of FOL. It can thus be concluded that (1) the benefits in cellular uptake of DOX by TPGS conjugation and by TPGS+FOL conjugation are significant at the level of 5-10% increase and (2) incubation time is also an important factor. It seems that the benefit due to TPGS conjugation decreases with time while the targeting effect due to TPGS+FOL conjugation increases with time, both of them becoming saturated with time.

The cell uptake differences between the free drug DOX and the conjugates TPGS-DOX-FOL and TPGS-DOX, is mainly due to the Multi-Drug Resistance (MDR) effect, that is caused by the over expression of transporter proteins such as P-glycoproteins which is an energy dependent drug efflux to reduce the intracellular drug levels. These transporters are capable of pumping out many anticancer drugs that diffuse into the plasma membrane. For the DOX, it is said that most of the drug would be effluxed out by P-glycoproteins except those bound to DNA after entering the cells (Zhang and Feng 2006). The TPGS is said to inhibit the action of P-glycoproteins and only less drug will efflux out from the cells attributing to higher uptake efficiency (Dintaman and

Silverman 1999). In the case of TPGS-DOX-FOL conjugate, further the uptake is mediated by the targeting effect of folate. It is evident that the P-gp efflux affected the uptake of free doxorubicin compared to the uptake of folate-targeted liposomal doxorubicin in a MDR cell line (Goren, Horowitz et al. 2000).

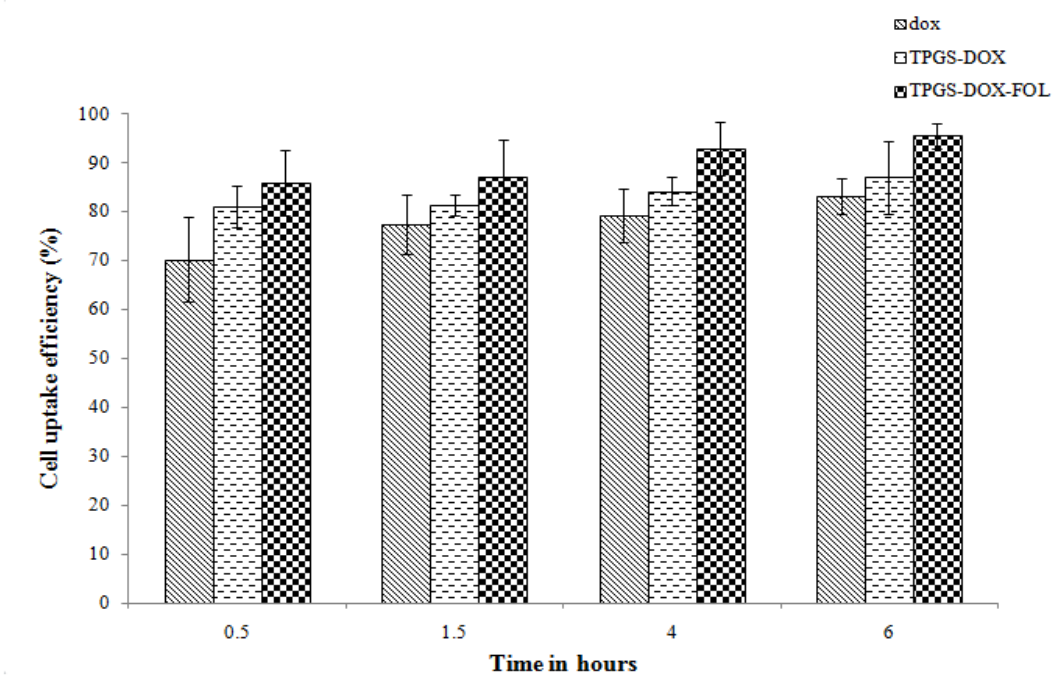


Fig 4-2 Cell uptake efficiency incubated with pristine DOX, TPGS-DOX or TPGS-DOX-FOL conjugate for 0.5, 1, 4, 6 h respectively at an equivalent DOX concentration of 1μg/mL in MCF-7 breast cancer cells (Mean±SD and n=6).

4.3.3 Confocal Laser Scanning Microscopy (CLSM)

The cellular uptake of TPGS, DOX, FOL, TPGS-DOX-FOL and TPGS-DOX conjugates by the MCF-7 breast adenocarcinoma cells were further visualized by confocal laser scanning microscopy (CLSM) and their images are shown in Fig 4-3. The five pictures in the figure show the confocal images of MCF-7 cancer cells after 4 h incubation with (a) TPGS-FITC, (b) DOX,

(c) FOL, (d) TPGS-DOX and (e)TPGS-DOX-FOL at the concentration of 1μg/mL TPGS, FOL and equivalent DOX concentration, respectively. Fig 4-3 (5a) shows the FITC fluorescence at λ_{ex} = 495 nm, λ_{em} = 520 nm, from which the TPGS was found distributed in the cytosol than the nucleus. This is because TPGS is an inhibitor of P-gp, which also improves the membrane interactions (Youk, Lee et al. 2005). Fig 4-3 (5b), Fig 4-3 (5d) and Fig 4-3 (5e) show the DOX fluorescence observed from the DOX, TPGS-DOX and TPGS-DOX-FOL respectively at λ_{ex} = 480 nm, λ_{em} = 580 nm. It can be seen from Fig 4-3 (5b) that DOX was found mainly present within the nucleus. The fluorescence in the cells is generally weak, which indicates a low DOX accumulation. This is due to the MDR effect caused by the P-glycoproteins, where most of the free DOX would be effluxed out of the cells and only the drug that binds to the DNA in the nucleus will remain inside the cell (Zhang and Feng 2006). Fig 4-3 (5c) shows the fluorescence from FOL, which was observed at λ_{ex} = 543 nm, λ_{em} = 590 nm. From this figure, the intense red fluorescence was observed around the nucleus and a broad distribution of fluorescence in the cytosol was also found. This suggests that the folic acid is endocytosed through the folate receptor-mediated mechanism, by attaching to the over expressed folate receptors found on the surface of the cancer cells (Lu and Low 2002). From Fig 4-3 (5d) for the TPGS-DOX, it can be found that the fluorescence is distributed more in the cytoplasm than in the nucleus, which shows a greater cellular uptake compared to the DOX. This is an indication that TPGS-DOX is able to avoid efflux by P-gp. Although the fluorescence is found mostly in the cytoplasm, which suggests that most of the DOX is still in conjugate form, the fluorescence in the nuclei indicates that some DOX has already been released by hydrolysis and intercalated with DNA. This result is similar to that obtained from the PAMAM-hyd-DOX conjugates which showed that the conjugate is able to release functional DOX (Lai, Lou et al. 2007). Fig 4-3 (5e) shows that in comparison to DOX and TPGS-DOX, the TPGS-DOX-FOL conjugate exhibited significantly greater cellular uptake with a broad intracellular distribution around the nucleus and the fluorescence appears to be distributed

more in the cytosol than in the nucleus. The intense fluorescence is generally due to the targeting effects of the FOL via receptor mediated endocytosis mechanism (Yoo and Park 2004). Lesser fluorescence in the nucleus was mainly due to slower release of the DOX from the conjugate.

