IN VITRO GENE AND DRUG DELIVERY AND TARGETING TO HUMAN GLIOMA CELLS BY LIPOPROTEIN MIMICS

by

GUANGLIANG PAN

(Under the direction of D. Robert Lu)

ABSTRACT

Malignant glioma represents a very difficult therapeutic challenge. One new therapeutic strategy is gene therapy, which involves the delivery to and expression of the therapeutic genes in the cancer cells. In this research, a novel artificial lipoprotein delivery system, which consists of nanoemulsion particles and the incorporated lipidized poly-L-lysine, was developed and evaluated. A model plasmid DNA, pSV-β-Gal, was carried by this system and transfected human glioma cell line SF-767 *in vitro.* The plasmid DNA was effectively delivered by this system and the reporter gene was expressed. Compared to Lipofectamine™ system, this new delivery system demonstrated similar transfection efficiency but a much lower cytotoxicity. Targeted delivery of therapeutics is another new strategy to treat glioma. The cellular uptake of a cholesterol-based anti-tumor compound, BCH, in liposomal formulation by normal neuron cells and glioma cells was compared. It was found that the cellular uptake of BCH by glioma cells was up to 11 times as high as that by normal neuron cells. In the presence of monoclonal anti-LDL receptor antibody in the culture medium, the cellular uptake of BCH in liposomal formulation by the glioma cells was greatly reduced. The effect of serum on the cellular uptake of BCH in liposome formulation by the glioma cells was also investigated by replacing normal serum with lipoprotein deficient serum. LDL could help the cellular uptake of BCH in liposome formulation by the glioma cells. In addition, the effect of divalent calcium ion and temperature on cellular uptake of BCH in liposome formulation was investigated. These results suggested that LDL receptor played an important role in the uptake of BCH in liposome formulation by the glioma cells. The cytotoxicity of another cholesterol-based anticancer drug, methotrexate-cholesterol, on the glioma cells was also evaluated. In addition, the application of biological protein nanostructure in targeted drug delivery was described.

INDEX WORDS: Gene delivery, Glioma, Nanoemulsion, Artificial lipoprotein, Plasmid DNA, β-Galactosidase, Poly-L-lysine, Lipidized, Transfection, Cytotoxicity, Endocytosis, BCH, Normal neuron cells, LDL receptor, Monoclonal anti-LDL receptor antibody, Lipoprotein deficient serum (LPDS), Calcium ion, Methotrexate-cholesterol

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To My Beloved Family

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Lipid-based formulations for drug delivery and targeting

Lipids are a diverse group of biological substances that are ubiquitously distributed in living organisms. They play very fundamental roles in the architecture and functionality of all living cells. Because they are primarily made up of non-polar groups, they are typically more readily to dissolve in non-polar solvents such as chloroform, acetone, and ether rather than in water. Based on the chemical composition and structure, there are three major classes of lipids, i.e., neutral lipids, phospholipids, and steroids. Because of their properties such as the ubiquity in the food-chain, being nature to the body, and their unique incorporation capabilities for both hydrophilic and hydrophobic compounds, the research on the utilization of lipids for drug delivery system has been for more than 30 years.

The most common lipid-based drug delivery systems are oily solution, suspension, emulsions, microemulsion, and liposomes. Pharmaceutical suspension is a particular class of dispersion or dispersion system in which the internal or suspended phase is dispersed uniformly with mechanical agitation throughout the external phase (suspending vehicle). It includes oral suspension, topical suspension, ophthalmic suspension, and parenteral suspension (Nash, 1996). Pharmaceutical emulsions are dispersions in which one phase (internal phase) exists as droplet within the other phase (external phase). Based on the size distribution and the approach of preparation, there are microemulsions, submicron emulsions, nanoemulsions, and self-emulsifying emulsions

(Block, 1996). Liposomes are microscopic spherical vesicles that form when phospholipids are hydrated. These vesicles consist of a lipid bilayer membrane that encloses an aqueous phase. Thus liposomes can be used to encapsulate both hydrophilic and hydrophobic drugs. They can be formulated as a solution, dry powder, aerosol, cream or lotion. Practically all of the conventional administration routes can be employed for liposomal formulations (Gregoriadis, 1998).

Lipid formulations provide many advantages for drug delivery and targeting. Depending on the nature of drug, lipid formulations exhibit versatility for different drugs. In addition, they can provide good and reproducible bioavailability for drugs. The renaissance in lipid-based formulation over the past 10 years has led to the development of novel lipid-based drug delivery systems and is hot research area. The rapid development in biotechnology and combinatorial chemistry make lipid-based formulations even more attractive in the delivery and targeting of biologics and hydrophobic new chemical entities.

