COMPARATIVE CELLULAR UPTAKE STUDIES OF A CARBORANE CHOLESTERYL ESTER BY HUMAN GLIOMA CELL LINES

by

YAN HUANG

(Under the Direction of D. Robert Lu)

ABSTRACT

Low-density lipoprotein (LDL) is the major cholesterol carrier in human plasma. To utilize the elevated expression of LDL receptor on many types of cancer cells, reconstituted LDL has been used as a potential carrier system to deliver anti-cancer drugs selectively to tumor cells. A new cholesterol-carborane conjugate (BCH) mimicking the native cholesteryl ester has been developed for boron neutron capture therapy (BNCT) in our laboratory. The compound is extremely hydrophobic and can be formulated into liposomes. Liposomes carry it to interact with LDL, resulting in the transfer of the drug into the LDL, and thereby, the uptake of BCH by cells via receptor-mediated endocytosis of the LDL. In this study, the cellular uptake of boron was evaluated in human glioma cell line SF-767 based on a series of comparative cell culture studies with BCH and p-carborane, either in DMSO or in conventional liposomes. The cellular uptake of boron from PEG-liposomal formulation was also evaluated. The dependence of uptake on incubation time and the cytotoxicity of the delivery systems were also investigated in the above experiments. The results indicated that the cellular uptake of boron by human glioma cell SF-767 was significantly higher (2.5-8.3 folds) from BCH than that from p-carborane when they were dissolved in DMSO. The encapsulation efficiency of BCH was 10 folds of that of p-carborane when they were formulated in liposomes. In cell culture studies with SF-767 and SF-763, there was no significant difference between the results of experiments (uptake of boron and cytotoxicity) using BCH-loaded conventional liposomal formulation and PEG liposomal formulation.

INDEX WORDS: Glioblastoma, Boron, Low-density lipoprotein (LDL), Liposome, Polyethylene glycol (PEG), Cellular uptake

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv

LIST OF TABLES vii

LIST OF FIGURES viii

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW 1

2 COMPARATIVE CELLULAR UPTAKE STUDIES OF A CARBORANE CHOLESTERYL ESTER BY HUMAN GLIOMA CELL LINES 12

2.1 Introduction 12

2.2 Materials and Methods 15

2.3 Results 21

2.4 Discussion 29

2.5 References 33

3 DETAILED EXPERIMENTAL RESULTS 37

3.1 Characterization of the liposomal formulations 37

3.2 Boron uptake with p-carborane and BCH dissolved in DMSO 40

3.3 Boron uptake with p-carborane and BCH conventional liposomal formulation 41

3.4 Boron uptake by two cell lines with BCH-loaded conventional and PEG liposomes 42

3.5 Cytotoxicity evaluation of DMSO, BCH-DMSO, p-carborane-DMSO, BCH-loaded conventional and PEG liposomal formulations.....43

APPENDICES

A CELL CYCLE, GROWTH CONTROL AND DEFENSIVE RESPONSES ..46

LIST OF TABLES

Page

Table 3.1: p-carborane encapsulation efficiency 40

Table 3.2: The effect of incubation time on the uptake of boron by SF-767 with BCH-DMSO and p-carborane-DMSO 41

Table 3.3: The effect of incubation time on the uptake of boron by SF-767 with conventional liposomal formulations containing p-carborane or BCH 41

Table 3.4: Cellular uptake of boron by SF-767 with BCH-loaded conventional and PEG liposomal formulations after 12, 24, and 36 hours of incubation 43

Table 3.5: Cellular uptake of boron by SF-763 with BCH-loaded conventional and PEG liposomal formulations after 12, 24, and 36 hours of incubation 43

Table 3.6: Cytotoxocity of DMSO, p-carborane-DMSO, and BCH-DMSO based on relative cell viability (%) in SF-767.....44

Table 3.7: Cytotoxocity of the liposomeal formulations based on relative cell viability (%) in SF-767.....44

Table 3.8: Cytotoxocity of the BCH liposomeal formulations based on relative cell viability (%) in SF-763 45

LIST OF FIGURES

Page

Figure 2.1: Molecular Structure of Cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH) 14

Figure 2.2: P-carborane encapsulation efficiency in conventional liposomes 23

Figure 2.3: Cellular uptake of boron by SF-767 with BCH-DMSO and p-carborane-DMSO after 12, 16, and 24 hours of incubation 24

Figure 2.4: Cellular uptake of boron by SF-767 with liposomal formulations containing p-carborane and BCH after 12, 16, and 24 hours of incubation 25

Figure 2.5: Cellular uptake of boron by SF-767 with BCH-loaded conventional and PEG liposomal formulations after 12, 24, and 36 hours of incubation 26

Figure 2.6: Cellular uptake of boron by SF-763 with BCH-loaded conventional and PEG liposomal formulations after 12, 24, and 36 hours of incubation 27

Figure 2.7: Cytotoxocity of DMSO, p-carborane-DMSO, and BCH-DMSO based on relative cell viability (%) in SF-767 28

Figure 2.8: Cytotoxocity of the liposomeal formulations based on relative cell viability (%) in SF-767 28

Figure 2.9: Cytotoxocity of the BCH liposomeal formulations based on relative cell viability (%) in SF-763 29

Figure 3.1: Size Distribution of Conventional Liposome containing BCH 38

Figure 3.2: Size Distribution of Conventional Liposome containing p-carborane 38

Figure 3.3: Size Distribution of PEG Liposome containing BCH.....39

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Glioblastoma multiforme (GBM) is the most common and most aggressive of the primary brain tumors and is highly malignant. GBM are usually treated with a combination of surgery, radiation, and chemotherapy. Although some advances have been achieved in diagnostic and surgical techniques, the median survival time has not been increased significantly with the current treatments. There are several factors contributing to the poor effectiveness of conventional treatments. Surgical removal of GBM is very difficult as GBM is usually embedded in normal tissues tightly. The systemic toxicity and lack of specificity of chemotherapeutic agents also limit the use of chemotherapy, as the radiation and chemotherapeutic agents do harm to normal tissues as well as malignant tumors.

Boron neutron capture therapy (BNCT) is an investigational form of a two-part radiation therapy which has the potential ability to selectively kill tumor cells embedded within normal tissue. In traditional chemotherapy, the strength of the chemotherapeutic agent is restricted by the survival ability of the surrounding cells. In BNCT, a chemical compound containing element ^{10}B is infused intravenously into the subject's body and concentrates more in tumor cells (e.g., glioblastoma or melanoma) than in corresponding normal cells (by requirement). Once tumor cells have been selectively loaded with boron in this approach, the brain is irradiated with neutrons from a nuclear reactor. Neutrons are atomic radiation particles, which have a minor harmful effect on tissue in the absence of

^{10}B. However, the absorption of these neutrons by the boron atoms in the tumor cells causes the boron atoms to emit alpha particles. Alpha particles can be lethal to the tumor cells within 10 μm of the site of capture reaction. The distance they travel is about the diameter of a tumor cell, so any surrounding normal cells are much less affected by the alpha radiation. Based on this mechanism, the generated alpha particles attack the tumor cells while at the same time have a less damaging effect on normal cells [1].

As mentioned before, successful BNCT depends on higher boron concentration at the tumor sites than the surrounding normal tissues. The boron concentration needs to stay at a high level until the neutron irradiation is applied, thus provides a much higher toxicity to the tumor cells than the normal cells. Development of drugs to provide better targeting to tumors cells is essential for the success of this process.

BNCT was first attempted at Brookhaven laboratory in the 1950s. Clinical trials at Brookhaven and another research facility at the Massachusetts Institute of Technology were halted in 1961 because of disappointing results. Two major factors contributed to this failure: (1) The low-energy neutron beam did not penetrate deeply enough into the patient's head to control tumor growth without harming surrounding healthy brain tissue; (2) Boron compounds used at that time did not preferentially accumulate in the tumor [2]. The first barrier was overcome by the introduction of an intermediate-energy neutron beam, called an epithermal neutron beam. The second barrier is till a bottleneck for the success of BNCT, although various boron compounds have been synthesized and tested for practical use in clinical treatment. It was reported in [18] that p-carboxybenzeneboronic acid and sodium decahydrodecaborate have very favorable properties for tumor localization. P-borophenylalanine (BPA) was reported to be

promising when applied to animals [4]. Boronated LDL was also studied for its potential use in BNCT. Although the localization capability has been improved significantly since the introduction of BNCT, research in boron compounds for better tumor targeting is still a main challenge for the success of BNCT.

It has been known that many cancer cells over-express certain cell surface receptors including transferrin receptor [4], folate receptor [5], and LDL receptor [4, 7-13], to meet the increased cell proliferation and growth requirements. One strategy of developing targeted drug delivery for cancer therapy is to take advantage of these over-expressed cell surface receptors. Structural and/or functional uniqueness of these receptors, together with the fact that they are over-expressed on the surface of the tumor cells, provides the specificity for drug targeting. By incorporating the corresponding ligands for the cancer cell surface receptors onto drug or drug carriers, anticancer drug can be specifically delivered to the cancer site.

Low-density lipoprotein (LDL) receptor has been identified for targeting certain type of cancer cells including human glioma cells. This is based on the fact that fast growing tumor cells need more cholesterol in order to synthesize new membrane, and LDL is the main transport carrier for cholesterol [12,14]. In a study on human malignant glioma cells, more than 80% of the cholesterol moiety of LDL was endocytosed via the LDL receptors [24]. It is also reported that that many tumors of various origins have an elevated level of LDL receptors compared with the corresponding normal cells. For example, it was recently reported that seven gliobastoma multiforme cell lines have a much higher level of LDL receptors than their corresponding normal cells. Two human glioma cell lines, SF-767 and SF-763, which will be used in our experiments, have an

average of 288,000 and 950,000 LDL receptors, while the corresponding normal cells have about 15,000 to 70,000 LDL receptors [4]. These results suggest that the elevated level of LDL receptors in the tumor cells provides an ideal candidate for the selective delivery of anticancer drugs to the tumor cells.

LDL is quasispherical endogenous nanoparticle. An LDL particle, whose size is about 22 nm in diameter, consists of a lipid core surrounded by a monolayer of phospholipids in which cholesterol and apolipoprotein B-100 (apo B) are incorporated [20]. LDL particles are endogenous with a long serum half-life of 2–4 days in humans. The nanoparticle size of LDL allows the drug to be delivered inside the targeted cells instead of the vicinity of the tumor cells compared with other drug carriers. The lipid fractions of LDL allow a substantial quantity of lipophilic drug(s) to be stored inside, and it has been shown that it is possible to incorporate cytotoxic drugs in LDL [21].

To take the advantage of the over expression of LDL receptors on the surface of tumor cells, a carborane mimic of cholesteryl ester for targeted drug delivery was synthesized in our laboratory. This compound, Cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH), is a carborane mimic of native cholesteryl ester and is very hydrophobic [36]. BCH has a cage structure consisting of 10 boron atoms and two carbon atoms. The two carbon atoms allow the formation of cholesteryl ester bond on one carbon atom and further chemical modification on the second one. Liposomes have been used widely in cancer chemotherapy as a non-covalently bound, biocompatible and biodegradable carrier [30]. We formulated the drug in liposomes to aid the drug dispersion in aqueous medium in the cell culture studies [30]. Liposomes can entrap the drug, BCH, effectively and carry it to interact with LDL in the blood circulation system.

It has been shown that liposomes have higher incorporation efficiency in loading lipophilic drugs [16]. As liposomes have high leakage in plasma or in lipoprotein solutions, they can interact with LDL and deliver the drug to LDL particles upon leakage in the medium. It is also shown that liposomes help to reduce the toxicity of certain anticancer drugs [15], thus provides an ideal candidate as the carrier of BCH in our experiments.

There are several factors that limit the use of liposomes to deliver drugs to tumor sites: (1) The rapid clearance by the reticuloendothelial system RES in the spleen and liver; (2) Their marked tendency to stay in mononuclear phagocyte system; (3) Their dose dependency [34]. Therefore, successful delivery by liposomes should meet the following requirements: (1) The liposomes should contain large quantities of drug and the drug should stay in the liposomes for a long period of time; (2) The liposomes should circulate in the blood for a long period of time; (3) They should be small enough to extravasate through the highly permeable vasculature of tumors.

The lipid bilayer of lipsomes interact with plasma protein may result in digestion by RES in the spleen and liver before they reach the targeting site and interact with LDL [30]. To avoid this RES clearance, polyethylene glycol (PEG) is added to the liposomal bilayer by grafting. PEG has been used in many therapeutic applications. It is a weakly anionic hydrophilic coating, which attracts a water shell. The coating of liposomes with PEG confers optimal protection to the vesicle from RES clearance. The PEG coating generates a steric barrier, which prevent hydrophilic interaction between plasma opsonins and the liposomal surface. Liposomes that are formulated to escape from being recognized by the RES, can remain in circulation for prolonged periods and may

minimize the problems associated with conventional liposomes [34]. The term "Stealth" liposomes was coined to describe this evasive property (stealth is a registered Trademark of Liposome Technology Inc, Menlopark, CA (USA) and polyethyleneglycol lipids (PEG-lipids) are commonly referred to as Stealth Lipids [33].

The purpose of this study was to determine whether BCH, the cholesterol-carborane conjugate would be a potential drug for BNCT use through a series of cell culture studies. We need to determine that whether the cellular uptake of boron is higher from BCH than p-carborane. LDL receptors have been identified for targeting to human glioma cells. Liposomes were chosen to solubilize the drug and carry it to interact with LDL, fulfilling the transfer of the drug to LDL and then the cancer cell. In the first group, DMSO was used to dissolve the compounds, p-carborane and BCH, for the determination of their cellular boron uptake, since the liposomes might introduce some factors potentially affecting the cellular uptake of the drug encapsulated. In the second group of study, p-carborane and BCH were formulated into liposomes to compare the cellular uptake. The encapsulation efficiency of p-carborane and BCH in the liposomes are also studied in the experiments. To overcome the drawbacks of conventional liposomes, PEG has been used in many therapeutic applications to make liposomes more hydrophilic and less able to bind opsonins from plasma [37], resulting in a prolonged circulating half-live, decreased clearance by RES, and increased accumulation in the cancer cells [34]. In the third group of study, we evaluated the feasibility of PEG liposome delivery system by comparing its BCH cellular uptake to that of the conventional liposomal formulation using two human glioma cell lines: SF-763 and SF-767. Some other factors affecting the boron cellular uptake, such as the cytotoxicity of the DMSO and the liposomes delivery

systems, and the dependence of the boron cellular uptake on incubation time were investigated as well.