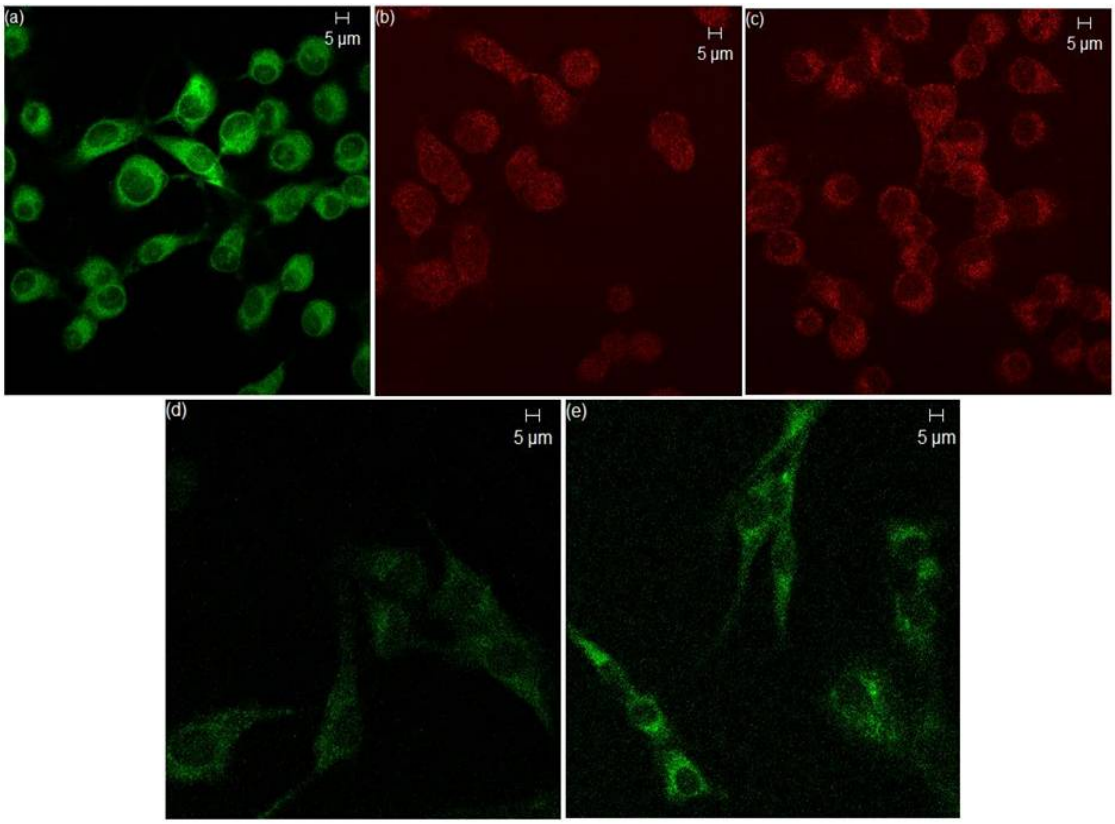
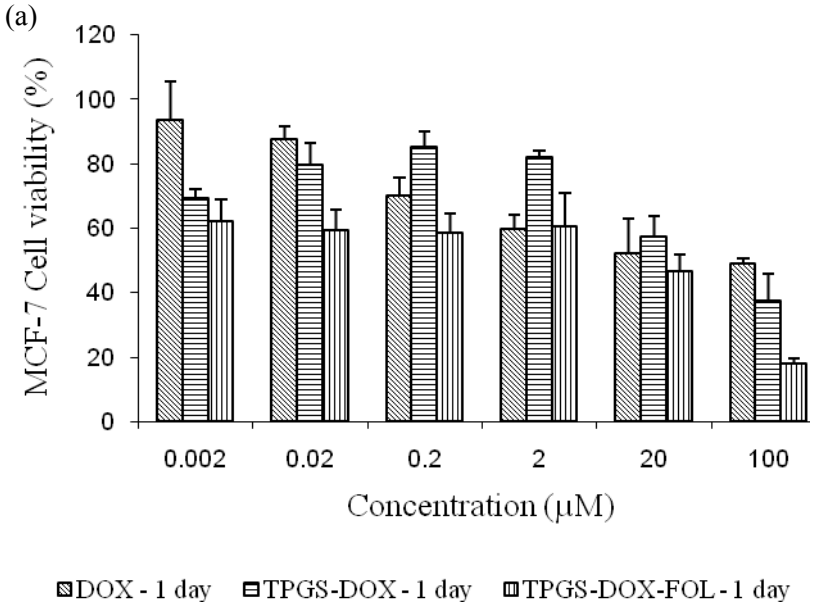


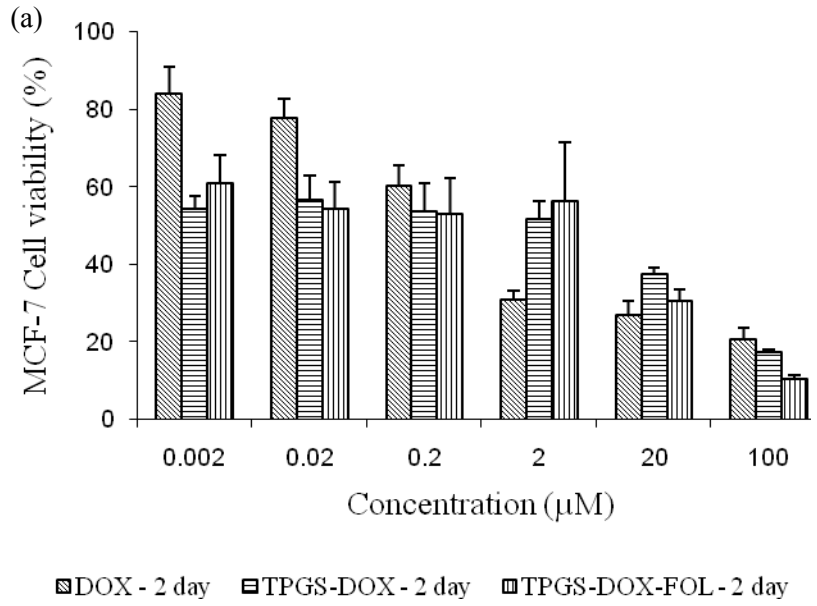
Fig 4-3 Confocal laser scanning microscopy (CLSM) of MCF-7 cells after 4 h incubation with (a) TPGS-FITC, (b) pristine drug DOX, (c) FOL, (d) TPGS-DOX conjugate and (e) TPGS-DOX-FOL conjugate at an equivalent DOX concentration of 1µg/mL

4.3.4 *In vitro* Cytotoxicity

The breast cancer cell line MCF-7 was used as an *in vitro* model to evaluate the cell cytotoxicity of the free DOX and the conjugates TPGS-DOX and TPGS-DOX-FOL. Fig 4-4 shows the

cellular viability of MCF-7 cells after (a) 24, (b) 48, (c) 72 hour culture at 37ºC with the DOX, TPGS-DOX and TPGS-DOX-FOL at the various equivalent DOX concentration of 0.002, 0.02, 0.2, 2, 20 and 100 μM respectively. The cellular viability was determined by the CCK-8 assay. The mortality is difference of the viability from 100%. It can be concluded from these three figures that (1) the benefits in resulting higher cytotoxicity by TPGS conjugation and by targeting effect of FOL are both significant and (2) the benefits are also dependent on the DOX concentration. For example, After 24 hour cell culture at 0.002 μM equivalent DOX concentration, the mortality of the MCF-7 cells were 9.36, 30.4 and 37.6% for the DOX, TPGS-DOX and TPGS-DOX-FOL, respectively, which shows 225% increase by TPGS conjugation and further 23.7% increase by targeting effect of FOL.