2. Emulsions and their pharmaceutical applications

Emulsions are generally considered to be dispersions, which are composed of two phases, i.e., an oil phase, and an aqueous phase. One of the two phases (internal phase) is dispersed as droplets with the other (external phase). Two most common structures of emulsions are oil-in-water (O/W) in which water is the external phase, and water-in-oil (W/O) in which oil is the external phase. The structure of emulsion is dependent upon the surfactant and the phase volume ratio. An O/W emulsion is generally formed in the presence of small amount of oil, while a W/O emulsion is more likely to be formed in the presence of small amount of water. The surfactant is the most important factor in the

formation of structure of emulsion. O/W emulsions can be formed with surfactants that have a hydrophilic lipophilic balance (HLB) range of 8-18, and W/O emulsions can be formed using surfactants with a HLB range of 3-8. If the internal phase itself comprises emulsified system, the polydisperse systems, such as oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) can be formed. Based on the size of emulsion droplets, emulsions can be subcategorized into microemulsion, submicron emulsions or nanoemulsions (<100 nm).

2.1 Emulsions for oral drug delivery

The interest in emulsions for drug delivery is mainly due to the possibility of dissolving lipophilic drugs in a safe and tolerable matrix. With the introduction of new automated synthesis methods, combinatorial chemistry, and high-throughput screening techniques, more and more active drug compounds have been synthesized; a large portion of these compounds are lipophilic. Among different routes of drug delivery, the oral route is the most convenient and accepted method. However, lipophilic drugs have very poor solubility in the gastrointestinal tract. On the other hand, the rapid advances in biotechnology leads to the discovery of many peptide and protein drugs, which cannot overcome the physical and enzymatic barrier imposed by the intestinal tract. Emulsion vehicles have the potential to improve the oral delivery of these drugs. The application of lipid emulsions for improving drug dissolution and oral absorption has been reviewed in many papers (Constantinides, 1995; Charman, 2000; Mizushima, 1996; Tomii, 2002). One good example of this is to utilize a microemulsion vehicle for oral drug delivery of cyclosporin A, which has been developed and widely used recently (Arumugam et al., 1998). The emulsion vehicles can not only improve drug solubilization, but also protect

the drug against enzymatic hydrolysis. It has been reported that microemulsions have the potential to deliver peptide drugs, whose delivery has been thought to be difficult because of their large molecular weight (Ritschel, 1993; Constantinides et al., 1994, 1995).

2.2 Emulsions for topical drug delivery

Besides the potential for oral drug delivery, emulsion vehicles can also be used to improve topical bioavailability of drugs (Friedman et al., 1987; Kriwet and Muller-Goymann, 1995; Ktistis, 1997; Osborne et al., 1991; Fevrier et al., 1991). The topical application of emulsions has been previously reviewed by Block (Block, 1995). It is believed that the improved bioavailability by emulsion vehicles is contributed by the prolonged absorption resulting from the reservoir effect of the dispersed phase. Increased skin penetration and drug flux across the skin were observed when emulsions were used as vehicles for the drugs (Fevrier et al. 1991; Thevenin et al. 1996).

2.3 Emulsion for parenteral drug delivery

Because of their high solubilizing capability and thermodynamic stability, emulsions are attractive vehicles for parenteral administration. Phospholipid-stabilized lipid emulsions by the parenteral route have been used as energy sources for more than three decades. These emulsions have also been viewed as vehicles for drug delivery, controlled drug release and targeting to specific sites in the body for more than ten years (Davis et al., 1987; Muller, 1991). The lipid emulsion particles undergo similar transport, distribution and clearance as the natural lipids in the body. Emulsion particles are primarily cleared by liver, adipose tissue, heart muscles, and lactating mammary glands. Thus they can serve as good drug delivery systems to these sites, especially for lipophilic drugs. The clearance of emulsion particles from the body is largely dependent on the

interaction with the reticuloendothelial system (RES). The critical factors that affect the clearance are the size and charge. The smaller the particle size, the slower the clearance by the RES. Charged particles are cleared more quickly than neutral particles. In addition, emulsion particles with higher molecular weight emulsifier are cleared more slowly than those with smaller molecular weight emulsifier.

Both two-phase emulsion systems, such as oil-in-water (O/W) and water-in-oil (W/O), and polydisperse emulsion systems, such as water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O), can be used for parenteral drug delivery. The choice of emulsions is dependent on the characteristic of the drug. For example, O/W is usually given as intravenous route as drug carrier. However, the pharmaceutical applications of emulsions are limited by the availability of suitable surfactants because most surfactants are toxic. Among the nontoxic surfactants, lecithin is the most widely used since it is naturally occurring biological surfactant and is a major component of cell membrane lipid. So it is the ideal surfactant to prepare pharmaceutically acceptable emulsion, especially for parenteral administration. Many researches have been conducted in the development of emulsion vehicles for parenteral use (Shinoda et al., 1991; Trotta et al., 1998; von Corswant et al., 1997).