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CHAPTER 2

COMPARATIVE CELLULAR UPTAKE STUDIES OF A CARBORANE CHOLESTERYL ESTER BY HUMAN GLIOMA CELL LINES

2.1 Introduction

There are 35-45% of primary brain tumors that are caused by malignant gliomas. Glioblastoma multiforme tumor cells are characterized by rapid cell proliferation and resistance to conventional treatments [3]. With the requirement that the anti-cancer drug should be located in tumor cells preferentially while maintaining low concentrations in the normal cells and blood, targeted drug delivery to tumor cells may provide a potential approach to enhance the efficiency of chemotherapy. Among many targeting systems, an endogenous cholesterol carrier, low density lipoprotein (LDL), has been found suitable as a potential carrier to deliver cytotoxic agents selectively to tumor cells [15]. This approach is based on the fact that rapid dividing tumor cells utilize a lot more cholesterol to construct their cell membranes as compared to the corresponding normal cells. It is known that up to 90% of cholesterol is obtained by cells via the receptor-mediated endocytosis of LDL [21,23]. Studies have also suggested that in human malignant glioma cells, more than 80% of the cholesterol was endocytosed via LDL receptor [24]. Correspondingly, it has been reported that many types of cancer cells have higher LDL receptor levels [6-11], including seven human glioma cells lines. These findings

suggested that LDL may serve as an ideal vehicle for delivery of therapeutic compounds into the desired tumor cells selectively [3].

LDL is a natural nanoparticle consisting of a lipid core surrounded by a monolayer of phospholipids. There are about 1500 molecules of cholesterol esters in one LDL lipid core, which can potentially be replaced with hydrophobic drugs. Therefore, the lipid property of LDL provides a means of encapsulating a substantial quantity of hydrophobic drug, then delivering and releasing them to targeted cancer cells through receptor-mediated endocytosis pathway.

To utilize this targeted delivery system, an anti-cancer compound, cholesteryl 1,12-dicarba-closo-dodecaborane-1-carboxylate (BCH) was designed and synthesized in our laboratory (see Figure 2.1). It's a cholesterol-carborane conjugate mimicking the native cholesteryl ester and extremely hydrophobic [25], which can be a good candidate for this approach. Carborane is a boron-rich compound, providing the ^{10}B element used for boron neutron capture therapy (BNCT) [15,26,27]. BNCT is a promising treatment for many malignant tumors, the mechanism is to convert the isotope ^{10}B into tumor-destroying and cytotoxic alpha particles and ^{7}Li nuclei under the irradiation of a beam of neutrons [15,16,28]. For BNCT to be successful, the boron must preferentially localize in the tumor cells, compared to the surrounding normal cells [28]. The new developed compound, BCH, has similar chemical and physical characteristics with native cholesteryl ester. They can be stored in the core of LDL and thus delivered to the cancer cells utilizing the elevated LDL receptors for targeted drug delivery [29].

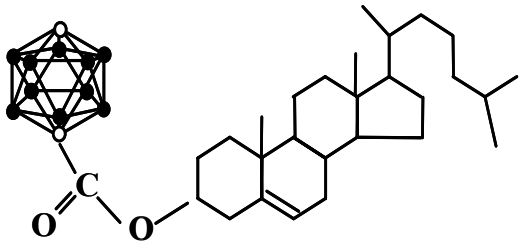

Figure 2.1 The Molecular Structure of Cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH)

Liposomes have been used widely in cancer chemotherapy as a non-covalently bound, biocompatible and biodegradable carrier [19]. We formulated the drug in liposomes to aid the drug dispersion in aqueous medium in the cell culture studies [30]. Liposomes can entrap the drug, BCH, effectively and carry it to interact with LDL in the blood circulation system. It was reported that the phospholipids in the liposomes can interact with LDL since they share similar physicochemical characteristics, resulting in the leakage of the drug, which diffuses into the LDL either into the core or the phospholipids monolayer, thus result in the transfer of the drug into the LDL [15]. However, systemically delivering the drug to targeted tumor cells using liposomes faces a serious obstacle: the liposomes after intravenous injection could be uptake and cleared by the reticuloendothelial (RES) in liver and spleen, which makes the long-term physicochemical stability of the liposomes unpredictable [19]. To avoid the RES uptake and prolong the liposome half-live in blood circulation, the most direct approach is to design a liposomal formulation that would not be readily recognized and removed [30]. It has been reported that polyethylene glycol (PEG) derivatized phospholipids can produce

a large increase in the pharmacological efficacy of encapsulated anti-cancer drug by prolonging the circulating half-live, decreasing the clearance by RES, and increasing the accumulation in the cancer cells [34]. Therefore, we designed and formulated a sterically stabilized liposomal formulation with PEG derivatized phospholipids for the purpose of enhancing the delivery efficiency.

The purpose of this study was to determine whether BCH, the cholesterol-carborane conjugate would be a potential drug for BNCT use through a series of cell culture studies. We need to determine whether BCH, either dissolved in DMSO or formulated in liposomes could result in higher cellular uptake of boron as compared to p-carborane. We need also to evaluate the feasibility of PEG liposome delivery system by comparing its boron cellular uptake from BCH to that of the conventional liposomal formulation using two human glioma cell lines: SF-763 and SF-767. Some other factors, such as the encapsulation efficiency of p-carborane and BCH in the liposomes, the cytotoxicity of the DMSO and the liposome delivery systems, and the dependence of the boron cellular uptake on incubation time were investigated as well.

2.2 Materials and Methods

2.2.1 Materials

BCH was synthesized in our laboratory. Dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cholesterol (CHOL), and the phospholipid, DL-α-dipalmitoyl phosphatidylcholine (DPPC) were purchased from Sigma Chemicals Co. (St. Louis, MO). The amphipathic lipid dipalmitoyl-

phosphatidylethanolamine-PEG 2000 (DPPE-PEG 2000) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Para-carborane was purchased from Aldrich Chem. Co. (Milwaukee, WI). Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), and the antibiotics, penicillin/streptomycin were purchased from BioWhittaker (Walkersville, MD). Dulbecco's phosphate buffered saline (PBS) and trypsin-EDTA solution were purchased from Sigma (St. Louis, MO). The human glioblastoma multiforme cells, SF-763 and SF-767, were obtained from the tissue bank of the Brain Tumor Research center (University of California-San Francisco, San Francisco, CA). The Analytical grade chloroform, methanol and other chemicals were purchased from J. T. Baker (Phillipsburg, NJ).

2.2.2 Preparation of liposomal formulation

The BCH and p-carborane conventional liposomal formulations composed of DPPC and CHOL (3:1) were prepared by solvent evaporation and hydration method. DPPE-PEG 2000 was added in the PEG liposomal formulation (DPPC: PEG = 15:2). Specifically, 57 mg DPPC, 10 mg cholesterol, and 5mg BCH were dissolved in 6ml 2:1 chloroform-methanol mixture in a round-bottom flask. For PEG liposome, the lipid consisted of 50.24 mg of DPPC and 25.1 mg of DPPE-PEG 2000. The solvent was then evaporated under vacuum in a rotary evaporator (RE 200 series, Yamato, Orangeburg, NY) until a thin film was formed on the flask wall. The film was dried by N_2, followed by hydration with 10ml pre-headed PBS. The flask was then shaken mechanically at 55°C overnight. Size reduction of the liposomes was conducted by 10-time repeated extrusion using the

LiposoFast extrusion Emulsiflex device (Avestin, Ottawa, Canada). To remove the unbound lipids or BCH, Econo-Pac 10 DG desalting column (Bio-Rad, Hercules, CA) was used for size exclusion chromatography. The particle size distribution of the liposomes was determined by the Nicomp submicron particle sizer (Model 370, NICOM Particle Sizing System, Santa Barbara, CA). Inductively coupled atomic emission spectrometry (ICP-AES) was employed to determine the boron concentrations in the formulations.

2.2.3 Cell culture studies

Human glioma cell lines (SF-763 and SF-767) were grown in Eagle's MEM supplemented with 10% fetal bovine serum and 1% antibiotic solution. Cultures were passed twice a week to maintain the exponential growth. After several passages from the stock culture, the 150 cm^2 plastic cell culture flasks containing 26 ml growth medium were seeded with approximately 3 x 10^6 cells and then placed in a humidified 5% CO_2 incubator at 37°C for about two days. At the 80% confluent cell growth stage, the medium was replaced and a specified quantity of formulation was added. After a pre-determined incubation period, the medium was removed; the cells were rinsed 3 times with PBS and then harvested with trypsin-EDTA solution. The cells were counted and centrifuged at 3600 rpm for 30 minutes. The supernatant was removed completely and the cell pellets were stored at 4°C until analysis.

2.2.4 Boron uptake study with p-carborane and BCH dissolved in DMSO

Since the liposomes might introduce some factors potentially affecting the cellular uptake of the drug encapsulated, DMSO was used to dissolve the compounds, p-carborane and BCH, for the determination of their cellular uptake. Certain amount of p-carborane and BCH was dissolved in DMSO, and the final boron concentration in both solutions was made to be identical as 100 μg/ml. The solutions were then placed under sonication for 30 minutes to dissolve the solutes completely.

The cells (SF-763) were seeded into the flasks and placed in the incubator at 37°C for two days. At the 80% confluent cell growth stage, the medium was replaced and 390 μl DMSO with p-carborane or BCH was added. The final boron concentration in the medium is 1.5 μg/ml. The cells were incubated with each solution for 12, 16, and 24 hours, respectively, and this was done in duplicates at each time point. After the specified incubation period, the cells were harvested as described above. Inductively coupled plasma-mass spectrometry (ICE-MS) was used for the sample analysis.

2.2.5 Boron uptake study with p-carborane and BCH in conventional liposomal formulation

To compare the cellular uptake of boron with the two formulations, p-carborane and BCH were formulated into the conventional liposomes, respectively. The final concentration of boron for the two formulations was 25.5 μg/ml. The cells (SF-767) were seeded into the flasks and placed in the incubator at 37 °C for two days. At the 80% confluent cell growth stage, the medium was replaced and 1 ml p-carborane or BCH liposomal

formulation was added. The final boron concentration in the medium was 0.98 μg /ml. The cells were incubated with each formulation for 12, 16, and 24 hours, respectively, and this was done in duplicates at each time point. After the specified incubation period, the cells were harvested as described above. Inductively coupled plasma-mass spectrometry (ICE-MS) was used for the sample analysis.

2.2.6 BCH uptake study by two cell lines with conventional and PEG formulations

To compare the cellular uptake of BCH with conventional and PEG formulations, two human glioma cells SF-763 and SF-767 were seeded into the flasks. The flasks were placed in the incubator at 37 °C for two days. At the 80% confluent cell growth stage, the medium was replaced, and 1 ml conventional or PEG liposomal formulation containing BCH (226 μg/ml, equal to 43.9 μg boron/ml) was added. The final boron concentration in the medium is 1.69 μg/ml. The cells were incubated with each formulation for 12, 24, and 36 hours, respectively, and this was done in triplicates at each time point. After the specified incubation period, the cells were harvested as described above. HPLC method specific for BCH was applied for the sample analysis.

2.2.7 Cytotoxicity evaluation of DMSO delivery system, BCH loaded conventional and PEG liposomal formulations

The cellular toxicity of DMSO, p-carborane in DMSO, BCH in DMSO, and BCH-loaded conventional and PEG liposomal formulations used in the above studies were determined

by using the method reported by Mosmann [35]. In 6-well plates, the cells (SF-767 and SF-763) were seeded at 6 x 10^4 in each well containing 1 ml of EMEM, then the plates were placed in a humidified 5% CO_2 incubator at 37°C for two days. The DMSO with drug and the liposomal formulations were added into each well with the same concentration as used in the cellular uptake experiments, specifically, 15 µl for DMSO and 39 µl for liposomal formulations. The viability of cells was measured after certain incubation time; the cell culture without drug added was used as control. Briefly, 100 µl MTT (1mg/ml) was added to each well, and the plates were incubated at 37 °C for 4 hours. The medium was removed and 1 ml 0.04 N HCl in isopropanol was added to all wells and mixed thoroughly until all the dark blue crystals were dissolved. The plates were read on a Water Analysis Specatrometer (Thermo Spectronic, Rochester, NY, USA) at the wavelength of 570 nm.

2.2.8 Analysis of the samples

A high-performance liquid chromatography (HPLC) method had been developed specific for BCH test. The analysis were performed using a Waters model 2690 separation module equipped with a column heater and a Waters Nova-Pak C-18 150×3.9 mm analytical column. The mobile phase used was 50:50 (v:v) methanol-isopropanol, and the flow rate was 0.5 ml/min. The column was kept at 40°C, and the UV detector wavelength was 202 nm. For HPLC analysis, the cell pellets were freeze-dried for 5 hours, and 0.5 ml mobile phase (50:50 methanol-isopropanol) was added to extract the BCH. The mixture was votexed every 30 minutes during a 3-hour period, followed by centrifugation at 3600

rpm for 30 minutes. The supernatant was then collected for analysis. BCH dissolved in the mobile phase (40 μg/ml) was used as the external standard in each test. Since p-carborane can not be analyzed by HPLC method, ICP-AES was employed to measure the boron content of the p-carborane formulations, no treatment was needed before the analysis. The aqueous sample was burned in an argon flame, and the intensity of the light emitted by the present element at specific wavelengths is proportional to the element concentration. The elemental spectrum and their concentrations were analyzed by a computer; an electronic internal standard was used for calibration.

ICP-MS was employed to measure the boron content of the cell pellet samples, as ICP-MS can provide higher sensitivity for elemental analysis. Indium in 2% nitric acid was used as internal standard. The cell pellets were dissolved in 400 μl concentrated nitric acid in a Teflon-lined digestion bomb, and then heated to 140°C for one hour to lysis the cells completely. The solution was diluted 10 times for test.

The content of boron per flask of cells was converted to boron concentration per gram of cells mathematically according to 10^9 cells = 1 g of cells, which was expressed by μg of boron/g cells.

2.3 Results

In this section, our experimental results are presented in (1) the characterization of the liposomal formulations, (2) the boron uptake with p-carborane and BCH dissolved in DMSO, (3) the boron uptake with p-carborane and BCH in conventional liposomal formulations, (4) the boron uptake by two cell lines with BCH-loaded conventional and

PEG liposomes, and (5) the cytotoxicity evaluation of DMSO, BCH-DMSO, p-carborane-DMSO, BCH-loaded conventional and PEG liposomal formulations.

2.3.1 Characterization of the liposomal formulations

The size distribution of the liposomes was examined by photon correlation spectroscopy. In terms of number-weighted Gaussian distribution analysis, the mean diameter is 36.8 nm for the conventional liposomes containing BCH, 48.4 nm for the conventional liposomes containing p-carborane, and 49.4 nm for the PEG liposomes containing BCH.