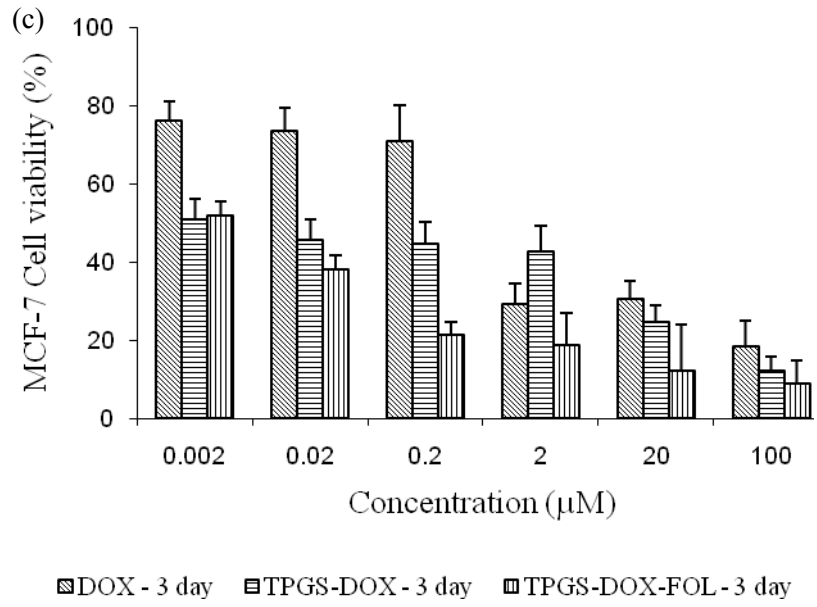


Fig 4-4 Cell viability of MCF-7 breast cancer cells after incubation with the TPGS-DOX conjugate and TPGS-DOX-FOL conjugate in comparison with that of the pristine DOX after (a) 24, (b) 48, and (c) 72 h at various equivalent DOX concentrations (Mean+SD and n=6)

After 24 hour cell culture at 100 μM equivalent DOX concentration, however, the mortality of the MCF-7 cells were 50.8, 62.6 and 82.0% for the DOX, TPGS-DOX and TPGS-DOX-FOL,

respectively, which shows 23.2% increase by TPGS conjugation and further 31.0% increase by targeting effect of FOL. A similar conclusion can be reached from the results obtained after 48 or 72 hour cell culture.

Alternatively, the *in vitro* cytotoxicity of a drug can be quantitatively evaluated by IC50, which is defined as the drug concentration needed to kill 50% of the cancer cells cultured with the drug in a designated time period, for example, in 24 hours etc. Table 4-1 lists the IC50 values of DOX in the form of the free drug, the TPGS-DOX and the TPGS-DOX-FOL conjugate after 24, 48, 72 h cell culture at 37°C respectively. It can be seen from this table that after 24 hour cell culture, the IC50 is 27.9 μM for the free DOX, 22.8 μM for TPGS-DOX and 0.590 μM for TPGSDOX- FOL, which implies that TPGS-DOX conjugate could be 22.4 % more effective than DOX and TPGS-DOXFOL could be 97.4% more effective than TPGS-DOX or 97.9% more effective than the DOX. The results after 48 and 72 hour cell culture showed the same significant enhancement of the TPGS-DOX and TPGS-DOX-FOL conjugates.

Table 4-1 IC_{50} values (in equivalent μM DOX level) of MCF-7 cancer cells cultured with the TPGS-DOX-FOL conjugate, TPGS-DOX conjugate and the pristine DOX in 24, 48 and 72 hrs

	IC50 (μM)		
Incubation time (hr)	DOX	TPGS-DOX	TPGS-DOX-FOL
24	27.1	22.8	0.590
48	0.620	0.1022	0.0641
72	0.530	0.01277	0.001811

Although, usually lower *in vitro* cytotoxicity is known for polymeric doxorubicin compounds such as hydroxypropylmethacrylate-copolymer conjugates (Duncan 1999; Kopecek, Kopeckova et al. 2000), doxorubicin-cephalosporin prodrugs (Veinberg, Shestakova et al. 2004), in this case, the TPGS-DOX conjugation results in lower IC_{50} values *in vitro* than the parent drug which might be due to the conjugation of the DOX with TPGS, that acts as an inhibitor of P-glycoprotein that is said to lower the MDR effect by decreased efflux of the drug (Dintaman and Silverman 1999). TPGS also has enhanced permeability due to the inhibition of P-glycoprotein. Also, TPGS possess great ROS (reactive oxygen species) generating ability (Youk, Lee et al. 2005) which enables it to facilitate increased cytotoxicity. Thus the TPGS-DOX is said to have a much lower IC_{50} values which can increase the therapeutic effect of the drug. Now, TPGS-DOX-FOL has much lower IC_{50} value than both the TPGS-DOX conjugate and the parent drug. This might be due to the targeting effect of the FOL, which enables increased amount of drug to enter the cancer cells and lesser amount of drug to enter the normal cells. It was also shown that the folate receptor-targeted formulation has lower IC_{50} value than the non-targeted formulations (Lu, Wu et al. 2007; Zhang, Huey Lee et al. 2007), which is similar in our case.

4.4 Conclusions

In vitro release of DOX from the conjugates, TPGS-DOX and TPGS-DOX-FOL, were found to be pH dependent, where the TPGS-DOX-FOL releases the drug in a slow and sustained manner that the TPGS-DOX, whose release is slower than the pristine DOX. TPGS-DOX-FOL conjugates gave higher cellular uptake efficiency that the TPGS-DOX conjugate and free DOX at all incubation times, which confirms the targeting effect of folate. The *in vitro* confocal laser scanning microscopy imaging showed that the TPGS-DOX-FOL conjugate was found distributed in the cytoplasm of MCF-7 cells, confirming the increased uptake by the cell. Also, the CLSM

images of TPGS and FOL showed that TPGS can inhibit P-glycoproteins, which resulted in its accumulation inside the cell and FOL can enter the cells by targeting. TPGS-DOX-FOL conjugate showed higher *in vitro* cytotoxicity in MCF-7 cells and achieved much lower IC_{50} values in comparison with TPGS-DOX and pristine DOX. As the time increases, the cytotoxicity also increases for TPGS-DOX-FOL conjugate. However, *in vivo* studies are required to confirm the *in vitro* results.