3. Liposomes and their pharmaceutical applications

Liposomes are colloidal particles in which a lipid bilayer membrane encapsulates part of the aqueous phase where they are dispersed (Bangham et al., 1965; Lasic, 1993). Based on their size and the number of bilayers, they are classified into three classes. Multilamellar vesicles (MLV) have a size range of 0.1-5.0 μm, large unilamellar vesicles

(LUV) have a size range 0.06 μm, and small unilamellar vesicles (SUV) have a size range of 0.02-0.05 μm.

The liposome was first proposed as a pharmaceutical delivery system for enzymes used to treat lysosomal storage disease (Roerdink et al. 1987). Since then, a variety of pharmaceutical applications of liposomes have been proposed. Liposomes have the unique advantages of being both nontoxic and biodegradable because they are composed of naturally occurring substances. In addition, the unique ability of liposomes to entrap both hydrophilic and hydrophobic drugs makes them attractive delivery systems for these drugs. The advancement in liposome formulation has made it possible to efficiently entrap drug molecules into liposomes. Utilization of liposomes as carriers for drugs to treat cancer (Ahmad et al. 1993; Mayhew et al., 1987), fungal diseases (Lopez-Berstein et al., 1985), leishmaniasis (Alving et al, 1978), and rheumatoid arthritis (RA) (Watson-Clark et al., 1998) was conducted in many different labs and some of liposome-based anticancer drugs, such as Doxil, Myocdet, have been commercialized. In addition, cationic liposomes are widely used for gene delivery research.

3.1 Liposome for anticancer drug delivery

Liposomal technology has been advanced significantly in the last two decades. These techniques mainly include the production of homogenous liposome particles by extrusion science, customized entrapment for controlled release, and the production of long circulating liposomes such as PEG-liposomes. These techniques can greatly enhance drug therapy, especially cancer therapy. In cancer therapy, cytotoxicity of anticancer drug is one of the major concerns. Liposomes can selectively deliver anticancer drugs to the

tumor site resulting in increased therapeutic index and reduced cytotoxicity to normal cells (Cabizon et al. 1982; Olson et al., 1982; Conley et al., 1993; Cowens et al., 1993).

The therapeutic effect of anticancer drugs is dependent on both tumor physiology and tumor cell heterogeneity. An ideal anticancer drug must access the target cancer cells in sufficient amount to cause cytostatic and cytotoxic effect. Among the commonly used anticancer drugs, only a limited number of them have been administered via drug carriers. Because of this, the therapeutic dose must be limited to schedules and amount to reduce the non-specific toxic effect and to allow the regeneration of blood cells and the cells of the immune system. Targeted drug delivery to cancer cells can be achieved by coating monoclonal antibody against tumor-cell specific antigens. For example, when anticancer drug doxorubicin was entrapped into monoclonal antibody coated PEG-liposomes, more effectiveness and less cytotoxicity was obtained to treat squamous-cell carcinoma in mouse model (Ahmad et al., 1993).

3.2 Liposomes for anti-infection drug delivery

Infections caused by bacteria and fungi are frequently met in clinical practice. Antibiotic treatment remains the major choice for these infections. However, antibiotic treatment failure often occurs due to the moderate antibiotic susceptibility of the microorganism, insufficient availability of antibiotic caused by the low half-life in blood, or the low dose limited by the toxic side effect. In order to increase the antibiotic concentration in the target site, liposomes can be used to achieve the site-specific delivery for the antibiotic. For example, long circulating liposomes can remain in the vascular compartment for prolonged period of time without the requirement of high lipid dose or rigid nature of the lipid bilayers (Woodle et al., 1994; Marjan and Allen, 1996). In order

to overcome the toxic side effects of some antibiotics, e.g., amphotericin B (AMB), the rationale to use liposomes as carriers for antibiotics is to achieve site-avoidance drug delivery. It is believed that the reduction of toxicity of the AMB in lipid formulation was caused by the reduced affinity of AMB to cholesterol in the human cell membrane, as compared to the lipids in the lipid carrier and the ergosterol in the fungus membrane. Used this way, high therapeutic index could be achieved (Leenders and De Marie, 1996; Hiemenz and Walsh, 1996).

Liposomes can also be used in the therapy of HIV infection. Drugs for HIV infection such as the reverse transcriptase inhibitor 3'-azido-3'-deoxythymidine (AZT) can be encapsulated into liposomes and targeted to cells and tissues infected with HIV-1 to enhance the efficacy and reduce the toxicity of the drugs. In order to overcome the low oral availability of water-insoluble drugs such as some protease inhibitors, liposomes can be used to entrap these drugs in their membrane phase and to deliver them intravenously or subcutaneously. For large molecular weight drugs such as antisense oligonucleotides and therapeutic genes, liposomes can not only effectively deliver these medicines but also protect them from the digestion by nucleases.