Based on the HPLC and ICP-AES analyses, the concentration of BCH in both conventional and PEG liposomes were the same, which was 226 μg/ml (equals 43.9 μg boron/ml). The encapsulation efficiency was 44%. The encapsulation efficiency of the p-carborane in conventional liposomes was much lower, which was 4.4%. In order to achieve the same boron concentration in p-carborane and BCH liposomes, a series of p-carborane liposomes were made since there's no previous data regarding its incorporation efficiency. The profile of the encapsulation efficiency of p-carborane is shown in Figure 2.2. It appeared that the boron concentration was not directly proportional to the amount of p-carborane added. The saturation was achieved at 25.51 μg boron/ml, and adding more p-carborane did not result in higher drug loading.

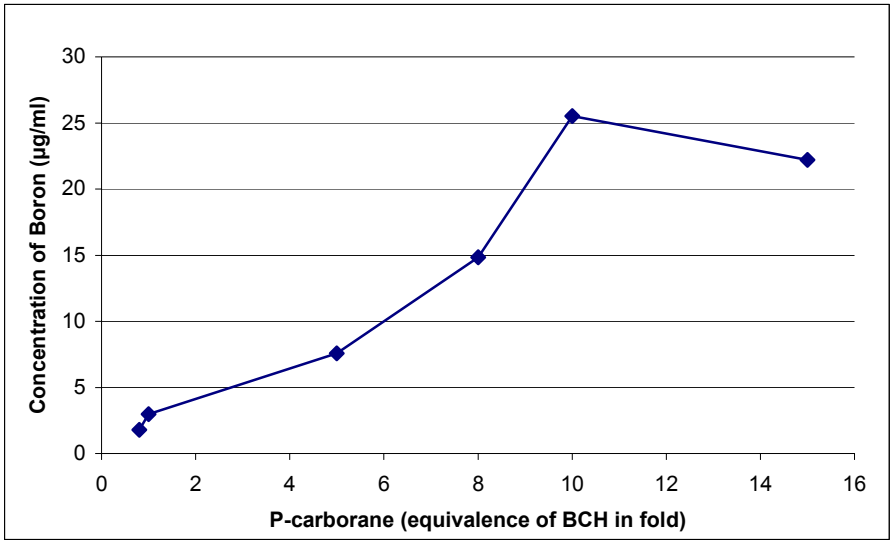

Figure 2.2 P-carborane encapsulation efficiency in conventional liposomes

2.3.2 Boron uptake with p-carborane and BCH dissolved in DMSO

To compare boron uptake with p-carborane and BCH dissolved in DMSO, SF-767 cells in 26 ml EMEM were incubated with 390 µl DMSO containing p-carborane or BCH (100 µg boron/ml) for 12, 16 and 24 hours. The data is shown in Figure 2.3. The uptake of boron with BCH-DMSO was significantly higher, which was 2.5, 3.1, and 8.3 fold as much as the uptake with p-carborane-DMSO after 12, 16, and 24 hours incubation, respectively. After 24 hours exposure to BCH-DMSO, the uptake reached the maximum, which was 247.29 ± 12.36 µg boron/g cells. The highest uptake with p-carborane-DMSO was 58.87 ± 11.27 µg boron/g cells, which was observed after 16 hours of incubation. The lowest uptake was 117.74 ± 14.97 µg boron/g cells with BCH-DMSO and 29.96 ± 7.62 µg boron/g cells with p-carborane-DMSO, respectively.

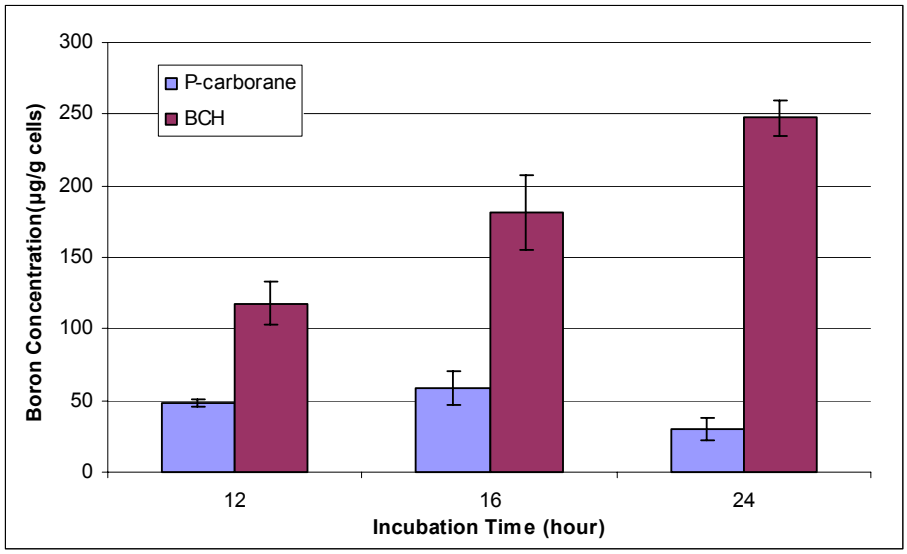

Figure 2.3 Cellular uptake of boron by SF-767 with BCH-DMSO and p-carborane-DMSO after 12, 16, and 24 hours of incubation

2.3.3 Boron uptake with p-carborane and BCH in conventional liposomal formulations

SF-767 cells in 26 ml EMEM were incubated with 1 ml conventional liposomal formulation containing p-carborane or BCH (25.5 µg boron/ml) for 12, 16 and 24 hours. The data is shown in Figure 2.4. There was no significant difference between the cellular uptake with p-carborane liposomes and that with BCH liposomes after 12 and 24 hours of incubation. The uptake with p-carborane liposomes was a little higher than that with BCH liposomes after 16 hours incubtion, and this was the highest during the 24-hour incubation with p-carborane liposomes, which was 173.86 ± 18.64 µg boron/g cells. The highest uptake with BCH liposomes was observed after 12 hours of incubation, which was 164.22 ± 26.24 µg boron/g cells, and further growth of cells resulted in lower uptake.

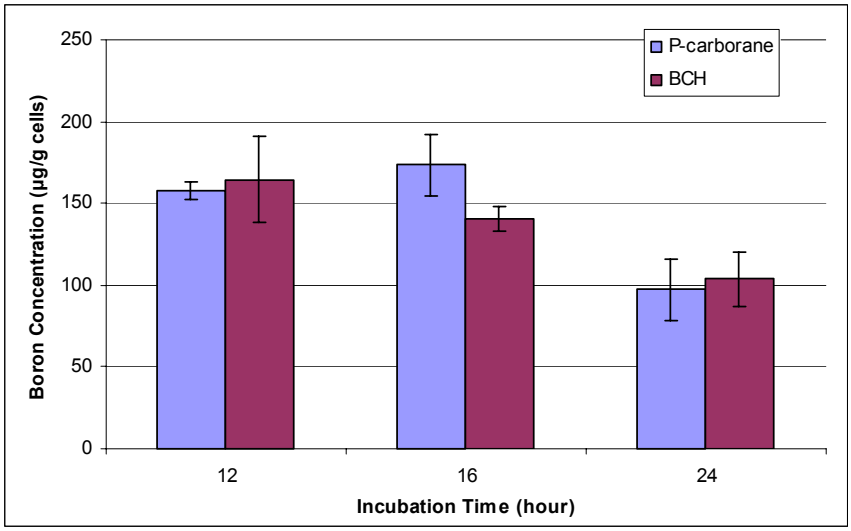

Figure 2.4 Cellular uptake of boron by SF-767 with liposomal formulations containing p-carborane and BCH after 12, 16, and 24 hours of incubation

2.3.4 Boron uptake by two cell lines with BCH-loaded conventional and PEG liposomes

SF-763 and SF-767 cells were incubated with 1 ml conventional or PEG liposomal formulation containing BCH (43.9 µg boron/ml) for 12, 24 and 36 hours. The data is shown in Figure 2.5 and Figure 2.6. There was no significant difference in the BCH uptake between the conventional and PEG liposomal formulations in both cell lines. The highest BCH uptake was observed in both cell lines after 24 hours of incubation with the two formulations, and the highest uptake with conventional liposomes was higher than that with PEG liposomes. Specifically, 255.71 ± 25.14 µg boron/ g cells with conventional liposomes and 228.17 ± 34.33 µg boron/ g cells with PEG liposomes for SF-763; for SF-767, that was 277.22 ± 36.82 µg boron/ g cells with conventional liposomes and 231.24 ± 2.69 µg boron/ g cells with PEG liposomes. The dependence of

the uptake on incubation time was similar for two formulations in both cell lines. For SF-763, the uptake with PEG liposomes was a little lower in comparison to the uptake with conventional liposomes during the 36-hour period of incubation. For SF-767, however, the uptake was rather low after 12 hours incubation with conventional liposomal formulation.

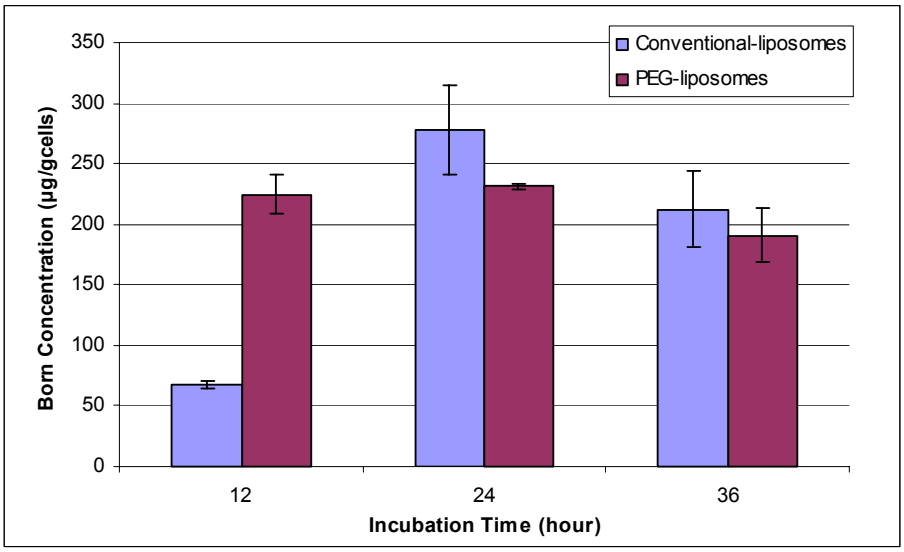

Figure 2.5 Cellular uptake of boron by SF-767 with BCH-loaded conventional and PEG liposomal formulations after 12, 24, and 36 hours of incubation

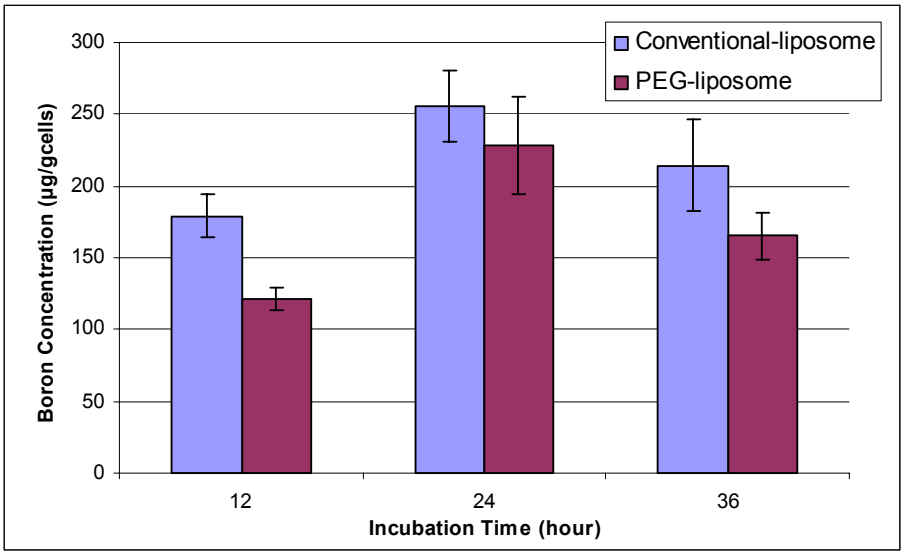

Figure 2.6 Cellular uptake of boron by SF-763 with BCH-loaded conventional and PEG liposomal formulations after 12, 24, and 36 hours of incubation

2.3.5 Cytotoxicity evaluation of DMSO, BCH-DMSO, p-carborane-DMSO, BCH-loaded conventional and PEG liposomel formulations

Cellular toxicity is one of the main concerns in the development of the drug delivery systems. The cytotoxicity of DMSO, p-carborane-DMSO, BCH-DMSO, and two liposomal formulations containing BCH were evaluated based on the relative cell viability, grown with and without the delivery system. The results were shown in Figure 2.7, Figure 2.8, and Figure 2.9. For DMSO delivery system, p-carborane-DMSO and BCH-DMSO both showed a little higher toxicity (lower cell viability) compared with DMSO, but the difference was not significant. For the liposome delivery systems, compared with the PEG liposomes, a higher cytotoxicity was observed from the conventional liposomal formulations in both SF-767 and SF-763.