CHAPTER 5: *IN VIVO* STUDIES ON PHARMACOKINETICS AND BIODISTRIBUTION OF THE TPGS-DOX-FOL CONJUGATE

5.1 Introduction

The *in vivo* pharmacokinetics (PK) and the *in vivo* biodistribution (BD) of the conjugates TPGS-DOX-FOL, TPGS-DOX and DOX were investigated in male SD rats and compared. The *in vivo* pharmacokinetics makes use of the non-compartmental model for analysis of the therapeutic effects of the conjugates. In the case of *in vivo* biodistribution, HPLC was used to detect the drug amount in the tissues, which is easy to handle with acceptable sensitivity and selectivity. Other few methods have also been developed for detecting the amount of drug in blood and tissues. The blood and the organs are collected from the rats after administration and is undergone liquid-liquid extraction after which the samples were detected using HPLC with fluorescence detection.

5.2 Materials and Methods

5.2.1 Animal Type

Male Sprague-Dawley (SD) rats weighing 150-200 g and 4-5 weeks old were provided by the Laboratory Animals Centre of Singapore and maintained at the Animal Holding Unit (AHU) of National University of Singapore. They were kept in well-ventilated rooms at a temperature of 25°C and a humidity of 50-60% under nature lighting conditions. All caring and handling procedures for rats and experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC), Office of Life Sciences, National University of Singapore under the authority of Animal Welfare Act (AWA).

Fig 5-1 Experimental SD rats, who had sacrificed their lives for the well being of human

5.2.2 *In vivo* Pharmacokinetics

5.2.2.1 Drug Administration and Blood Collection

The rats were randomly assigned to three groups, each of four rats. One group is for the *i.v.* administration of free DOX, one group for TPGS-DOX conjugate and the other for TPGS-DOX-FOL conjugate. The free DOX and both the conjugates, TPGS-DOX and TPGS-DOX-FOL, were diluted in normal saline containing 1.9% w/v NaCl to obtain the required injection volume of 1-1.5 mL. Intravenous injection was given via the tail vein at a 5mg/kg equivalent drug dosage. All the rats were observed for mortality, general condition and potential clinical signs. The blood samples were collected in heparinized tube at 0, 10, 30 min, 1, 2, 4, 8, 12, 24, 48, 72 h after the administration of the DOX and the conjugates.

5.2.2.2 Sample Analysis

Plasma samples were harvested by centrifugation at 1500xg for 10 mins and stored at -20°C until analysis. Liquid-liquid extraction was performed prior to the HPLC analysis. Briefly, the plasma (100 μL) was mixed with 100 μL of 10 mM phosphate-buffered saline (pH 7.8). The drug was extracted by dichloromethane-isopropanol (4:1, v/v) on a vortex-mixer for 1 min. Upon centrifugation at 2000xg for 15 mins, the upper aqueous layer was removed by aspiration and the organic layer was transferred to a glass tube and evaporated under nitrogen at room temperature overnight. The residue was then dissolved in 100 μL of the HPLC mobile phase (1/15 M KH_2PO_4/CH_3CN=75:25 v/v, pH 4.16 adjusted with H_3PO_4) by vortex and transferred to auto sampler vials containing limited-volume inserts (100 μL). The standards were prepared using blank plasma with a series of concentration of commercial DOX (0.025 μg/mL – 10 μg/mL) followed by the same procedure as the blood samples were treated. The drug concentrations in samples were calculated using a standard calibration curve. For the HPLC analysis, the drug concentration in plasma was determined using Agilent 110 Series installed with Agilent Eclipse XDB-C18 column with 5 μm pore size. The mobile phase was delivered at a rate of 1 mL/min. 20 μL of the sample were injected into the instrument and the column effluent was detected with a fluorescent detector (λ_{ex} = 470 nm, λ_{em} = 585 nm) (Watson, Stewart et al. 1985).

5.2.2.3 Pharmacokinetic Parameters

Non-compartmental Analysis (NCA), done by Kinetica Software (Thermo Electron Corporation, USA), provides an estimate of the kinetic parameters of a drug based on statistical moment theory. As for the specific parameters, the maximum drug concentration (C_{max}) and the corresponding time (t_{max}) can be observed from the plasma concentration *vs* time curve. The

elimination half-life ($t_{1/2}$), an important index, can be calculated as $\ln 2/\lambda_n$, in which λ_n is the elimination constant obtained via log-linear regression analysis of the terminal phase of the profile. The area under the curve (AUC) and area under the first moment (AUMC) can be figured out using log-linear trapezoid rule. The mean residence time (MRT) is calculated as AUMC/AUC. Apparent volume of distribution at steady state (V_{ss}) and plasma clearance (CL) were obtained as Dosage × $AUMC_{inf}/(AUC_{inf})^2$ and Dosage/AUC_{inf}, respectively, in which $AUMC_{inf}$ and AUC_{inf} mean the corresponding value from 0 to infinity.

5.2.3 *In vivo* Biodistribution

5.2.3.1 Drug Administration and Tissue Collection

The SD rats were randomly assigned to three groups, i.e. Group A with *i.v.* injection of the pristine DOX suspension, Group B of the TPGS-DOX conjugate and Group C of the TPGS-DOX-FOL conjugate at the equivalent 5 mg/kg, respectively. Each group has 4 sets corresponding to 4 time points, and each set with 3 rats. Before drug administration, commercial DOX or TPGS-DOX conjugate were diluted in normal saline containing 1.9% w/v NaCl to obtain an estimated injection volume of 1-1.5 ml. Intravenous injection was given via the tail vein at a 5 mg/kg equivalent drug dosage. All animals were observed for mortality, general condition and potential clinical signs. Animals in each set were sacrificed by cardiac stick exsanguinations at 0.5, 2, 8 and 24 h respectively after the injection and tissues (heart, spleen, stomach, lung, intestine, kidney and liver) were collected. The tissues were then washed with saline and stored at -80^{o}C prior to analysis.

5.2.3.2 Sample Analysis

For the analysis, the tissues were freeze-dried, homogenized. After that, 30 mg organ for each was mixed with 300 μL PBS, followed by extraction and HPLC analysis as the blood samples done. The standards of different organs needed to be prepared using the blank tissues collected from the rats without any drug administration. A series of commercial DOX was added in the different blank organs respectively and then the standards were treated in the same way as the samples were analyzed. The drug level in organs was figured out using the standard calibration curve.

5.2.4 Statistics

Statistical analysis was conducted by using Student's t-test with $p<0.05$ as significant difference.