3.3 Liposome for vaccine delivery

Liposomes have been widely used to encapsulate and deliver peptide and protein antigens (Harding et al., 1991; Collins et al., 1992; Walker et al., 1992; Wassef et al., 1994). The rationale to use liposomes as immunological vectors is that liposomes are able to sequester antigens and release them slowly (Allison and Gregoriadis, 1974; Gregoriadis and Allison, 1974). In addition, liposomes resemble the structure of natural immune systems (Garcon and Six, 1991). They have the potential to stimulate both

antibody and T-cell responses simultaneously by gaining entry to both the MHC class I and class II pathways.

4. Lipid-based formulation for gene delivery

Gene therapy can generally be defined as a process by which genetic information is delivered to cells to correct, repair or modify the gene-derived malfunction. It has most often been discussed in the context of treating lethal and disabling diseases. Since the aim of gene therapy is to eradicate the cause rather than the symptoms of disease, it is believed to be the therapy of 21st century. The rapid advances in human genomics and molecular biology have revealed the wide relationships between diseases and genetic component. This makes the promise of gene therapy continue to grow. As a gene medicine, it consists of three basic components: a gene encoding a specific therapeutic protein, a plasmid-based gene expression system that controls the functioning of the gene within a target cell, and a gene delivery system that controls the effective delivery of the gene expression system to the target cells within a specific organ (Mahato et al., 1999). A diagram of gene delivery and gene expression system is shown in Figure 1.1. The major challenge to gene therapy is how to effectively deliver the gene to the target tissue and then transport the gene to the nucleus of cells for DNA replication and transcription.

There are two major gene delivery systems, i.e. viral and nonviral vectors. The main viral vectors include adenovirus, retrovirus, lentivirus, adeno-associated virus, and herpes simplex virus. Most viral vectors show high transfection efficiency, however, their applications are limited by the immune response and possible mutagenesis caused by the insertion of viral vectors into the host chromosome. As an alternative gene delivery system, nonviral vectors provide a safer profile. In addition, nonviral vectors are easier to manufacture and control. The most commonly investigated nonviral vectors include

naked DNA, cationic liposomes, polymers, and protein based cations. The limitation to the current available nonviral vectors for gene delivery lies in the relative low transfection efficiency.

Among the nonviral gene delivery system, liposomes are the most widely studied because their phospholipid compositions can facilitate the delivery of DNA by promoting membrane fusion. Liposomes are classified according to charge, phospholipid composition, pH-sensitivity, and structure. Because of their positive surface charge, cationic liposome can condense the negatively charged DNA by electrostatic interaction and will enhance the cellular uptake via endocytosis. In addition, cationic liposomes can protect the DNA from the attack by DNases. Many investigations have shown that cationic liposome can efficiently deliver DNA into target cells (Gao and Huang, 1996; Zhou and Huang, 1994; Zabner et al., 1995; Xu and Szoka, 1996; Zelphati and Szoka, 1996; Tan et al., 2001). Currently, the major challenges for cationic liposomes for gene delivery are the systemic barriers and cellular barriers. In addition, the toxicity of cationic lipids also greatly limits the application of these delivery systems.

Besides the liposomal formulations, other lipid-based formulations may provide new opportunities for gene delivery. By mimicking the natural chylomicron remnants, an emulsion system was developed to deliver the hydrophobic DNA complex to liver of mice and the delivered DNA was successfully expressed (Hara et al., 1997). This is the first nonviral vector that resembles a natural lipoprotein carrier. Recently, Kim et al. developed a new gene delivery system called Terplex system, which is based on a complex formed by natural low-density lipoprotein (LDL) and stearyl-poly-L-lysine (Kim et al., 1997; 1998). Through hydrophobic interaction, stearyl-poly-L-lysine can be

incorporated into the LDL particles. The assembled complex possessed positive charge and was able to carry negatively charged DNA and successfully deliver the DNA into vascular smooth muscle cells. Since this system requires natural LDL, its application was limited by the availability of natural LDL.

Based on the chemical composition and structure of natural LDL, an artificial lipoprotein based gene delivery system has been proposed in our lab. Similar to the structure of natural lipoproteins, this artificial lipoprotein delivery system consists of nanoemulsion cores made of natural lipids and a polar shell, and lipidized poly-L-lysine, which replaces the surface protein as in natural lipoproteins. With the proper weight ratio of poly-L-lysine to the lipids in a nanoemulsion, the artificial lipoprotein delivery system will be able to carry DNA for gene delivery. The comparison of Terplex system and artificial lipoprotein system is shown in Figure 1.2. Since the lipids used in the system are all natural substances, the cytotoxicity of this delivery system was expected to be low. Another advantage of this system is that it can be readily assembled using commercially available materials including phospholipids, cholesterol and poly-L-lysine. In addition, the chemical composition, particle size, and type of surface poly-peptide or surface protein can be controlled and optimized, allowing widely-diversified gene or drug delivery and targeting. The development and evaluation of this system for gene delivery to human glioma cell line will be reported in Chapter 2.