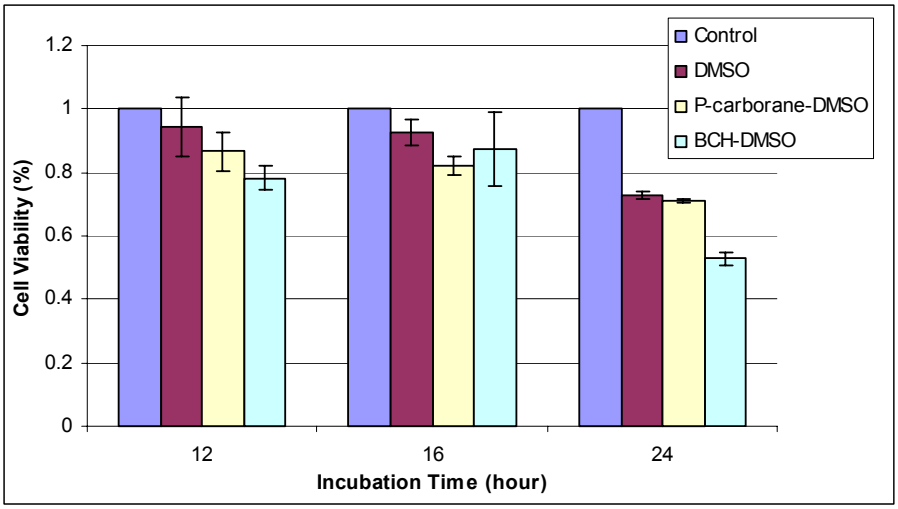

Figure 2.7 Cytotoxocity of DMSO, p-carborane-DMSO, and BCH-DMSO based on relative cell viability (%) in SF-767

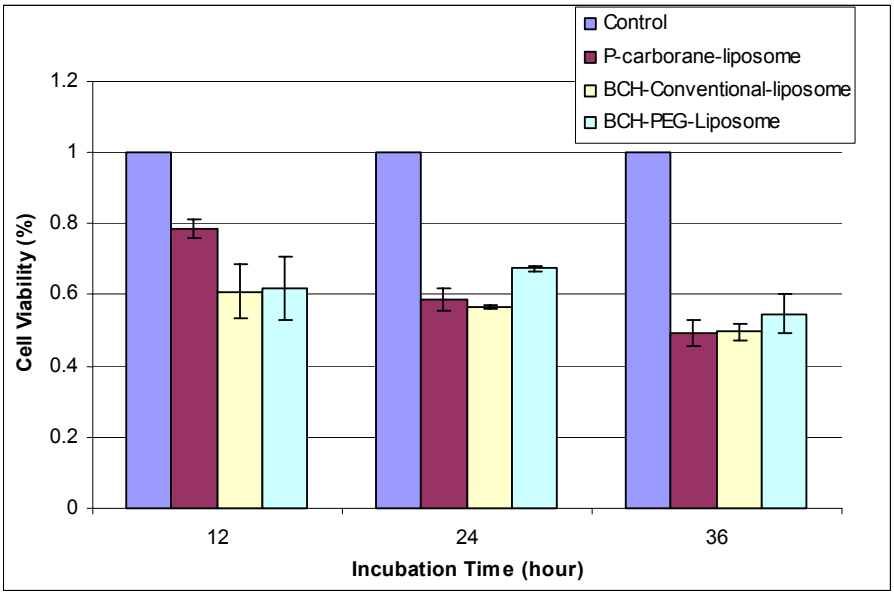

Figure 2.8 Cytotoxocity of the liposomal formulations based on relative cell viability (%) in SF-767

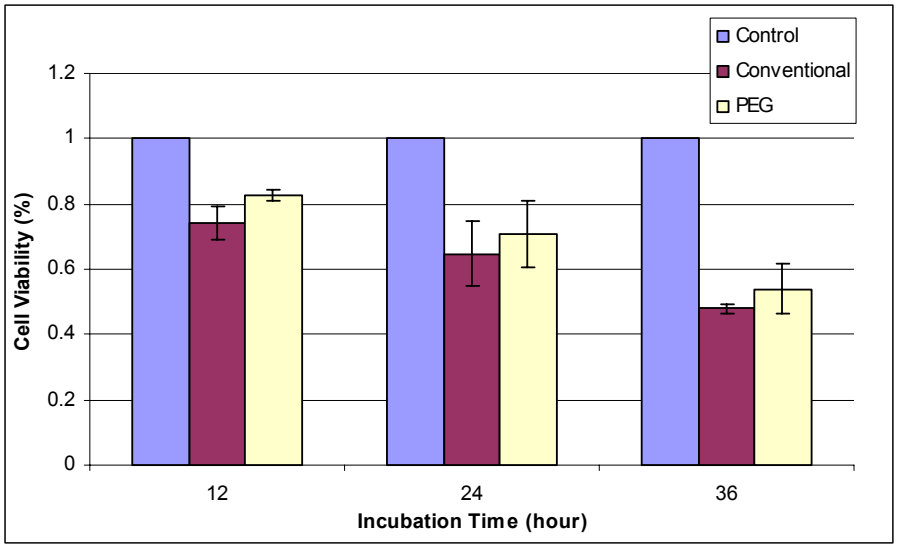

Figure 2.9 Cytotoxocity of the BCH liposomal formulations based on relative cell viability (%) in SF-763

2.4 Discussion

The success of cancer chemotherapy largely depends on the availability of techniques to selectively localize the compound in the cancer cells while maintaining low concentration in the neighboring normal cells and blood [36]. Numbers of targeted drug delivery approaches have been applied to enhance the chemotherapy efficacy and have showed great potential. One of them is based on the fact that aggressively growing tumor cells consume a lot more cholesterol to synthesis their cell membranes as compared to the corresponding normal cells. It has been found that LDL receptor is over-expressed in many types of cancers including human malignant glioma cells [8-12]. In recent years, our laboratory has been trying to utilize the LDL pathway for targeted delivery of boron compound into the glioblastoma multiforme tumor cells, thereby to achieve a higher efficacy in the boron neutron capture therapy (BNCT).

LDL is a natural nanoparticle consisting of a lipid core surrounded by a monolayer of phospholipids. There are about 1500 molecules of cholesterol esters in one LDL lipid core, which can potentially be replaced with hydrophobic drugs. We have designed and synthesized a bornated cholesterol compound BCH (cholesteryl 1, 12-dicarba-closo-dodecaborane-carboxylate) (see figure 1) [36], which resembles the native cholesteryl esters and may substitute them in the LDL. This cholesterol-carborane conjugate is very hydrophobic, and thus can be formulated into liposomes, to interact with LDL in the biological system, and further to be delivered to cancer cells through receptor-mediated endocytosis pathway [15,36].

In this paper, the cellular uptake of boron was evaluated in human glioma cell lines SF-767 and SF-763 based on a series of comparative cell culture studies with BCH and p-carborane. The results indicated that, when using DMSO as the solvent, the uptake of boron using BCH was significantly higher (up to 8.3 fold after 24 hours of incubation) than that using p-carborane. The values for BCH were 117.74 ± 14.97 μg boron/g cells, 181.18 ± 26.35 μg boron/g cells, and 247.29 ± 12.36 μg boron/g cells after 12, 16, 24 hours of incubation, all of which were much higher than the required lever for successful BNCT (25 μg boron/g cells). However, the mechanism regarding how it happened is not clear. Most conceivably, since BCH is the derivative of native cholesterol ester, the similarity in physicochemical properties might allow it to interact with and enter the LDL much more efficiently. Thus it could be delivered to cells with a large quantity. Another possibility is that BCH might be recognized and be taken by the cell membrane more easily than p-carborane because of the similarity with native cholesteryl esters. The boron uptake mechanisms of BCH and p-carborane need further exploration.

Since BCH is extremely hydrophobic, liposomes were chosen to solubilize the compound for the cell culture studies. In the second group of study, p-carborane and BCH were formulated into liposomes to compare the cellular uptake. It was found that the encapsulation efficiency of BCH was much higher than that of p-carborane. The encapsulation efficiency for BCH and p-carborane were 44% and 4.4%, respectively. Therefore, in order to load the same boron content in p-carborane liposomes as that in BCH liposomes, the amount of p-carborane required in the process is as much as 10 fold (in moles) of BCH. This might be caused by the more hydrophobic structure of BCH, which allows it to be entrapped into liposomes more easily. However, for the BCH and p-carborane liposomes containing same content of boron, the uptake was very similar, which was 173.86 ± 18.64 μg boron/g cells at most for p-carborane liposomes and 164.22 ± 26.24 μg boron/g cells for BCH liposomes.

Although liposomes have been used widely as a drug carrier in cancer chemotherapy, systemically delivering drug to targeted tumor cells using conventional liposomes is limited by a big problem: the liposomes after intravenous injection could be recognized and cleared by the reticuloendothelial (RES) in liver and spleen [19]. To avoid this, we formulated a sterically stabilized liposomal formulation with PEG derivatized phospholipids. PEG has been used in many therapeutic applications. It has been found that the polymer can alter the surface of the liposomes, to make it more hydrophilic and less able to bind opsonins from plasma [37], resulting in a prolonged circulating half-live, decreased clearance by RES, and increased accumulation in the cancer cells [34]. Therefore, the drug delivery efficiency could be greatly enhanced.

In the third group of study, we evaluated the feasibility of using PEG liposomes to deliver BCH by the cellular uptake studies in two cell lines: SF-767 and SF-763. The results indicated that under most circumstances, the uptake of boron from conventional liposomal formulations was a little higher that that from PEG liposomal formulations. However, the difference was not significant and both conventional and PEG liposomal formulations could deliver more than enough boron to the tumor cells for the success of BNCT (>>25 μg boron/g cells). For SF-767, the highest uptake from conventional liposomes was 277.22 ± 36.82 μg boron/g cells, while the highest uptake from PEG liposomes was 231.24 ± 2.69 μg boron/g cells. For SF-763, the highest uptake from conventional liposomes was 255.71 ± 25.14 μg boron/g cells, and the highest uptake from PEG liposomes was 228.17 ± 34.33 μg boron/g cells. For both formulations, the highest uptake was observed after 24 hours' incubation in both the two cell lines. This indicated that the BCH uptake was apparently associated with certain growth stage of the cells.

Cellular toxicity is one of the major concerns in the development of drug delivery system. Although liposomes have been used as a feasible and well-founded drug carrier in the cancer therapy for some years, there are still many controversies about it [19]. It has been reported that high dosage of liposomes might cause some cytotoxicity to cells [30]. The dependence of the cytotoxicity on the dosage of DMSO and liposomes is not clear yet. It appeared that all of the delivery systems showed somewhat cytotoxicity. However, the cytotoxicity could be reduced by manipulating the dosage regimen or improving the drug loading efficiency. PEG liposomal formulations were found to have less cytotoxicity in comparison to the conventional liposomal formulations, which might be another advantage of using this delivery system.

In conclusion, BCH in DMSO appears to be taken up by human glioma cell SF-767 much more effectively in comparison to p-carborane (2.5-8.3 fold). The encapsulation efficiency of BCH is much higher than that of p-carborane (10 fold) once they are formulated into liposomes. PEG liposomal formulation can provide satisfactory cellular uptake of boron with reduced cytotoxicity. For BCH, either DMSO delivery system or liposomes delivery system can produce more than enough boron uptake for successful BNCT with acceptable cytotoxicity. Therefore, the chlesterol-carborane conjugate BCH would be a promising drug candidate for BNCT.

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CHAPTER 3

DETAILED EXPERIMENTAL RESULTS

In this chapter, the experimental results discussed in chapter 2 are presented in more detail. Detailed data about characterization of the liposomal formulations, boron uptake with p-carborane and BCH dissolved in DMSO, boron uptake with p-carborane and BCH conventional liposomal formulations, boron uptake by two cell lines with BCH-loaded conventional and PEG liposomes are included in the following sections 3.1 to 3.4. Detailed results from cytotoxicity evaluations of DMSO, BCH-DMSO, p-carborane-DMSO, BCH-loaded conventional and PEG liposomal formulations are included in section 3.5. The reason to not include these data in the previous chapter is to ensure the appropriate organization for a future publication.

3.1 Characterization of the liposomal formulations

The size distribution of the liposomes was examined by photon correlation spectroscopy. In terms of number-weighted Gaussian distribution analysis, the mean diameter is 36.8 nm for the conventional liposomes containing BCH, 48.4 nm for the conventional liposomes containing p-carborane, and 49.4 nm for the PEG liposomes containing BCH. The size distribution of the conventional liposomes containing BCH, the conventional

liposomes containing p-carborane, the PEG liposomes containing BCH were shown in Figure 3.1, 3.2, and 3.3 respectively.

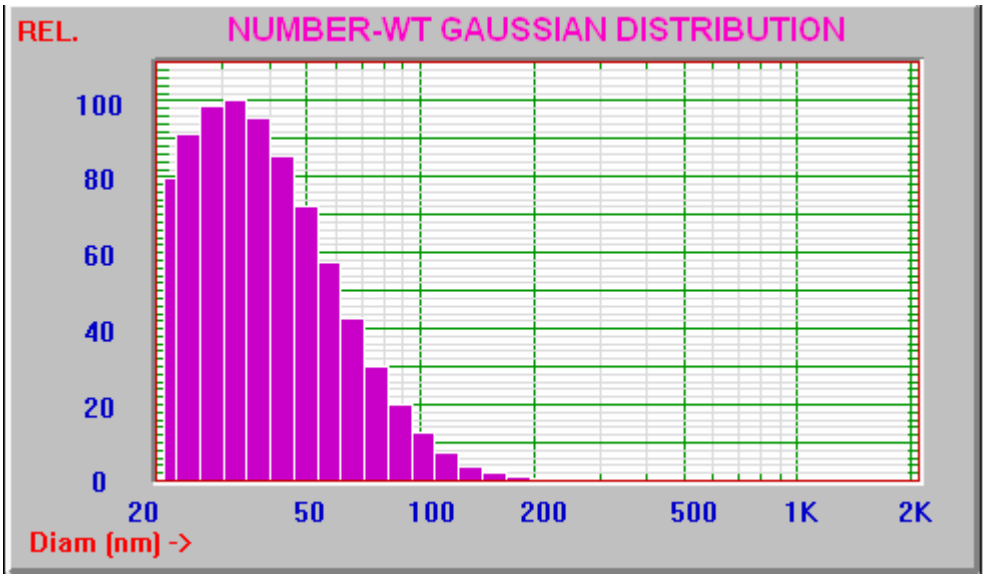


Figure 3.1 Size Distribution of Conventional Liposome containing BCH

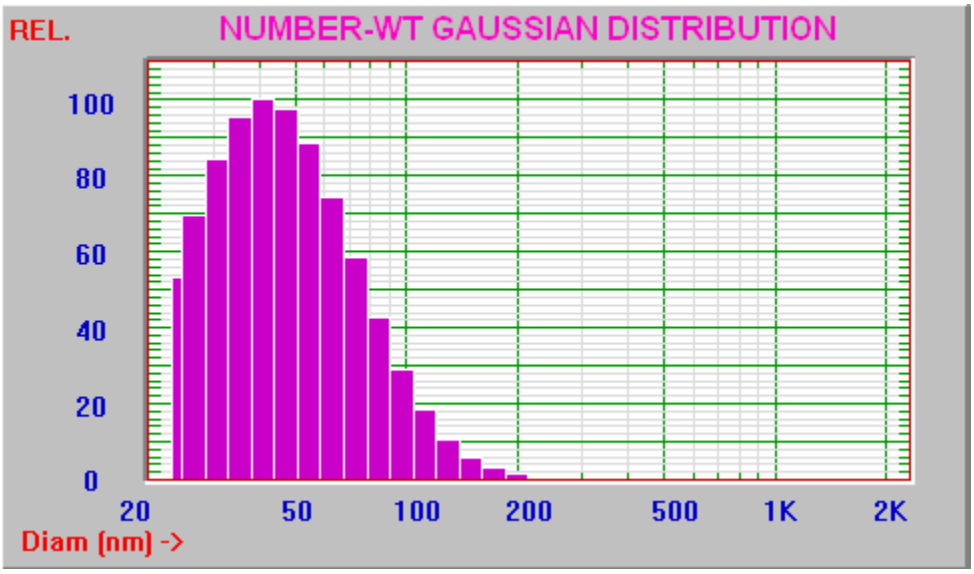
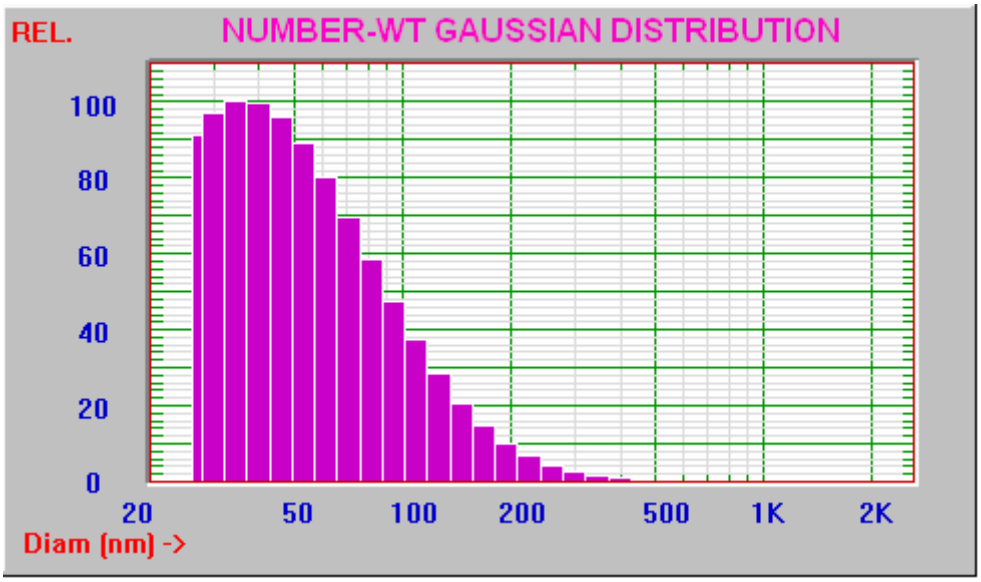