5.3 Results and Discussion

5.3.*1 In vivo* Pharmacokinetics

The DOX concentration in the plasma was found after *i.v.* injection of the commercial DOX, TPGS-DOX and TPGS-DOX-FOL in male SD rats at 5mg/kg dose up to 72 hours, which is shown in Fig 5-2. We find that the free DOX remains in plasma with shorter circulation time due to its short half-life. But, TPGS-DOX conjugates showed a much longer circulation time. This is due to the action of TPGS that is said to enhance the circulation time and hence the half-life of the pristine drug (Hanauske 2005). TPGS in a parental formulation has been used in clinical trials and has been the subject of a pharmacokinetic study (Lissianskaya 2004). In contrast to these, TPGS-DOX-FOL conjugate has a shorter circulation time than the TPGS-DOX conjugate and a longer circulation time than the free DOX. This might be attributed to the targeting property of

folate, resulting in increased cellular uptake and decreased presence of the drug in plasma that leads to shorter half-life than the TPGS-DOX conjugate. The amount of drug in the plasma for TPGS-DOX-FOL conjugate is greater than the one for pristine DOX and lower for TPGS-DOX conjugate. However, all the values fall below the peak concentration (8913.728 ng/mL) with the Maximum Tolerated Dose (MTD) administration (8 mg/kg) of pristine DOX (Houba, Boven et al. 2001). The lowest effective level was also found from the literature to be 5.44 ng/mL (Gavenda, Sevcik et al. 2001).

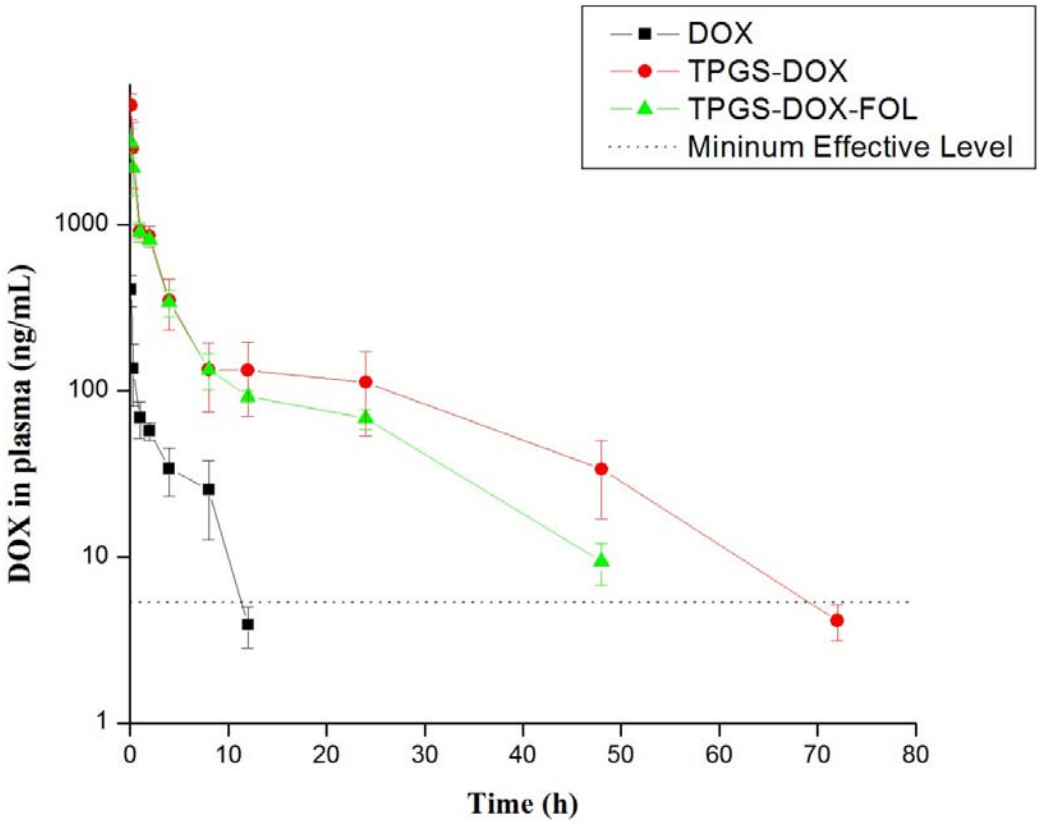


Fig 5-2 Pharmacokinetic profile of the pristine DOX, TPGS-DOX conjugate and TPGS-DOX-FOL conjugate after intravenous injection in rats at an equivalent dose of 5 mg/kg (mean±SD and n=4)

Table 5-1 shows the pharmacokinetic parameters of the pristine DOX and the conjugates, TPGS-DOX and TPGS-DOX-FOL. It can be seen that the half-life of the drug was 10.5±1.14 h for TPGS-DOX-FOL conjugate, 10.2±1.15 h for TPGS-DOX conjugate and 2.69±0.13 h for pristine DOX, which is in good agreement with the literature value (Gao, Lee et al. 2005). Here, the folate targeted conjugate has almost the same half-life period as the TPGS-DOX conjugate, which has 3.79 times longer half-life than DOX and is comparable to the previous similar work (Cao and Feng 2008). The mean residence time of the drug in plasma was found to be for 9.05±0.69 h TPGS-DOX-FOL conjugate, 12.5±1.89 h for TPGS-DOX conjugate and 3.8±0.26 h for DOX. Here, the mean residence time of TPGS-DOX is 3.29 times longer than that of the DOX, which is almost similar to that of the half-life period. In contrast, the mean residence time of the TPGS-DOX-FOL conjugate is 2.38 times longer than the pristine DOX and 1.38 times longer than the TPGS-DOX conjugate. Actually, increased plasma circulation time gives a positive and increased targeting effect (Seymour 1995). Area under the plasma (blood) concentration curve (AUC), which is the key therapeutic index, was found to be 6948±634 h.ng/mL for the TPGS-DOX-FOL conjugate which is 15 times greater than DOX, 9203±1534 h.ng/mL for the TPGS-DOX conjugate which is about 20 times greater than DOX and 480±145 h.ng/mL for the DOX. The Total clearance for the conjugate TPGS-DOX-FOL is 0.724±0.06 L/h/kg, for the conjugate TPGS-DOX is 0.55±0.09 L/h/kg and for the DOX is 11.2±3.7 L/h/kg. Since the AUC is directly proportional to the dose and inversely proportional to the elimination (Mehvar 2004), the total clearance for the conjugates TPGS-DOX-FOL, TPGS-DOX and pristine DOX was found to be acceptable. The clearance for the folate targeted conjugate is found to be 1.3 times faster than the TPGS-DOX and 15 times slower than the DOX. These results show enhanced pharmacokinetic properties for TPGS-DOX conjugate and almost not much difference in the folate targeted conjugate. The volume of distribution at steady state was found to be 8.24±1.85 L/kg for TPGS-DOX conjugate and 43.5±13.7 L/kg for DOX, which is 5.3 times greater value. Here, the volume

of distribution for the TPGS-DOX-FOL conjugate is 10.9±0.4 L/kg, which is about 4 times smaller than the DOX and 1.3 times smaller than TPGS-DOX. Although the conjugate TPGS-DOX show enhanced pharmacokinetic effects than the other polymer-drug conjugates (Senter, Svensson et al. 1995; Veronese, Schiavon et al. 2005), the TPGS-DOX-FOL was found to have comparably similar pharmacokinetic properties of TPGS-DOX conjugate than the pristine DOX and greater antitumor activity due to the folate targeting.