5. Receptor-mediated drug delivery and targeting to cancer cells

The aim of drug targeting is to restrict the access of pharmacological agents to selected cells within a tissue. The first step involved in this process is the recognition and interaction of the carrier with specific target cells. The second step is the delivery of the

therapeutics into the target cells with little or no uptake by the non-target cells (Poste, 1983). This process is either cell-surface receptor mediated by ligand-receptor interaction or cell-surface epitope mediated by antigen-antibody interaction. The cellular targeting is largely dependent on the specificity of the target cell surface proteins.

In the past several decades, research on cancer treatment is still discouraging in both laboratory and the clinic (Brun et al., 1997; Dunton, 1997). One reason for this is that the cytotoxic anticancer therapy is largely dependent on the rapid dividing rate of cancer cells and the anticancer drugs mostly act on the DNA, tubulin, and enzymes such as the topoisomerase that are important to the DNA replication. Such drugs will also act on the normal host cells and result in severe side effects. This will not only limit the dose to kill the cancer cells but also cause the induction of drug resistance and metastasis. Targeting the cytotoxic drugs to cancer cells becomes an apparent approach to solve these problems in cancer therapy.

One of the most common methods for anticancer drug targeting is to employ the monoclonal antibody against the tumor-associated antigens (Hellstrom and Hellstrom, 1997; Wick and Groner, 1997). Cytotoxic drugs could be attached to the lysine residues that are distributed over the entire protein surface or the thiol groups generated by the reduction of interchain disulfides at the hinge region (Jinno et al., 1996).

It is well known that mammalian cells have developed various mechanisms to internalize specific substrates or targets into the cells. These mechanisms are collectively termed as endocytosis which comprises phagocytosis, pinocytosis, receptor-mediated endocytosis (clatherin-mediated), and potocytosis (non-clatherin-mediated) (Mukherjee et al., 1997; Lehr, 1994). Receptor-mediated endocytosis is a highly specific biological

process which requires that specific binding and interaction between ligand and receptors. Such a process is initiated by the binding of an exogenous ligand to a receptor across the cell membrane with the binding domain oriented to the outside of the cell. When the ligand binds to receptors, which cluster in domains in the plasma membrane known as coated pits. These domains eventually invaginate to form coated vesicles. The loss of coating with the aid of chaperone proteins will result in the formation of endosome, which may further fuse with lysosome, in which the contents in the vesicles will be degraded enzymatically.

The cell surface receptors are a group of complex transmembrane proteins. They play very important roles in regulating cellular functions such as growth differentiation, metabolism, secretion, contraction, and migration (Hirt et al. 1993). Although various receptors are expressed in different types of cells, they share common structural features, i.e. all the receptors have an extracellular ligand binding domain, a single hydrophobic transmembrane domain, and a cytosolic domain that encodes for the endocytosis and other functional signals (Schwartz, 1995). Since different cell types have different types and levels of receptor and specific binding of ligand with receptor is dependent on the extracellular binding domain, this provides the basis for targeted drug delivery. A specific ligand can serve as a homing device for specific cell types by recognizing its cell surface receptors.

It has been found that many cancer cells over-express some cell surface receptors such as transferrin receptor (Plant et al. 1989; Huwyler et al., 1996), folate receptor (Garin-Chesa et al. 1993; Ross et al. 1994; Lee and Low, 1994; Reddy et al., 1998), and LDL receptor (Vitols et al, 1984, 1985, 1992; Rudling et al. 1990; Jung-Testas et al,

1992; Maletinska et al., 2000), to meet the increased cell proliferation and growth requirement. So one strategy to develop targeted delivery system for cancer therapy is to take advantage of these over-expressed cell surface receptor by conjugating the drug with the ligands or by incorporating their corresponding ligands to a drug carrier such that the anticancer drug can be specifically delivered to the cancer site.

Cholesterol is an essential component of cell membranes in mammalian cells. Cells obtain cholesterol by taking up plasma LDL, the main cholesterol carrier in the blood, via LDL receptor-mediated endocytosis (Brown and Goldstein, 1986) (Figure 1.3), or by de novo synthesis of cholesterol. Rapidly dividing cells, e.g. cancer cells, require more cholesterol for membrane growth. Elevated LDL receptors have been found in many cancer cells (Vitols et. al, 1984, 1985, 1992; Rudling et. al. 1990; Jung-Testas et. al, 1992; Maletinska et. al., 2000). This phenomenon provides the basis for the design of targeted drug delivery systems. In our lab, a cholesterol-based anti-tumor compound, cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH) was synthesized for the targeted drug delivery for boron neutron capture therapy (BNCT) (Ji et al., 2002). Early research has demonstrated that BCH could be taken up by glioma cells SF-763 and SF-767 and accumulated in high concentration when BCH was formulated into liposome (Peacock et al., 2003). However, as a targeted drug delivery system for cancer therapy, drugs should be delivered preferentially to cancer cells. So the research on the comparison of cellular uptake of BCH by normal neuron cells and cancer cells was conducted and the results are reported in Chapter3.