Figure 3.2 Size Distribution of Conventional Liposome containing p-carborane



Based on the HPLC and ICP-AES analysis, the concentration of BCH in both conventional and PEG liposomes are almost the same, which was 226 μg/ml (equals 43.9 μg boron/ml). The encapsulation efficiency was 44%. The encapsulation efficiency of the p-carborane in conventional liposomes was much lower, which was 4.4%. In order to achieve the same boron concentration in p-carborane and BCH liposomes, a series of p-carborane liposomes were made since there's no previous data regarding its incorporation efficiency. The profile of the encapsulation efficiency of p-carborane is shown in Table 3.1. It appeared that the boron concentration was not proportional to the amount of p-carborane added. The saturation was achieved at 25.51 μg boron/ml, and adding more p-carborane did not result in higher loading.

Table 3.1 p-carborane encapsulation efficiency

Fold of Equivalent Molar of BCH	Boron Concentration (μg boron/ml formulation)
0.8	1.80
1.0	2.98
5.0	7.59
8.0	14.84
10.0	25.51
15.0	22.20

3.2 Boron uptake with p-carborane and BCH dissolved in DMSO

SF-767 cells in 26 ml EMEM were incubated with 390 μl DMSO with p-carborane or BCH (100 μg boron/ml) for 12, 16 and 24 hours, and the data is shown in Table 3.2. The uptake of boron with BCH DMSO was significantly higher, which was 2.5, 3.1, and 8.3 fold as much as that with p-carborane DMSO for 12, 16, and 24 hours incubation, respectively. After 24 hours exposure to BCH DMSO, the uptake reached the maximum, which was 247.29 ± 12.36 μg boron/g cells. The highest uptake with p-carborane DMSO was 58.87 ± 11.27 μg boron/g cells, which was observed after 16 hours of incubation. The lowest uptake was 117.74 ± 14.97 μg boron/g cells with BCH DMSO and 29.96 ± 7.62 μg boron/g cells with p-carborane DMSO, respectively.

Table 3.2The effect of incubation time on the uptake of boron by SF-767 with BCH-DMSO and p-carborane-DMSO

Incubation Time (Hour)	Cellular Boron Uptake (μg boron/g cells)	
	p-carborane	BCH
12	47.95 ± 2.63	117.74 ± 14.97
16	58.87 ± 11.27	181.18 ± 26.35
24	29.96 ± 7.62	247.29 ± 12.36

3.3 Boron uptake with p-carborane and BCH conventional liposomal formulation

SF-767 cells in 26 ml EMEM were incubated with 1 ml conventional liposomal formulation containing p-carborane or BCH (25.5 μg boron/ml) for 12, 16 and 24 hours. The data is shown in Table 3.3. There was no significant difference between the cellular uptake with p-carborane liposomes and BCH liposomes after 12 and 24 hours of incubation. The uptake with p-carborane liposomes was a little higher than that with BCH liposomes for 16 hours' incubation, and it was the highest during the whole incubation with p-carborane liposomes, which was 173.86 ± 18.64 μg boron/g cells. The highest uptake with BCH liposomes was observed for 12 hours of incubation, which was 164.22 ± 26.24 μg boron/g cells, the further growth of cells resulted in lower uptake.

Table 3.3 The effect of incubation time on the uptake of boron by SF-767 with conventional liposomal formulations containing p-carborane or BCH

Incubation Time (Hour)	Cellular Boron Uptake (μg boron/g cells)	
	p-carborane	BCH
12	158.08 ± 5.43	164.22 ± 26.24

16	173.86 ± 18.64	140.82 ± 7.71
24	97.20 ± 18.39	103.63 ± 16.44

3.4 Boron uptake by two cell lines with BCH-loaded conventional and PEG liposomes

SF-763 and SF-767 cells were incubated with 1 ml conventional or PEG liposomal formulation containing BCH (43.9 μg boron/ml) for 12, 24 and 36 hours, and the data is shown in Table 3.4, 3.5. It appeared that there was no significant difference between the BCH uptake with conventional and PEG liposomal formulations in both the two cell lines. The highest BCH uptake was observed in both cell lines after 24 hours of incubation with the two formulations, and the highest uptake with conventional liposomes was higher than that with PEG liposomes. Specifically, 255.71 ± 25.14 μg boron/ g cells with conventional liposomes and 228.17 ± 34.33 μg boron/ g cells with PEG liposomes for SF-763; for SF-767, that was 277.22 ± 36.82 μg boron/ g cells with conventional liposomes and 231.24 ± 2.69 μg boron/ g cells with PEG liposomes. The dependence of the uptake on incubation time was similar with two formulations in the two cell lines. For SF-763, the uptake with PEG liposomes was a little lower in comparison to the uptake with conventional liposomes during the whole period of incubation. For SF-767, however, the uptake was rather low at 12 hours incubation with conventional liposomal formulation.

Table 3.4 Cellular uptake of boron by SF-767 with BCH-loaded conventional and PEG liposomal formulations after 12, 24, and 36 hours of incubation

Incubation Time (Hour)	Cellular Boron Uptake (μg boron/g cells)	
	Conventional	PEG
12	67.28 ± 3.43	224.69 ± 16.23
24	277.22 ± 36.82	231.24 ± 2.69
36	212.55 ± 30.88	190.98 ± 22.17

Table 3.5 Cellular uptake of boron by SF-763 with BCH-loaded conventional and PEG liposomal formulations after 12, 24, and 36 hours of incubation

Incubation Time (Hour)	Cellular Boron Uptake (μg boron/g cells)	
	Conventional	PEG
12	179.11 ± 15.31	121.10 ± 7.81
24	255.71 ± 25.14	228.17 ± 34.33
36	214.23 ± 32.16	165.03 ± 16.86

3.5 Cytotoxicity evaluation of DMSO, BCH-DMSO, p-carborane-DMSO, BCH loaded conventional and PEG liposomal formulations

The cytotoxicity of DMSO, p-carborane-DMSO and BCH-DMSO were evaluated based on the relative cell viability grown with and without the delivery system. The results were shown in Table 3.6. For DMSO delivery system, p-carborane-DMSO and BCH-DMSO both showed a little higher toxicity (lower cell viability) in comparison to DMSO, but the difference was not significant.

Table 3.6 Cytotoxocity of DMSO, p-carborane-DMSO, and BCH-DMSO based on relative cell viability (%) in SF-767

Incubation Time (Hour)	Relative Cell Viability (%)		
	DMSO	P-carborane-DMSO	BCH-DMSO
12	0.95 ± 0.09	0.87 ± 0.06	0.78 ± 0.04
16	0.93 ± 0.04	0.82 ± 0.03	0.87 ± 0.12
24	0.73 ± 0.01	0.71 ± 0.01	0.53 ± 0.02

The cytotoxicity of liposomal formulations containing BCH or p-carborane was evaluated based on the relative cell viability grown with and without the delivery system. The results were shown in Table 3.7, 3.8. A higher cytotoxicity was observed from the conventional liposomal formulations in both SF-767 and SF-763. There was no significant difference of cytotoxicity between p-carborane conventional liposomes and BCH conventional liposomes in SF-767.

Table 3.7 Cytotoxocity of the liposomeal formulations based on relative cell viability (%) in SF-767

Incubation Time (Hour)	Relative Cell Viability (%)		
	P-carborane conventiona liposomes	BCH conventional liposomes	BCH PEG liposomes
12	0.79 ± 0.02	0.61 ± 0.08	0.62 ± 0.09
24	0.58 ± 0.03	0.57 ± 0.01	0.67 ± 0.01
36	0.49 ± 0.04	0.50 ± 0.02	0.55 ± 0.05

Table 3.8 Cytotoxocity of the BCH liposomeal formulations based on relative cell viability (%) in SF-763

Incubation Time (Hour)	Relative Cell Viability (%)	
	Conventional	PEG
12	0.74 ± 0.05	0.83 ± 0.02
24	0.65 ± 0.10	0.71 ± 0.10
36	0.48 ± 0.01	0.54 ± 0.07

APPENDIX

CELL CYCLE, GROWTH CONTROL AND DEFENSIVE RESPONSES[1]

[1] Yan Huang, Guangliang Pan, Svein ∅ie and D. Robert Lu, submitted to "Cellular Drug Delivery: Principle and Practice" (D.R. Lu and S. Øie, Eds.) by Humana Press in 2003.

1 Normal and Abnormal Cell Growth

1.1 Cell cycle

The cells of living organisms experience one of the three fates, i.e., live with reproducing, live without reproducing, and die. A cell reproduces by carrying out a series of processes in which it duplicates its contents and divides itself into two daughter cells. The cycle of duplication and division is known as the cell cycle, which involves four major events: cell growth, DNA replication, chromosome segregation into two identical sets, and cell division. The entire process can be divided into two fundamental parts: interphase (including G_1, S, G_2 phases) and mitosis (M phase) [1-2], as illustrated in Figure 1.

Interphase starts with a gap phase 1, or G_1 phase, during which the cell grows and prepares for the initiation of DNA replication. After the cell enters S phase, DNA synthesis starts and the whole chromosomes are replicated. In order to prepare for cell division, the cell enters the second gap period, or G2 phase, and continues to grow and synthesize proteins such as mitosis promoting factors for the next phase. The culmination of cell cycle is the triggering of the mitosis phase during which the chromosome segregation and cell division take place. Interphase can be further subdivided into 5 phases: prophase, prometaphase, metaphase, anaphase, and telophase. During mitosis, chromosomes are condensed and nuclear envelope is broken down. The mitotic spindle is then formed and the chromosomes move to the opposite poles followed by cell division.

Typically, interphase lasts much longer time than the mitosis phase. For example, interphase lasts about 23 hours, while mitosis lasts only about 1 hour for human cell with a total cycle time of 24 hours. Although S phase and M phase are two major phases in a cell cycle, the two gap phases, G1 an G2, monitor the internal and external environment to ensure that conditions are suitable and preparations are complete for S and M phase, respectively. The length of G1 phase is most variable among all the phases and it is greatly dependent upon external conditions and extracellular signals. If extracellular conditions are unfavorable, cells delay progress through G_1 and may even enter a quiescent state known as G_0, in which they can remain metabolically active but no longer proliferate, unless called on to do so by appropriate extracellular signals. If extracellular conditions are favorable and signals for growing and dividing are present, cells in early G_1 or G_0 progress through a commitment point near the end of G_1 known as the restriction point. After passing this point, cells are committed to DNA replication, even if the extracellular signals that stimulate cell growth and division are removed.

1.2 Intracellular and extracellular control of cell-cycle

Normally cell cycle is a rather complicated and precisely coordinated process. In eukaryotes, it is mainly controlled by enzymes known as cyclin-dependent kinases (Cdks) that serve as the control system to coordinate the major transitions in a cell cycle. Coordination of the timing and order of these processes is achieved by regulatory system that represents the checkpoints in the major transitions in the cell cycle. Two key checkpoints are at the G_1/S and G_2/M phase transitions. G_1 and G_2 cyclins and their associated catalytic subunits, Cdks, are responsible for controlling the transition. In most cells, the control system responds to various signals from both inside and outside of the

cell. Intracellularly, the control system monitors progression to ensure that the transitions are properly timed and ordered. For example, it is critically important that the cell does not begin mitosis until replication of the genome has been completed. Extracellularly, the control system analyzes its environmental conditions and regulates the progression according to the extracellular signals. Cdk activities oscillate as the cell progresses through the cell cycle. These oscillations lead directly to cyclical changes in the phosphorylation of key components of the cell-cycle process, resulting in the initiation of different cell-cycle events. For example, an increase in Cdk activity at the beginning of mitosis leads to protein phosphorylation that controls spindle assembly, nuclear envelope structure, and chromosome condensation. The Cdk activities are in turn controlled by a combination of small inhibitory proteins know as cyclin dependent-kinase inhibitors (CKIs). It should be noted that a variety of other mechanisms also contribute to the control of Cdk activity, e.g., some Cdks exemplified by Cdk7 are able to control the activities of other Cdks, which make the control system a complex Cdk regulatory network [3].

Extracellular signals contribute to the cell cycle control by helping the cells determine whether there is need and whether it is ready to divide to ensure that the organism and its organs achieve and maintain an appropriate size. They are classified into three types based on the effects of these regulators on the progression in a cell cycle: cell division, cell growth, and cell survival [1-2]. By removing the restriction point that restricts the cell cycle progression in G1 phase, mitogens can stimulate the cell division rate. On the other hand, growth factor can promote the cell mass increase by stimulating

the biosynthesis while inhibiting the biodegradation of macromolecules. Survival factors can inhibit the process of apoptosis and lead to the increase in cell numbers.