Table 5-1 Pharmacokinetic parameters of the TPGS-DOX-FOL conjugate, TPGS-DOX conjugate and the pristine DOX through *i.v.* injection at an equivalent dose of 5 mg/kg

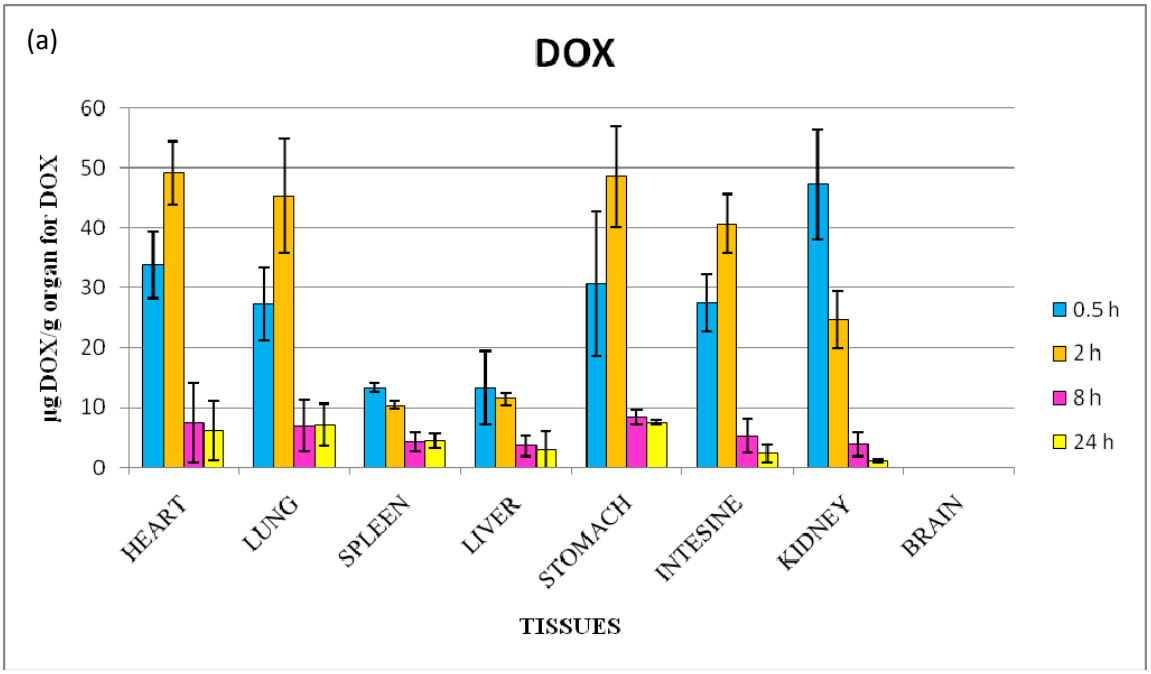
Parameter	*DOX*	*TPGS-DOX*	*TPGS-DOX-FOL*
$T_{1/2}$[a] (h)	2.69±0.13	10.2±1.15	10.5±1.14
MRT[b] (h)	3.8±0.26	12.5±1.89	9.05±0.69
$AUC_{0-\alpha}$[c] (h.ng/mL)	480±145	9203±1534	6948±634
CL_{tot}[d] (L/h/kg)	11.2±3.7	0.55±0.09	0.724±0.06
V_{dss}[e] (L/kg)	43.5±13.7	8.24±1.85	10.9±0.4

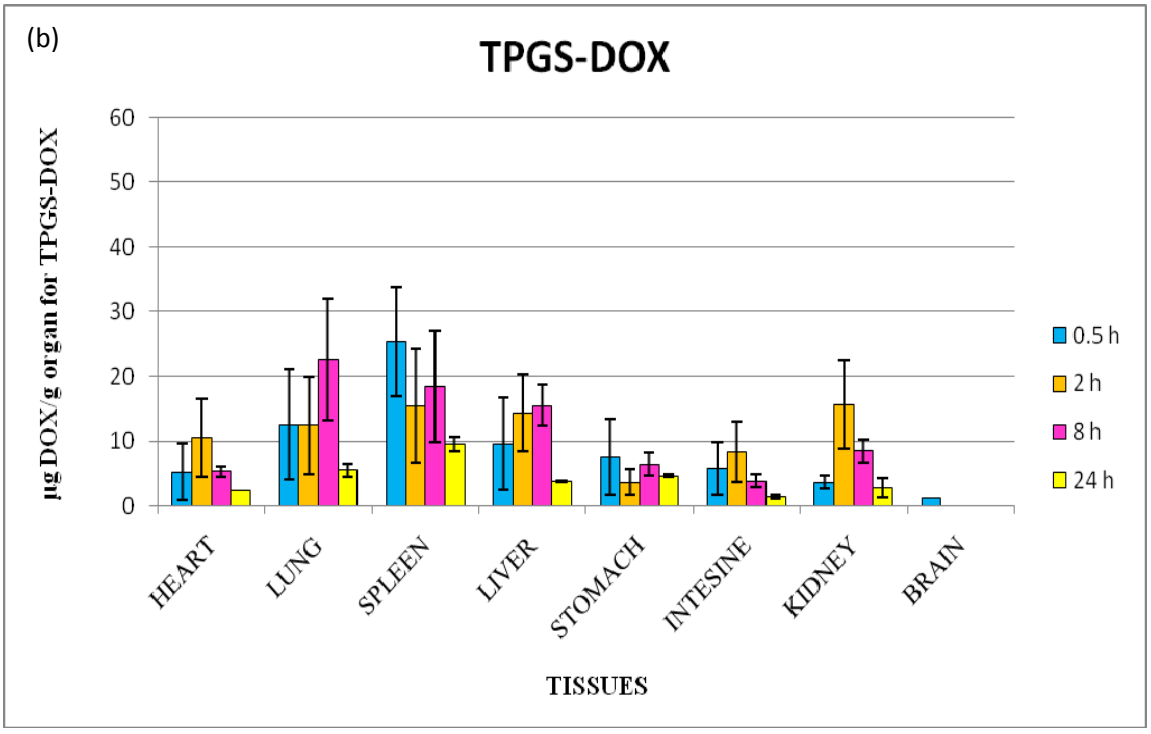
[a] half-life time; [b] mean residence time; [c] area under the curve; [d] total clearance; [e] volume of distribution at steady state.

5.3.2 *In vivo* Biodistribution

The biodistribution of DOX in tissues, which includes heart, lung, spleen, liver, stomach, intestine, kidney and brain, were investigated at time periods of 0.5, 2, 8 and 24 h. The amount of DOX distributed in the tissues for DOX, TPGS-DOX and TPGS-DOX-FOL conjugates are shown in Fig 5-2 (a), (b) and (c). The peak concentration for DOX accumulation was found at 0.5 or 2 h. Whereas, the peak concentration for TPGS-DOX and TPGS-DOX-FOL conjugates were

similar and is detected at 2 or 8 h. The conjugation of TPGS has significantly affected the biodistribution. The results for free DOX and TPGS-DOX conjugate were in agreement with the earlier work done (Cao and Feng 2008).