It has been reported that glioma cell lines SF-763 and SF-767 express high level of LDL receptor on their cell surface (Maletinska et al., 2000). In order to clarify whether

the high uptake of BCH in liposome formulation by glioma cells is related to the elevated LDL receptors, monoclonal antibodies against LDL receptors can provide direct evidence whether LDL receptors are involved in the cellular uptake of BCH in liposome formulations. Therefore, monoclonal anti-LDL receptor antibody was prepared and purified. The purified product was applied during the experiments of cellular uptake of BCH in a liposome formulation. BCH uptake by glioma cells in the presence and absence of the monoclonal anti-LDL receptor antibody was determined and compared. On the other hand, since the prerequisite for the receptor-mediated endocytosis is ligand-receptor binding, the depletion of ligand will further demonstrate the role of receptor-mediated pathway in the cellular uptake of BCH in the liposome formulation. Different combinations of normal human serum and lipoprotein deficiency serum was supplemented in the culture medium and the cellular uptake of BCH in liposome formulation was determined. Since LDL-receptor mediated endocytosis is divalent metal ion and temperature dependent, their effects on the cellular uptake of BCH in liposome formulation by the glioma cell were also evaluated. These results are recorded in Chapter 4.

Although BCH could be preferentially delivered to cancer cells as compared to normal neuron cells in the in vitro experiment, the anticancer activity of the delivered boron compound can only be demonstrated by neutron irradiation, which was not available in our research because of the unavailability of a neutron resource. In order to demonstrate further whether the cholesterol-drug conjugate can be beneficial for the drug targeting, a chemotherapeutic agent may provide direct evidence. Methotrexate is an antimetabolite used in the treatment of cancer. Since methotrexate molecule has carbonyl

group, it can form an ester bond with the hydroxyl group in the cholesterol molecule. In this research, methotrexate- cholesterol conjugate was synthesized and its cytotoxicity to glioma cell line SF-767 was evaluated and compared with that of methotrexate. The result of this project is reported in Chapter 5.

6. Biological protein nanostructures and targeted drug delivery

Targeted delivery of drugs to specific cells involves the specific interactions between drugs or drug carriers and the cell surface proteins through ligand-receptor interactions or antigen-antibody interactions. Targeted drug delivery to specific molecular complexes or organelles within a cell requires the specific interactions of drug with the targeted complexes to lead to the therapeutic effect. In the biological systems, these interactions generally occur on various types of biological nanostructures of protein origin and understanding and utilization of the biological nanostructures could lead to significant improvement in drug targeting and drug carriers.

The biological protein nanostructures primarily include protein-lipid, protein-protein, protein-carbohydrate, and protein-nucleic acid complexes. Proteins, are smaller nanoscale molecules with typical size range between 1 and 20 nm (1). Through sophisticated interactions with other biomolecules, these protein nanostructures are formed and widely distributed in human body. For example, low-density lipoproteins (LDL), with a diameter of 25-28 nm, are protein-lipid complexes. They are the major circulatory nanostructures in the blood. As a drug carrier, these protein-lipid complexes offer a certain advantage of being endogenous nanostructures that do not trigger immunological response. They can also escape recognition and elimination by the reticuloendothelial system (RES). On the other hand, glycoproteins, i.e. protein-

carbohydrate complexes, are vital structural and regulatory proteins in viruses and can serve as important therapeutic targets for antiviral drug development. Telomerase, a protein nanostructure formed from protein and nucleic acid, is activated only in cell immortalization and cancer progression. Thus telomerase is an ideal therapeutic target for anticancer therapy. Because protein nanostructures are so critical to various biological and physiopathological activities, they have received wide attention in recent years in the development of drug targeting strategies as drug carriers and therapeutic targets. In the appendix, two aspects of biological protein nanostructures regarding their involvement in targeted drug delivery will be discussed: (1) biological protein nanostructures as targeting drug carriers and (2) biological protein nanostructures as therapeutic targets for new drug development.

In summary, my research objectives are:

1. To develop and evaluate the nanoemulsion system for in vitro gene delivery to glioma cells.
2. To compare the cellular uptake of BCH by normal cells and glioma cells.
3. To elucidate the mechanism of BCH uptake by glioma cells.
4. To evaluate and compare the cytotoxicity of methotrexate and methotrexate-cholesterol on glioma cells
5. To review the role of biological protein nanostructures in targeted drug delivery.