1.3 Abnormal growth of cell

Since cell cycle is tightly regulated by the intracellular and extracellular signals, it normally runs smoothly. However, cell mutation is a routine process in human body. Although most of cell mutations are efficiently corrected by human body, some of them can cause permanent disruption. Mutations, especially mutations of key regulatory genes, may cause much more rapid cell division than that for normal cells and lead to a growing mutant clone. If the mutant cells continue to grow vigorously and totally out of control, cancer can be developed. The cancer cells do not obey the normal cell division restraints and they can invade the territories of neighboring cells. They can even transfer to other organs that are far away from them through body circulation. Cancers can be classified into two major categories based on their original cell or tissue type: carcinomas and sarcomas. The former refers to cancers arising from epithelial cells, and the latter refers to those arising from connective tissue or muscle cells. Many cancers do not fit into either of these two categories, e.g., various leukemias derive from hemopoietic cells, and others derive from the cells of nervous system.

The development of cancer can be viewed as a multistep process involving mutations and selection for cells with progressively increasing capacity of proliferation, survival, invasion, and metastasis. It starts with tumor initiation, which is the result of genetic alteration leading to abnormal proliferation of a single cell. The tumor progression turns an initial mild disorder of cell behavior gradually into a full-blown cancer. Clonal selection, a process similar to natural selection that a new clone of tumor

cells has evolved on the basis of its parent, gaining increased growth rate, survival, invasion and metastasis ability, which makes the tumors become more rapidly growing and increasingly malignant.

A cell must acquire a whole range of aberrant properties as it evolves to turn an abnormal cell into cancer. Different cancers may require different combinations of properties. One of the common characteristics of cancer cells is that they disregard the external and internal signals that normally keep cell proliferation under tight control. A primary distinction between cancer cells and normal cells is that normal cells display density-dependent inhibition of cell proliferation. Normal cells proliferate to reach certain density and then cease proliferating, while cancer cells are insensitive to these signals. Cancer cells are also less stringently regulated, and insensitive to cell contact inhibition, which makes them tend to be invasive and to be able to metastasize. In order to develop cancer, cancer cells tend to have longer life span as compared to their normal cell counterparts. Since cancer cells are usually blocked at an early stage of differentiation, they fail to differentiate normally, which is related to the abnormal proliferation. Another important property of cancer cells is that they are genetically unstable, which makes it possible for further genetic alterations required for neoplasia and malignancy.

1.4 Cell cycle and cancer drug targeting

Components of the cell cycle machinery are frequently altered in cancer cells. Deregulated Cdk activity, combined with aberrant checkpoint control (at the G_1/S and G_2/M boundaries), leads to undesirable cell proliferation. The Cdks offer multiple mechanisms for intervention in the transformed state. In S phase, Cdk2/cyclin A is important for phosphorylation and inactivation of the E2F/DP1 transcription factor.

Inhibition of Cdk2/cyclin A results in elevated E2F concentrations, which leads to S phase arrest and apoptosis.

Among various regulatory proteins in a cell cycle, Cdks are essential for driving the cell through each stage of a cell cycle. Thus it is not surprising that Cdks are important targets for therapeutic intervention in various proliferative diseases including cancer. By modulating Cdks, it is possible to block cell cycle progression, induce apoptosis, promote differentiation, inhibit angiogenesis, and modulate transcription. For example, flavonoid can cause cell cycle arrest at G_1 or G_2 and can inhibit the activation and the activity of several Cdks [4]. Since flavonoid affects Cdk activity directly, it is called direct modulators. Other direct modulators include staurosporines, paullones, indirubins, roscovitine, olomoucine, and purvalanol [3]. On the other hand, indirect modulators such as proteasome inhibitors, can block G_1 or G_2 transition. They have offered a promising new approach to treating cancers. The 26S proteasome regulates the turnover of proteins involved in cell cycle control and apoptosis. It is relevant to human cancer because many intracellular proteins regulated by the ubiquitin-mediated proteasome degradative pathway govern the cell cycle, tumor growth, and survival. Lactacystin is one such inhibitor that was found to arrest umbilical vein cells at the G_1 phase of the cell cycle and to induce the nuclear accumulation of p53 in these cells [5].

The variety of mechanisms controlling the major transitions in cell cycle provide many possibilities for drug targeting along the cell cycle such as drugs arresting at G_0/G_1, drugs arresting at G_1/S, and drugs arresting at G_2/M. For example, the anticarcinogenic potential of grape seed polyphenols were found to involved in the modulation of mitogenic signaling, induction of G_1 arrest, and apoptotic cell death [6]. Okadeic acid

was found to arrest plasmacytoma cells at both G_2/M and S phases and induces vimentin expression in these cells [7], thereby induces apoptosis and cause differentiation of tumor cells. Although okadeic acid has not been tested in the clinic, it merits further development for the treatment of human proliferative diseases like cancers from the modulatory effects in cell cycle.

2 Genetic Process and Control

2.1 DNA-RNA-Protein process overview

The growth and development of all cells and living organisms are dependent on the faithful transmission of genetic information from parent to offspring. Thus accurate replication of DNA is essential for all cells and organisms. DNA replication goes through a process called DNA templating, in which the nucleotide sequence of a DNA strand is copied by complementary base-pairing (A with T, and G with C) into a complementary DNA sequence. The replication is done through a semi-conservative process as the resulting DNA contains a newly created strand and a strand from the parent DNA. DNA repair will follow to correct the mistakes in DNA replication through one of the two major DNA repair pathways: (i) direct reversal of chemical reaction responsible for the mistake, and (ii) removal of the damaged bases followed by their replacement with newly synthesized DNA. The replication and repair mechanisms ensure that the daughter DNAs carry exactly the same genetic information as that in their parent DNA.

When a cell needs a particular protein, the nucleotide sequence of the appropriate portion in DNA molecule is first transcribed into mRNA. Once mRNA is produced, it can be used to synthesize the corresponding protein. Each group of three consecutive nucleotides in RNA is called a codon, and each codon specifies either an amino acid or a

stop codon in the translation process. The translation of the nucleotide sequence of an mRNA molecule into protein takes place in the cytoplasm on a large ribonucleoprotein assembly called a ribosome, a complex catalytic machine made of the ribosomal proteins and the ribosomal RNAs (rRNAs). This process is generally divided into three stages, i.e., initiation, elongation, and termination. To initiate translation, a small ribosomal subunit binds to the mRNA molecule at a start codon (AUG) that is recognized by a unique initiator tRNA molecule. Elongation phase of protein synthesis follows, and each aminoacyl-tRNAs bearing a specific amino acid binds sequentially to the appropriate codon in mRNA by forming complementary base pairs with the tRNA anticodon. Each amino acid is added to the C-terminal end of the growing polypeptide. In the direction of 5'-to-3', the mRNA molecule progresses through the ribosome codon by codon until stop codon is reached. After the release factor binds to ribosome, translation is terminated and complete polypeptide is released. The flow of genetic information from DNA to RNA to protein is illustrated in Figure 2.

2.2 The control of gene expression and drug targeting

Different cell types in a multicellular organism differ dramatically in both their structures and functions, resulting from the different sets of RNA and protein molecules synthesized and accumulated.

The control of gene expression is exerted at multiple levels by changes of DNA content or position and changes in gene activity. The four main levels of gene activity control are transcriptional control, post-transcriptional control, translational control, and post-translational control. Gene expression is a very complicated process, and many regulatory factors including proteins and nucleic acids are involved in this process. For

example, it was recently discovered that many small RNAs play an important role in gene expression control by affecting mRNA degradation [8]. In theory, all regulatory factors in this process can serve as the therapeutic targets for targeted drug development. These regulated steps and the corresponding drug targeting strategies are discussed in the following sections.

2.2.1 Transcriptional Control

In eukaryotic cells, transcription is a complex process, in which many proteins work together to transcribe DNAs into mRNAs. It is the most regulated step in the genetic path from DNA to proteins. The DNA sequence to which RNA polymerase binds to initiate transcription of gene is called promoter. Multiple proteins are required by the RNA polymerase to act as initiation factors. Some of these proteins bind to promoter sequences and direct the polymerase to the transcription start site. Transcription is initiated by eukaryotic RNA polymerase II, and it can be further stimulated by activators and inhibited by repressors. Transcription can also be regulated by some extracellular signals such as hormones.

In recent years, researchers have found that gene expression can be altered by the change of the chromatin shape, although the reason of such change is still mysterious. For example, in many chromosomes, loops or puffs of exposed DNA are sites of heavy transcription. Genes can be amplified when there is a heavy demand for a protein coded by a gene or set of genes, as the cell can make multiple copies of the genes so that more mRNAs can be simultaneously made.

The complicated control network of transcription provides numerous possibilities for drug targeting. Triple helix-forming oligonucleotides, for example, can be used to

inhibit transcription initiation. The inhibition of binding of transcription activating factors by triplex formation can modulate the level of transcription of the target gene. This provides rationale for the development of new tools for cellular biology and of new therapeutic approaches to control gene expression at the transcriptional level [9].

2.2.2 Post-transcriptional control

In eukaryotes, transcription of protein-coding genes yields pre-mRNAs, which are spliced and edited into functional mature mRNAs that are used to guide protein synthesis. In eukaryotic cells, nascent pre-mRNAs are associated with a complex set of hnRNP proteins before transcription is completed. One function of these RNA-binding proteins is to help the formation of the structures recognized by RNA-processing factors.

In multicellular organisms, most pre-mRNAs are spliced to remove non-coding introns. Small nuclear ribonucleoprotein particles (snRNPs, U_1, U_2, U_4, U_5, and U_6) play a key role in this process. The snRNPs associate with splice sites to form a spliceosome (composed of many proteins and snRNPs), in which the splicing reactions occur. In this process, U_1 of snRNP is first bound to yield 5' splice site of pre-mRNA. The recognition of 5' splice sites is based on base pairing between the 5' splice site consensus sequence and a complementary sequence at the 5' end of U_1 snRNP. After U_2 snRNP binds to the branch point by a similar mechanism, a complex consisting of U_4/U_6 and U_5 snRNPs is then incorporated into the spliceosome.

Since most pre-mRNAs contain multiple introns, different mRNAs can be produced from the same gene by different combinations of 5' and 3' splice sites. The possibility of joining exons in varied combinations provides exponential ways of getting mature RNA from the same pre-mRNA. This process, called alternative splicing, increases the

diversity of proteins that can be expressed from a single transcription unit. Alternative splicing plays an important role in gene expression process, and it is estimated that at least 35% of all human genes are alternatively spliced [10].

Although the exact mechanism about splicing is not known, it is shown that several proteins contribute to the selection of splice site and can affect the use of alternative splice site in a pre-mRNA molecule. Sequence-specific RNA-binding proteins, for example, have been shown to bind near specific splice sites to either inhibit or activate splicing to the nearby site. As transcriptional activators consist of DNA-binding domain and activation domain, which are generally encoded in separate exons, alternative splicing enables the production of many activators and repressors from the same gene. It has been shown that alternative splicing can be modified by using antisense oligonucleotides, and this provides a very promising potential chemotherapeutic target for cancer and other proliferative diseases [10].

2.2.3 Control of mRNA degradation

In mammalian cells, the levels of both rRNA and tRNA are relatively stable, while the stability of different mRNAs may vary greatly. These unstable mRNAs often code for regulatory proteins whose levels need to be changed rapidly to respond to various signals. For example, the hormone lymphokine produced by lymphocytes, which coordinate cell-cell interactions between the cells involved in the immune response of mammals, are synthesized and secreted in short bursts. Correspondingly, the mRNAs encoding these proteins must be synthesized and degraded in a very short period of time, and the switches in this controlling machinery must be turned on and off promptly.

The degradation of most eukaryotic mRNAs is completed by gradually shortening of their poly-A tails. It is recently demonstrated that some small RNAs contribute to mRNA degradation in RNA interference (RNAi). The exact mechanism about this process is still unknown, but scientists believe that enzyme complex called RISC uses the sequence information in small RNAs (miRNAs and siRNAs) that are combined to identify and degrade mRNAs with complementary sequence [8].

The degradation of mRNAs can also be affected by extracellular signals. For example, mRNA encoding for transferrin receptors, is regulated by iron level within the cell. In the presence of adequate amounts of iron, transferrin receptor mRNA is rapidly degraded as a result of specific nuclease cleavage at a sequence near its 3' end. Otherwise, the mRNA is stabilized, resulting in increased synthesis of transferrin receptor and more iron uptake by the cell. This regulation is mediated by a protein that binds to specific sequences (called the iron response element, or IRE) near the 3' end of transferrin receptor mRNA, inhibiting the mRNA from cleavage.

2.2.4 Translational control

Translation completes the flow of genetic information within the cell from DNA to protein. It involves in a complicated procedure where the information encoded in RNAs is translated into proteins. The translation process is generally divided into three stages: initiation, elongation, and termination. Ribosomes, which are composed of proteins and rRNAs, are the sites of protein synthesis for mammalian cells. During translation, the rRNA plays the dominant role in determining the overall structure of the ribosome, forming the binding sites for the tRNAs, matching the tRNAs to codons in the mRNA,

and providing the peptidyl transferase enzyme to link amino acids together during translation.

Translational control can be achieved by binding repressor proteins, which block translation to specific mRNA sequences. It is shown that malignant transformation in cancer could be caused by the increased translation of a subset of mRNAs encoding important proteins for cell growth and proliferation, which usually possess regulatory sequences that render their translation more sensitive to changes in the activity of translation initiation factors. By repressing the translation of the mRNA which is required for cancer development, cancer cell growth and proliferation could be effectively inhibited. It has been reported that translation control in cancer cell could be an excellent target for anticancer drugs development [11].

2.2.5 Post-translational control

One important function of proteins is to serve as enzymes, and the regulation of enzyme activity plays an important role in governing cellular behavior. The catalytic activity of enzymes can be altered by changing their conformations. This can be achieved by binding small molecules, such as amino acids or nucleotides, to regulate enzyme activity. Protein activity can also be regulated by the interactions between the polypeptide chains that form proteins, and many activators and repressors function through protein-protein interactions. Proteins are targets of most traditional drugs, which use small molecules to inhibit functions of particular proteins [12].

Just as other molecules in the cells, the levels of proteins are determined by the difference between the rates of synthesis and the rates of degradation. Different rates of protein degradation affect the behavior of the cells, serving as an important factor for cell

regulation. For example, regulatory molecules, such as transcription factors usually have shorter half-lives to allow their levels to change quickly in response to various signals from both inside and outside of cells. The ubiquitin/proteasome-dependent protein degradation pathway plays an essential role in both cell proliferation and cell death in human cancer cells [13]. The knowledge that proteasome function is required for tumor cell survival has prompted the design, synthesis and evaluation of various pharmacological proteasome inhibitors. Both in vitro and in vivo, experimental results have demonstrated the potential use of proteasome inhibitors as novel anticancer drugs.