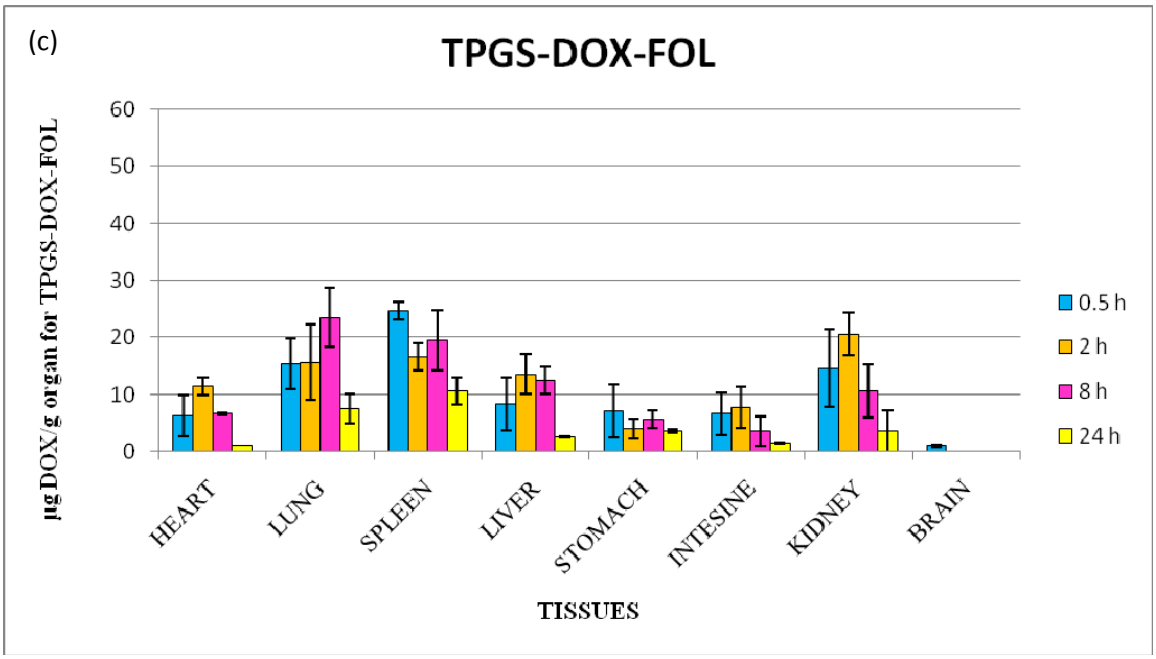


Fig 5-3 The amount of DOX (μg/g) in heart, lung, spleen, liver, stomach, intestine, kidney and brain after i.v. administration at 5mg/kg equivalent dose of (a) the free DOX, (b) the TPGS-DOX conjugate, (c) the TPGS-DOX-FOL conjugate (mean±SD and n=3)

The highest concentration for DOX was found in heart (49.23±5.27 μg/g organ at 2 h), followed by stomach (48.66±8.44 μg/g organ at 2 h), kidney (47.23±9.27 μg/g organ at 0.5 h), lung (45.32±9.43 μg/g organ at 2 h), intestine (40.75±4.93 μg/g organ at 2 h), spleen (13.34±0.74 μg/g organ at 0.5 h) and liver (13.32±6.13 μg/g organ at 0.5 h). Comparatively, the TPGS-DOX conjugate have a peak DOX concentration in heart (10.45±6.03 μg/g organ at 2 h), stomach (7.53±5.93 μg/g organ at 0.5 h), kidney (15.66±6.76 μg/g organ at 2 h), lung (22.54±9.43 μg/g organ at 8 h), intestine (8.24±4.67 μg/g organ at 2 h), spleen (25.34±8.51 μg/g organ at 0.5 h) and liver (15.55±3.25 μg/g organ at 8 h), which is 4.7-, 6.4-, 3-, 2-, 4.9- fold increase respectively. The spleen and the liver of the conjugates increased by 1.9- and 1.2- fold than the DOX. For the TPGS-DOX-FOL conjugate, the peak concentration in the tissues were found to be 11.52±1.54 μg/g organ at 2 h for heart, 7.15±4.65 μg/g organ at 0.5 h for stomach, 20.64±3.66 μg/g organ at 2

h for kidney, 23.55±5.24 μg/g organ at 8 h for lung, 7.75±3.65 μg/g organ at 8 h for intestine, 24.64±1.53 μg/g organ at 0.5 h for spleen, 13.63±3.54 μg/g organ at 2 h for liver, which is 4.3-, 6.8-, 2.3-, 1.9-, 5.3- fold lesser than DOX respectively and the concentration for spleen and liver are 1.8-, 1- fold higher than for DOX respectively. Unlike other tissues, brain has a very less amount of DOX accumulated and thus for 0.5 h, the amount of DOX for the conjugates TPGS-DOX and TPGS-DOX-FOL are 1.12±0.02 μg/g organ and 1.04±0.13 μg/g organ.

Table 5-2 AUC values (μg.h/g) of biodistribution in various organs after *i.v.* injection of free DOX or TPGS-DOX (T-D) or TPGS-DOX-FOL (T-D-F) conjugates to SD rats at 5 mg/kg equivalent dose

	HEART	**LUNG**	**SPLEEN**	**LIVER**	**STOMACH**	**INTESTINE**	**KIDNEY**
DOX	396±233	392±178	231±85	169±129	430±55	246±82	186±53
T-D	140±49	322±130	751±116	263±86	404±348	103±32	201±77
T-D-F	129±12	372±108	796±202	222±56	218±121	114±34	283±182

Table 5-2 summarizes the AUC values of DOX in various tissues after intravenous injection of the free DOX or TPGS-DOX or TPGS-DOX-FOL conjugates. Comparing the TPGS-DOX and TPGS-DOX-FOL conjugates with free DOX, the AUC values for liver and spleen of the conjugates is higher than DOX, which is 1.5- and 1.3- fold for liver and 3.2- and 3.4- fold for spleen respectively. The AUC values are lower for heart and intestine comparing both the conjugates with the free DOX i.e. for heart, TPGS-DOX is 2.8- fold and TPGS-DOX-FOL is 3-

fold lower than DOX and for intestine, TPGS-DOX is 2.4- fold and TPGS-DOX-FOL is 2.2- fold lower than DOX. Also, the AUC values in kidney for conjugates TPGS-DOX and TPGS-DOX-FOL is 1.08- and 1.5- fold higher than free DOX respectively.