Figure Legends:

Figure 1.1. Working diagram of gene therapy

Figure 1.2. Comparison of terplex system and artificial lipoprotein system

Figure 1.3. LDL receptor-mediated endocytosis pathway in cells.

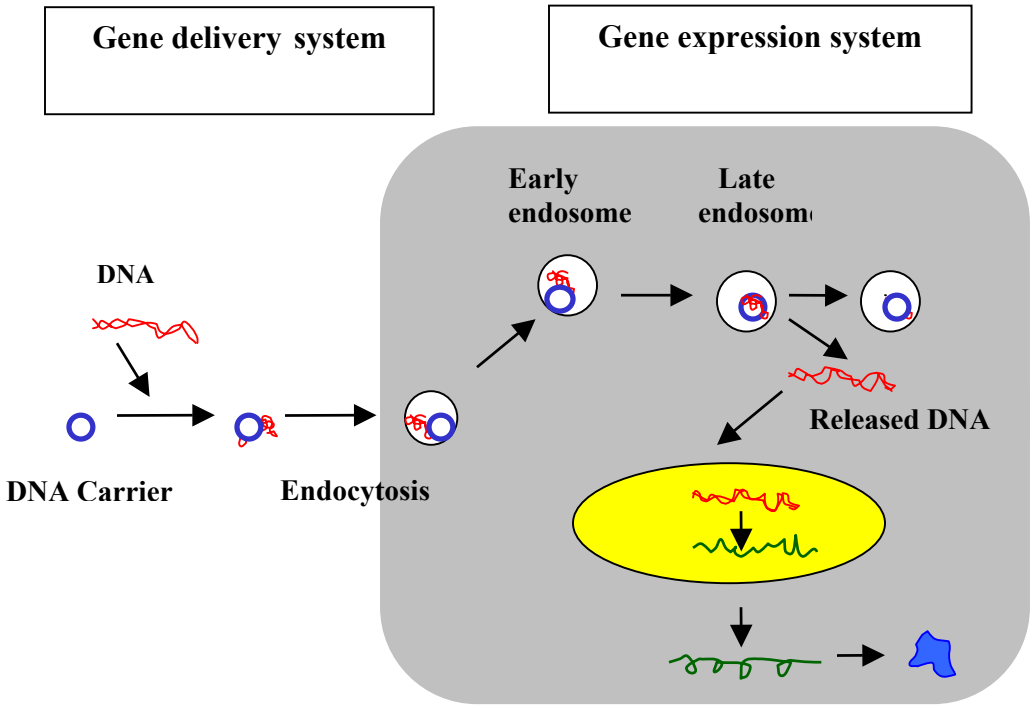
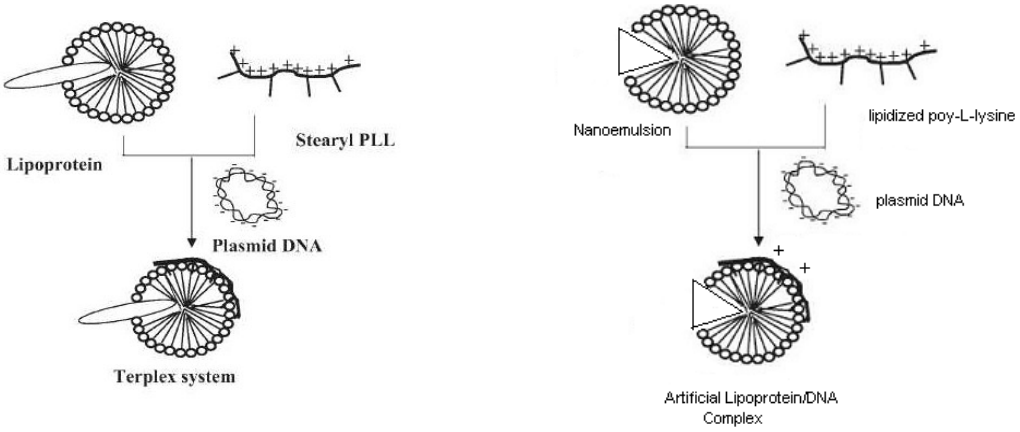

Gene delivery system
Gene expression system
Early endosome
Late endosom
DNA
Released DNA
DNA Carrier
Endocytosis

Figure 1.1


Lipoprotein
Stearyl PLL
Plasmid DNA
Terplex system
Nanoemulsion
lipidized poy-L-lysine
plasmid DNA
Artificial Lipoprotein/DNA
Complex

(a) (b)