2.2.6 Expression of receptor protein and drug targeting

It has been known that many cancer cells over-express certain cell surface receptors including transferrin receptor [14], folate receptor [15], and LDL receptor [16-17], to meet the increased cell proliferation and growth requirement. One strategy of developing targeted drug delivery for cancer therapy is to take advantage of these over-expressed cell surface receptors. By incorporating the corresponding ligands for the cancer cell surface receptors onto drug or drug carriers, anticancer drug can be specifically delivered to the cancer site.

Tumor growth and development is tightly related to the tumor neovascularization, the growth of new blood vessels. The endothelial cells play a very important role in this process, and therefore, they are attractive targets for cancer therapy. More importantly, angiogenic endothelial cells over-express certain proteins on the cell surface, which can be recognized only by certain peptide sequence [18-19]. Thus, targeting angiogenic endothelial cells becomes a very promising strategy in the treatment of cancers.

3 Cellular Defensive Response Systems

3.1 Human immune system

Our body, like other living organisms, is in an environment encountered by many foreign invaders. Most of the foreign invaders that confront the human immune system are microscopic pathogens including fungi, parasites, bacteria, and viruses. All foreign microbes in the human body display special markers, and it is these special markers that can be recognized as harmful and identified for destruction by the immune system. In order to survive in an environment with various types of pathogens, the body must have well-developed mechanisms to resist the infection by pathogens. These defenses can be categorized into two types: innate immune responses and adaptive immune responses [20].

Innate immunity refers to antigen-nonspecific defense mechanisms and is the immunity that one is born with. It serves as the first line of defense for the body which is switched on immediately after an infection begins, and do not depend on the host's prior exposure to the pathogen. Adaptive immunity, however, operates later in an infection, is highly specific to the pathogen that has induced them, and is much more powerful. However, the adaptive immune responses are slow to develop on first exposure to a new pathogen. It may take a week or so before the responses are effective. The key difference between the two immune responses is that innate immune responses are not specific to a particular pathogen while the adaptive immune responses are.

The function of immune system is to remove or destroy invading pathogens and any toxic molecules they may produce. It is a crucial task for a healthy immune system to tell what is foreign and what is own. This applies to both innate and adaptive immune

systems, although it is more important to adaptive immune system as it is more powerful and more destructive. Occasionally, the system fails to make this distinction and reacts destructively against the host's own molecules. Such autoimmune diseases can be fatal. On the other hand, tolerance can be problematic in some other cases like cancer. To the immune system, the difference between a cancer and a normal cell is so small that the immune system largely tolerates cancer cells rather than attacking them. In order to use the immune system to attack cancer cells, stimulations must be made to the immune system strongly enough to overcome this tolerance. Although the relation between the cancer and the immune system is still not clear, it is believed by most scientists that the cancer cells either have generated tolerance in the immune system or have developed ways of resisting immune recognition. In terms of cancer treatment, we need to identify ways to break the tolerance or circumvent resistance mechanisms [21]. Cancer vaccine is such a solution and will be discussed in later section.

3.2 Innate immunity system

Innate immunity consists primarily of a chemical response system including complement, endocytosis, and phagocytosis. Macrophages, for example, detect and engulf extracellular molecules and materials, clearing the system of both debris and pathogens. The innate immune responses in vertebrates are also required to activate adaptive immune responses.

Innate immune responses rely on the body's ability to recognize conserved features of pathogens that are not considered as self, as the pathogen surface has various classes of common pathogen-associated immunostimulants like many types of molecules on microbial surfaces and the double-stranded RNA of some viruses [1-2]. These molecules

can be recognized by some dedicated receptors in the host, which are collectively called pattern recognition receptors. The cell-surface receptors have two functions, one of which is to initiate the phagocytosis of the pathogen, and the other is to stimulate innate immune responses.

Phagocytic cells display a variety of cell-surface receptors that enable them to recognize and engulf pathogens. These include pattern recognition receptors such as TLRs (Toll-like receptors). There are two kinds of phagocytic cells, i.e., macrophages and neutrophils. Macrophages are usually long-lived cells residing in tissues throughout the body. They patrol the tissues of the body and are among the first cells to encounter invading microbes. Neutrophils, on the other hand, are short-lived cells, which are abundant in blood but are not present in healthy tissues. They are rapidly recruited and dispatched to sites of infection by both activated macrophages and molecules released by the microbes themselves. Phagocytic cells trigger inflammatory responses to help fight infection and begin to activate adaptive immune system. Adaptive immune system plays a major role after this point.

In recent years, macrophage-mediated gene delivery was studied for cancer treatment by arming macrophages with the ability to express a therapeutic gene. In experiment, a hypoxia-regulated adenoviral vector was used to transduce human macrophages with either a reporter or a therapeutic gene encoding human cytochrome P4502B6. Infiltration of transduced macrophages into a tumor spheroid results in the induction of gene expression. They have significant tumor cell killing ability in the presence of cyclophosphamide via activation by P4502B6. It is also shown that this can be further targeted to tumors through hypoxia regulated gene expression [22].

It has been found that macrophages play very important roles in HIV dissemination to the whole immune systems. When they are infected by human immunodeficiency virus (HIV), they keep live allowing HIV to live and replicate for a long time. Since macrophages expresses scavenger receptor proteins such as modified LDL receptor in the cell surface [23], they have been considered as very important therapeutic target for HIV infection. During the development of disease atherosclerosis, lipid-loaded macrophages appear in the blood vessel wall. Because of the scavenger receptors in these cells, they can serve as ideal therapeutic targets for this disease.

3.3 The Adaptive immune system

Adaptive immune system is a more advanced and powerful system. The adaptive immune system is called upon by the innate immune system to respond to pathogens. Unlike innate immune responses, the adaptive responses are highly specific to the particular pathogen that induced them, and the protection is also long-lasting. There are two broad classes of such responses: antibody responses and cell-mediated immune responses, which are carried out by different classes of lymphocytes, called B cells and T cells, respectively.

In the process of immune response, the pattern recognition receptors present on the surface of various types of host cells activate intracellular signaling pathways in response to the binding of pathogen-associated immunostimulants. This leads to the production of extracellular signal molecules that promote inflammation and help activate adaptive immune responses if needed.

3.3.1 B cells and antibody-mediated drug targeting

B cells respond to antigens by secreting antibodies. In antibody responses, the antibodies circulate in the bloodstream and permeate to other body fluids where they bind specifically to the foreign antigen that stimulated their production. Binding of antibody inactivates viruses and microbial toxins by blocking their ability to bind to the receptors on host cells, thus blocks viruses from entering cells. Antibody binding also marks invading pathogens for destruction by making it easier for phagocytic cells of the innate immune system to ingest them.

There are two phases in the development of B cells, i.e., antigen independent phase and antigen dependent phase. The first phase involves the generation of diversity and the acquisition of rearranged H- and L-chain genes for synthesizing IgM antibody as a surface receptor protein. In antigen dependent phase, a B cell uses one of its receptors to bind to its matching antigen that the B cell engulfs and processes. The B cell then displays a piece of the antigen, bound to a class II major histocompatibility complex (MHC) protein on the cell surface. The whole complex binds to an activated helper T cell. This binding process stimulates the transformation of the B cell into an antibody secreting plasma cell (Figure 3).

Each B cell clone makes antibody molecules with a unique antigen-binding site. Initially, during the development of B cell in the bone marrow, the antibody molecules are inserted into the plasma membrane, where they serve as receptors for antigen. In peripheral lymphoid organs, antigen binding to these receptors, together with costimulatory signals provided by helper T cells, activates the B cells to proliferate and differentiate into either memory cells or antibody-secreting effector cells. The effector

cells secrete antibodies with the same unique antigen-binding site as the membrane-bound antibodies. There are five classes of antibodies in mammals, namely IgM, IgD, IgG, IgA, IgE, each of which mediates a characteristic biological response following antigen binding.

Since antibodies are the product of immune response of B cells to immunogens, Antibodies can be used either as therapeutics or ligands for drug targeting. As proteins like Her2 are abundant on surface of cancer cells, antibodies against such proteins can be armed with a toxin, or made to carry an enzyme that cleaves a harmless 'prodrug' into a toxic molecule. The second case is especially useful as one molecule of enzyme can then generate a large number of toxic molecules. One virtue of this strategy is that the toxic drug generated enzymatically can then diffuse to neighboring tumor cells, increasing the odds that they can be killed, even if the antibody did not bind to them directly [1-2].

3.3.2 T-cells in adaptive immune system

The diverse responses of T cells are collectively called cell-mediated immune reactions. Most adaptive immune responses, including antibody responses, require helper T cells for their initiation. Most importantly, unlike B cells, T cells can help eliminate pathogens that reside inside host cells. Activated T cells react directly against a foreign antigen that is presented to them on the surface of a host cell. For example, killer T cells possibly kill a virus-infected host cell, thereby eliminating the infected cell before the virus has a chance to replicate. T cells are also responsible for orchestrating, regulating and coordinating the overall immune response. For example, helper T cells alert B cells to start making antibodies (Figure 3).

Like antibody responses, T cell responses are exquisitely antigen-specific. But T cell responses differ from B cell responses in at least two crucial ways. First, they differ in their mechanism of antigen recognition. T cells depend on antigen-presenting cells in peripheral lymphoid organs that present antigen to them. Protein antigens are partly degraded inside the antigen-presenting cell, and are then carried to the surface of the presenting cell on special molecules called MHC proteins, which bind the fragments and present them to T cells. The second difference is the mechanism that T cells degrade the antigen. Once activated, effector T cells act only at short range, either within a secondary lymphoid organ or at the site of infection. They interact directly with other cells in the body, during which they either kill or signal in some way. Activated B cells, by contrast, secrete antibodies that can act far away.

Based on their functions in the immune system, T cells can be categorized into two classes, i.e., cytotoxic T cells and helper T cells. The former kill infected cells directly by inducing them to undergo apoptosis, while the latter help activate B cells to make antibody responses and macrophages to destroy microorganisms. The helper T cells also stimulate the activation of cytotoxic T cells. They are dependent on the unique cell surface molecules, MHC, to help them recognize antigen fragments. They have receptors on their cell surface like antibodies, which recognize fragments of foreign proteins that are displayed on the surface of host cells in association with MHC proteins. Both are activated in secondary lymphoid organs by antigen-presenting cells, which express peptide-MHC complexes, costimulatory proteins, and various cell-cell adhesion molecules on their cell surface. Two kinds of MHC proteins, MHC-I and MHC-II, play crucial roles in presenting the antigens molecules to the cytotoxic T cells and helper T

cells respectively. MHC-I are expressed in almost all vertebrate cells while MHC-II are normally expressed in those cells interacting with helper T cells, such as dendritic cells, macrophages, and B lymphocytes [12].

MHC proteins have a single peptide-binding groove, which can bind a large and characteristic set of small peptide fragments derived from proteins. After they have formed inside the target cell, the peptide-MHC complexes are transported to the cell surface. Complexes containing a peptide derived from a foreign protein are recognized by T cell receptors, which interact with both the peptide and the walls of the peptide-binding groove. The MHC molecules appear to be the keys to understanding and manipulating T cells, including both T-4 and T-8 cells. Based on the knowledge concerning the presentation of peptides by MHC molecules, a reliable method for predicting peptides that can bind to MHC, thus induces T cell immunity, can be of great value to modulating immune responses.

T cells play very important roles in human immune systems. However, they are subject to be attacked by foreign invaders, e.g., T-4 cells are the primary target of the HIV-1 virus. Since T-4 cells are responsible for the effective immune activity of macrophages, B lymphocytes, and other T-lymphocytes, when they are depleted because of the HIV infection, the patient develops AIDS. The intensive study of the HIV replication has provided insight into the fundamental cellular mechanisms. One very important example is the discovery of protein TAT, an 86 amino acid transcriptional activator protein. TAT can be synthesized in one HIV infected cell and transited into a neighboring cell without the requirement for the receptor-mediated event. This

phenomenon has provided a great advantage to use TAT or fragment of TAT for efficient gene and drug delivery [24-25].

3.3.3 Diversity and specificity of adaptive immune system

The most remarkable feature of adaptive immune system is that it can respond to millions of different foreign antigens in a highly specific manner. There are two important issues related to adaptive immune system: (i) the generation of diversity, (ii) the specificity of the antibody and MHC molecules.

The immune system has the capacity to recognize and respond to a large amount of antigens. This extreme diversity can be generated in at least three possible ways: (i) multiple genes in the germ line DNA; (ii) variable recombination during the differentiation of germ line cells into B-cells; (iii) mutation during the differentiation of germ line cells into B-cells. The diversity of MHC molecules is generated in a similar process.

Once the diversified antigen binding receptors are generated, how could the immune system pick the appropriate ones to respond to a specific antigen? This can be explained by the clonal selection theory. The adaptive immune system is composed of millions of lymphocyte clones, with the cells in each clone sharing a unique cell-surface receptor that enables them to bind a particular antigen. With the binding of antigen on the cell surface receptors, the lymphocytes proliferate and differentiate into functional lymphocytes. Lymphocytes have three possible fates: (i) some lymphocytes proliferate and differentiate into memory cells, which are able to respond faster and more efficiently the next time the same pathogen invades to prevent the body from the same illness in the future; (ii) lymphocytes that would react against self molecules are either induced to alter

their receptors, induced to kill themselves, inactivated, or suppressed, ensuring self-tolerance; (iii) not to react at all and dies. In this process, both B and T cells circulate continuously between the blood and lymph until they encounter their specific foreign antigen in a peripheral lymphoid organ. After that, they proliferate and differentiate into effector cells or memory cells [26].

3.4 Artificially Acquired Immunity

Based on the key properties of the immune system, we can defend our body using artificially acquired immunity. We can artificially get the immunity either passively or actively. Passive immunity is usually achieved by injecting antibodies into the body that need them. For example, administering tetanus antitoxin to a patient is a way of conferring passive immunity [27]. Passive immunization is effective very quickly, but it lasts only a short time. It is mainly used to protect people when they are particularly vulnerable, such as immediately after exposure to a serious disease.

The purpose of vaccines is to stimulate the immune response like antibody formation or T cell responses without subjecting a person to the risk of actual infection [27]. Most traditional vaccines rely on the vaccine's capacity to evoke antibody formation, but more attention is paid to vaccines evoking T cells responses, especially for HIV vaccines and cancer vaccines. Recent developments in vaccine research have provided many ways to obtain active immunity including conjugate vaccines, subunit vaccines, recombinant vector vaccines, naked DNA techniques, and vaccine presentation [27]. Conjugate vaccines are used to deal with certain bacteria having special outer coats which disguise antigens so that the immature immune systems are unable to recognize these harmful bacteria. Proteins or toxins from a second type of organism that is easier for an immature

immune system to recognize are linked to the outer coats of the disease-causing bacteria. This enables an immature immune system to respond and defend against the disease agent. Subunit vaccines can be made by using a fragment of the microbe, because fragment of the microbe is able to trigger the immune system while has much fewer side effects. Subunit vaccines made from *Streptococcus pneumoniae* have been used to protect against pneumonia. A recombinant subunit vaccine for hepatitis B virus infection made by inserting a tiny portion of the hepatitis B virus' genetic material into common baker's yeast is also in clinical trial.