DOX is a lipophilic molecule and as a consequence it rapidly penetrates into tissues. Therefore, normal tissue DOX tissue levels are higher after DOX administration and causes side effects. The prodrug is said to deliver decreased amount of drug to the tissues, which proves to lower the side effects (Houba, Boven et al. 2001). TPGS, which is has hydrophilic property, when conjugated to DOX to produce TPGS-DOX and can prevent diffusion of the prodrug into the tissues. This is confirmed by the high peak concentrations of TPGS-DOX and its rapid clearance from normal tissues. The folate targeted conjugate, TPGS-DOX-FOL have almost similar distribution as the TPGS-DOX conjugate. The amount of drug for TPGS-DOX-FOL conjugate in kidney is little higher than the TPGS-DOX, which is due to the overexpression of the folate receptors on the apical membrane of the proximal tubules (Lu and Low 2002). The conjugation of DOX to TPGS decreased the accumulation of drug in the heart, which is an advantage of the prodrug because this organ is the site of cumulative dose-limiting toxicity of DOX. The TPGS-DOX-FOL also has similar amount of drug. Thus both the conjugates limit one of the important side effect of DOX, namely cardiotoxicity. The gastrointestinal toxicity is also considered as a serious side effects of DOX (Mishra and Jain 2000). Since the amount of DOX in stomach and intestine for TPGS-DOX-FOL and TPGS-DOX conjugates are much lower than for the DOX, this limits the gastrointestinal side effects as well. Eventhough, the DOX amount in brain is negligible for DOX, it is found in very small amount for TPGS-DOX and TPGS-DOX-FOL conjugates. This confirms that the conjugates can cross the blood-brain barrier. Thus, the TPGS-DOX-FOL prodrug significantly reduced the serious sideffects of DOX and showed increased efficacy that the DOX.

5.4 Conclusions

The *in vivo* pharmacokinetics results show that TPGS-DOX has higher AUC than the original DOX as well as the TPGS-DOX-FOL conjugation. The half-life of both the conjugates, TPGS-DOX and TPGS-DOX-FOL were found to be similar and higher than for the pristine DOX, due to the TPGS conjugation and very minimum effect of folate. As the time increases, the amount of drug in the plasma was found to be maximum for the TPGS-DOX conjugate, followed by TPGS-DOX-FOL and then finally free DOX. The peak values of DOX for the TPGS-DOX conjugate in the tissues were reduced, especially in heart, stomach and intestine, which imply decreased side effects. In the case of TPGS-DOX-FOL conjugate, the peak value in the heart is almost similar and a bit lower in the stomach and intestine, which is confirmed to be advantageous in reducing systemic cytotoxicity, especially preventing cardiotoxicity.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The main objective of this work is to show the active targeting effects of folate conjugated to a prodrug and prove that active targeting is better than the passive targeting. This formulation for cancer targeted chemotherapy has been synthesized by conjugating the anticancer drug DOX to folic acid and Vitamin E TPGS. They are analyzed using FT-IR and ^{1}H NMR for confirmation of conjugation, *in vitro* release, cellular uptake, cellular cytotoxicity, *in vivo* pharmacokinetics and biodistribution. There are many works on active targeting of the prodrug or polymer-drug conjugates, mainly using folate and herceptin. Also, TPGS acts as a novel amphiphilic polymer to overcome many barriers in cancer chemotherapy including uptake, plasma half-life and distribution of drug in the tissues. Thus it can be used to improve the therapeutic effects of the drug.

From chapter 3, the TPGS-DOX-FOL conjugate was synthesized via the reaction between the TPGS-DOX, which is prepared by the interaction of the succinoylated TPGS and amine group of DOX, and FOL-NH-NH_2, which is formed by the interaction of NHS ester of folate and hydrazine hydrate. The conjugates TPGS-DOX and TPGS-DOX-FOL were characterized by FT-IR and ^{1}H-NMR to study the molecular structure and to confirm the conjugation. This shows successful synthesis of the conjugates. The drug loading in the case of TPGS-DOX and TPGS-DOX-FOL was found to be 6 wt% and 13 wt%, which seems comparable to other polymer-DOX conjugates. From chapter 4, *in vitro* release of DOX from the conjugates, TPGS-DOX and TPGS-DOX-FOL, were found to be pH dependent, where the TPGS-DOX-FOL releases the drug in a slow and sustained manner that the TPGS-DOX, whose release is slower than the pristine DOX.

TPGS-DOX-FOL conjugates gave higher cellular uptake efficiency that the TPGS-DOX conjugate and free DOX at all incubation times, which confirms the targeting effect of folate. The *in vitro* confocal laser scanning microscopy imaging showed that the TPGS-DOX-FOL conjugate was found distributed in the cytoplasm of MCF-7 cells, confirming the increased uptake by the cell. Also, the CLSM images of TPGS and FOL showed that TPGS can inhibit P-glycoproteins, which resulted in its accumulation inside the cell and FOL can enter the cells by targeting. TPGS-DOX-FOL conjugate showed higher *in vitro* cytotoxicity in MCF-7 cells and achieved much lower IC_{50} values in comparison with TPGS-DOX and pristine DOX. As the time increases, the cytotoxicity also increases for TPGS-DOX-FOL conjugate. However, *in vivo* studies were required to confirm the *in vitro* results. From chapter 5, the *in vivo* pharmacokinetics results show that TPGS-DOX has higher AUC than the original DOX as well as the TPGS-DOX-FOL conjugation. In the case of TPGS-DOX-FOL conjugate, the peak value in the heart is almost similar and a bit lower in the stomach and intestine, which is confirmed to be advantageous in reducing systemic cytotoxicity, especially preventing cardiotoxicity.

Among all, this work demonstrated that folate, conjugated to the earlier developed prodrug could enhance the cellular uptake and the cytotoxicity of the drug, due to its targeting effect towards the folate receptors, which are over expressed on the cancer cells. It is also said to have a slower and sustained release due to the increased number of bonds in TPGS-DOX-FOL conjugate than the TPGS-DOX conjugate and pristine DOX. The IC_{50} values for the TPGS-DOX-FOL conjugate was found to be much lower than for TPGS-DOX conjugate and free DOX making it a great clinical promise for drug delivery. Further, *in vivo* pharmacokinetics revealed that TPGS-DOX conjugate had higher retention time in plasma when compared to TPGS-DOX-FOL, which has a retention time higher than the DOX. This is due to the folate targeting that enhances cellular uptake and decreases the drug concentration in plasma. In the biodistribution, both the conjugates have higher and similar amount of drug in heart, which greatly reduced the side effect of DOX.

Also the amount of drug in the stomach and the intestine was found to be in reduced amounts, impairing the gastrointestinal side effect of DOX as well.

6.2 Recommendations

Further, the TPGS-DOX-FOL conjugate can be improved by the following suggestions.

- To use different tumor models in animals and evaluate the anticancer effect of TPGS-DOX-FOL conjugate
- To apply the TPGS-DOX-FOL conjugate in clinical phase I test for further investigation in therapeutic effects for the treatment of cancer
- To develop folate targeted polymer-drug conjugate using TPGS with other hydrophobic drugs like Paclitaxel, Docetaxel or hydrophilic drugs.

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