A vaccine vector is a weakened virus or bacterium into which harmless genetic material from another disease-causing organism can be inserted. The vaccinia virus, for example, can be used to make recombinant vector vaccines as it is relatively large and has ample room to accept additional genetic fragments. A vaccinia virus with several genes from the HIV is currently being tested as a vaccine against AIDS. In addition, naked DNA technique has also been used to obtain active immunity. This is achieved by incorporating "naked DNA" encoding certain proteins from a disease-causing organism into the body's own cells. The proteins encoded by the DNA work as antigens to stimulate the immune system. In this way, the DNA will have an effect similar to that of a live, attenuated vaccine and produces antigens for years. The exclusion of genes critical to the disease-causing organism's survival also assures that the vaccines are safe and do not actually cause disease.

Vaccine presentation deals with presenting the vaccine to the immune system, which is closely related to drug delivery. Microspheres, tiny spheres containing bits of antigenic material, show promise in that they can release small doses of vaccine over extended

periods of time as they gradually dissolve in the body. This makes two or more doses of vaccine in one administration possible.

3.4.1 HIV vaccine

The focus of HIV vaccine research has progressed from HIV surface antigens and the role of antibodies in early stages to the importance of cytotoxic T cells. Subunit vaccines for HIV based on viral surface proteins, such as gp120, have the advantage of being safe and simple to prepare. It remains to be confirmed whether these vaccines can elicit antibodies capable of neutralizing primary HIV isolates. A vaccine candidate based on gp120 from two different HIV clades recently entered Phase III testing in the United States. Vectored vaccines employ non-HIV viruses (e.g. avian pox viruses) engineered to carry genes encoding one or more HIV epitopes. A related vector comparison study is ongoing and is evaluating three potential products to determine which vector produces the most robust immune response. By combining a canarypox-vectored product with a subunit vaccine, a hybrid vaccine has been studied to determine if a robust cellular and humoral response to HIV can be elicited. In addition, attenuated live or whole-killed HIV vaccines have shown promise in non-human primates.

3.4.2 Cancer vaccines

Cancer vaccines work primarily to prevent cancer from recurring by alerting the body for certain characteristics of cancer cells and killing the remaining cancer cells. Although there are some promising results reported, the research in this area is still in a very early stage. It has been shown that the most effective anti-tumor immune responses are achieved by stimulating T cells, which can recognize and kill tumor cells directly.

Cancer vaccine can be produced from cancer cells, parts of cells, or pure antigens [27]. Tumor cell vaccines use killed cancer cells removed during surgery. The antigens on the killed tumor cell surfaces can stimulate a specific immune system response. As a result, cancer cells carrying these antigens are recognized and attacked. To increase the effectiveness of the vaccine, killed tumor cells may be further enhanced with nonspecific adjuvants. The advantages of using whole tumor cells is that they may expose the immune system to a large number of important cancer antigens, some of which may have not been identified yet. There are two basic kinds of tumor cell vaccines: autologous and allogeneic. The former takes advantage of the tumor cells from the patient itself, while the latter utilizes cells from someone else [28].

Dendritic cells are the most important antigen-presenting cells. When exposed to a foreign molecule, they can take up the foreign substance and display it, and stimulate the immune responses. This process is rather specific, as each patient's own dendritic cells must be used as the foundation of the vaccine. It is recently shown at Duke Comprehensive Cancer Center that RNA instead of protein can be used to make dendritic vaccines, which have dramatically expanded the scope of cancer vaccines, making the technology broadly applicable [29].

By creating antigens that are easier for the immune system to recognize, antigen vaccines stimulate the immune system using individual antigens, rather than the whole tumor cells that contain many thousands of antigens. While antigen vaccines may be specific for a certain type of cancer, they are not patient specific like cell vaccines. Although large amount of work needs to be accomplished before making significant progress, cancer vaccine holds a promise in the battle with cancers.

4 Concluding remarks

As the basic structural and functional unit of all living organisms, cell is very complicated machinery with highly ordered structure and precisely controlled biological processes. Any deviation of the cell structure or function from the normal state can cause diseases in human body. In order to correct the cellular malfunction, drugs must be efficiently delivered to the cells to produce pharmacological effect. The understanding of cell cycle, growth control, and cellular defensive response becomes essential for the design and development of therapeutics for targeted cellular drug delivery. The rapid development in human genomics, cell biology, and molecular biology will lead to the complete understanding of various cellular processes and more and more targeted drug delivery systems will be developed in near future.

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Figure Legends:

Figure 1. A typical diagram of cell cycle

Figure 2. Diagram of genetic information flow (DNA-RNA-Protein)

Figure3. Activation of B-cell and T-cell

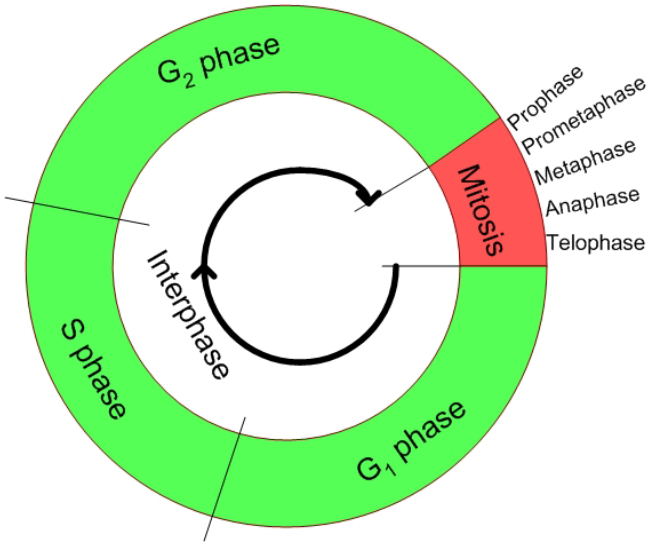
G2 phase
Prophase
Prometaphase
Metaphase
Anaphase
Telophase
Mitosis
Interphase
S phase
G1 phase

Figure A.1

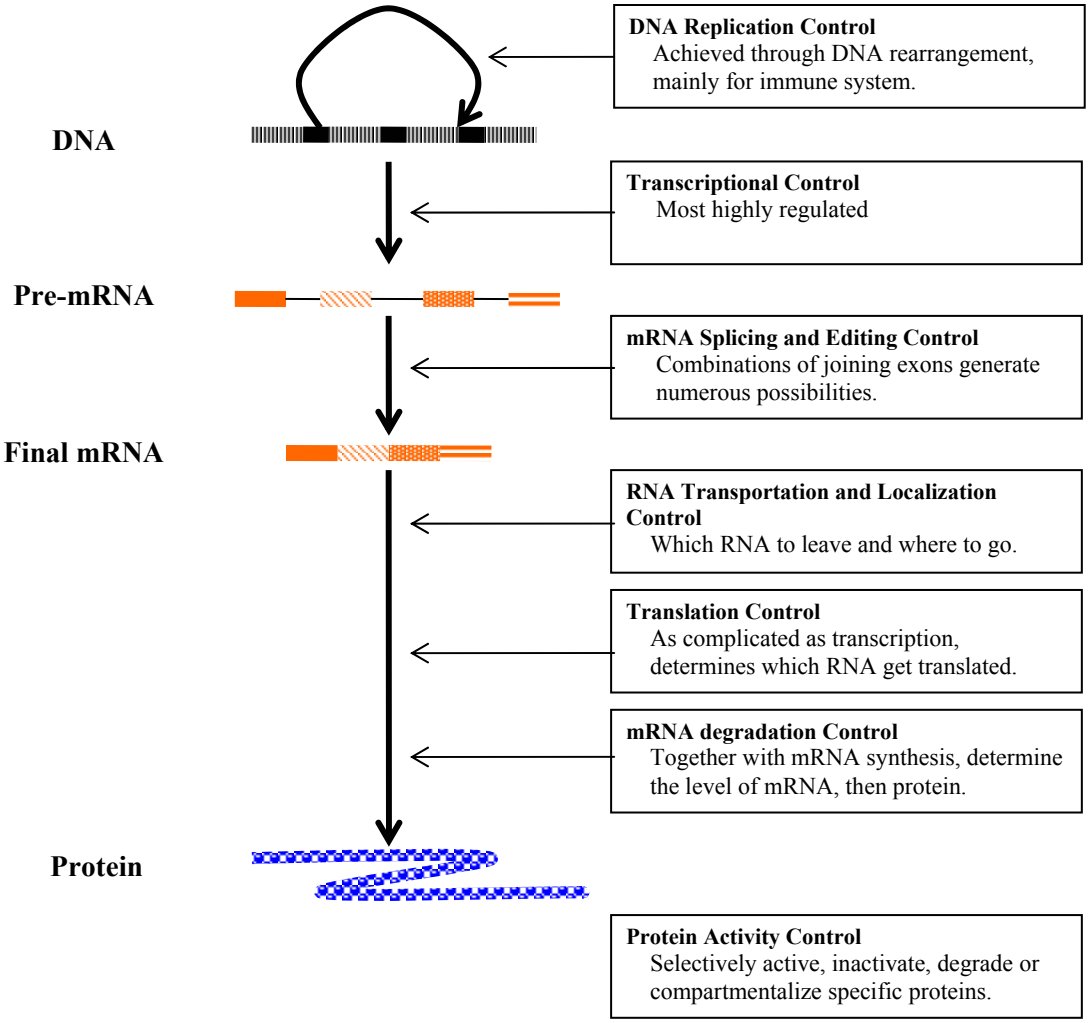
DNA
Pre-mRNA
Final mRNA
Protein
DNA Replication Control
Achieved through DNA rearrangement, mainly for immune system.
Transcriptional Control
Most highly regulated
mRNA Splicing and Editing Control
Combinations of joining exons generate numerous possibilities.
RNA Transportation and Localization Control
Which RNA to leave and where to go.
Translation Control
As complicated as transcription, determines which RNA get translated.
mRNA degradation Control
Together with mRNA synthesis, determine the level of mRNA, then protein.
Protein Activity Control
Selectively active, inactivate, degrade or compartmentalize specific proteins.

Figure A.2

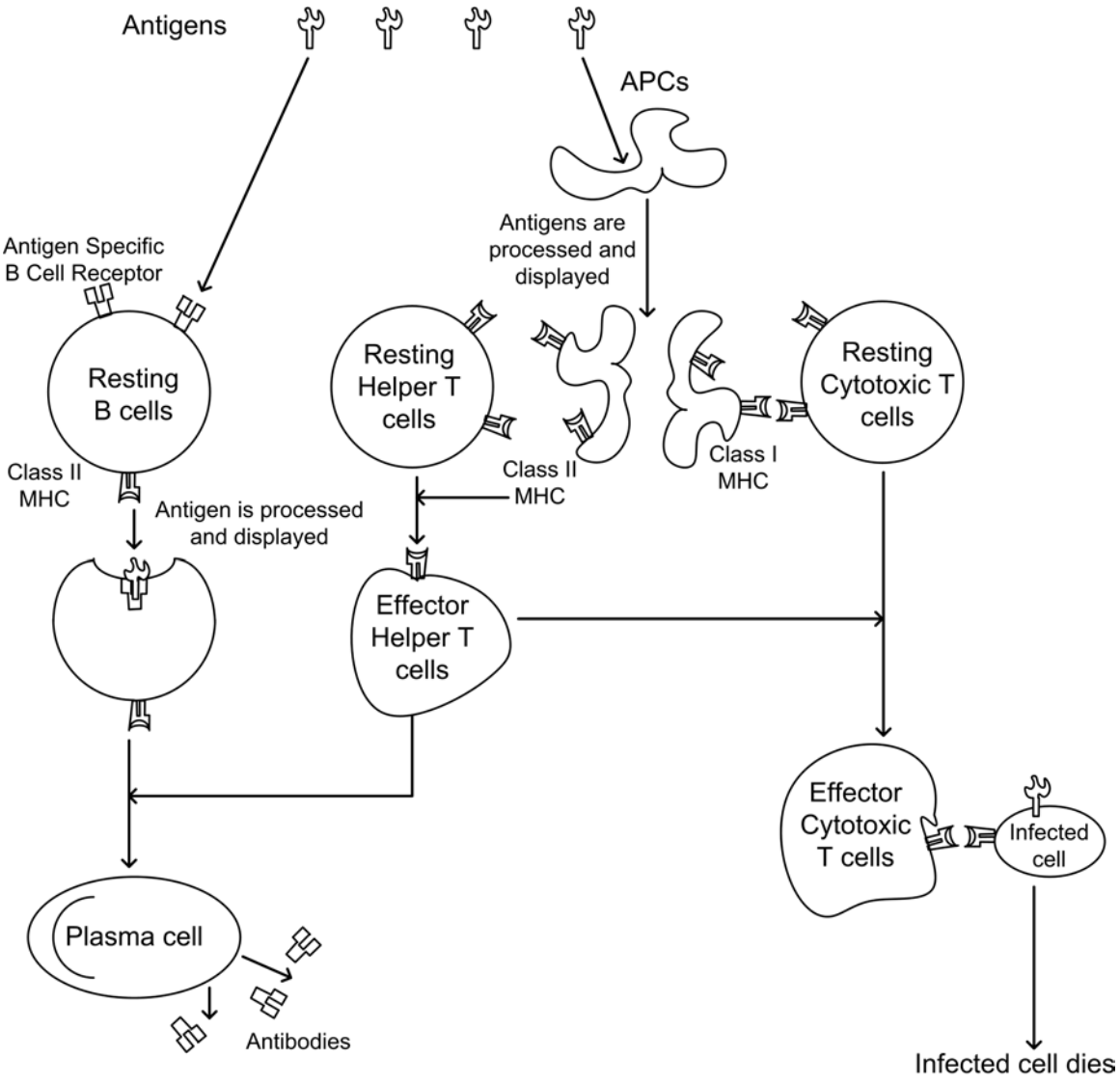
Antigens
APCs
Antigens are processed and displayed
Antigen Specific B Cell Receptor
Resting B cells
Resting Helper T cells
Resting Cytotoxic T cells
Class II MHC
Antigen is processed and displayed
Class II MHC
Class I MHC
Effector Helper T cells
Effector Cytotoxic T cells
Infected cell
Plasma cell
Antibodies
Infected cell dies

Figure A.3