Figure 1.2

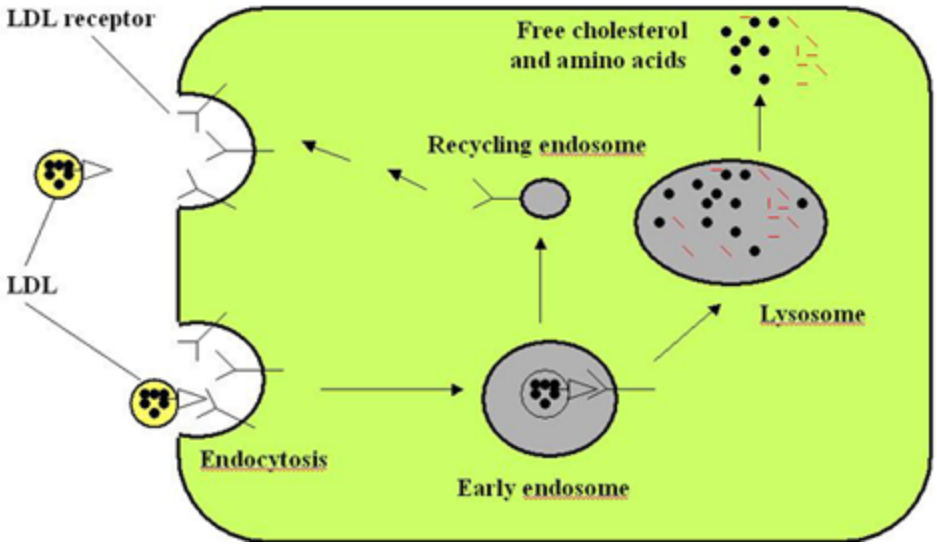

LDL receptor
Free cholesterol
and amino acids
Recycling endosome
LDL
Lysosome
Endocytosis
Early endosome

Figure 1.3

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CHAPTER 2

IN VITRO GENE TRANSFECTION IN HUMAN GLIOMA CELLS USING A NOVEL AND LESS CYTOTOXIC ARTIFICIAL LIPOPROTEIN DELIVERY SYSTEM[1]

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ABSTRACT

Purpose. To develop and evaluate a novel artificial lipoprotein delivery system for in vitro gene transfection in human glioma cells. **Methods**. A nanoemulsion was formulated with lipid compositions similar to natural lipoproteins. The oil phase of the nanoemulsion was composed of triolein (70%), egg phosphatidylcholine (22.7%), lysophosphatidylcholine (2.3%), cholesterol oleate (3.0%) and cholesterol (2.0%). To replace the surface protein as in natural lipoprotein, poly-L-lysine was modified to add palmitoyl chains at a basic pH and was incorporated onto the nanoemulsion particles through hydrophobic interaction. A model plasmid DNA, pSV-β-Gal containing a reporter gene for β-galactosidase was carried by the nanoemulsion/poly-L-lysine particles. The charge variation of the complex was examined by agarose gel electrophoresis and zeta potential measurement. In vitro transfection was conducted on human SF-767 glioma cell line using this new system. After standard X-Gal staining, transfected cells were observed under light microscope. The effect of chloroquine on the transfection was examined and, finally, the cytotoxicity of this new system was evaluated in comparison with commercial Lipofectamine™ gene transfection system. **Results**. The plasmid DNA was effectively carried by this artificial lipoprotein delivery system and the reporter gene was expressed in the glioma cells. Transfection efficiency was significantly increased by the treatment of chloroquine, indicating that endocytosis possibly was the major cellular uptake pathway. Compared to Lipofectamine™ system, this new delivery system demonstrated similar transfection efficiency but a much lower cytotoxicity. In the experiment, the cell viability was up to 75% using this system compared to only 24% using Lipofectamine™ system. **Conclusion**. A new artificial lipoprotein delivery system was developed for in vitro gene transfection in tumor cells. The new system showed similar transfection efficiency but a much lower cytotoxicity compared with the commercial Lipofectamine system.

KEY WORDS: Gene delivery, transfection, glioma, palmitoyl poly-L-lysine, nanoemulsion

ABBREVIATION: PLL, poly-L-lysine; p-PLL, palmitoyl poly-L-lysine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ONPG, o-nitrophenyl-β-D-galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside; LDL, low-density lipoprotein; PBS, phosphate-buffered saline

INTRODUCTION

Gene transfection can be defined as the delivery to and subsequent expression of functional genetic material in specific cells to manipulate their intrinsic genetic profiles. During the last decade, research involving gene transfection has been expanding very rapidly and many gene delivery systems have been developed to efficiently transfect various cells in both in vitro and in vivo experimental conditions. To be an effective gene delivery system, it must be able to carry sufficient amount of genetic material and express the genetic information in specific cells resulting in a significant changes in genetic profiles. In general, genetic materials can be carried and expressed in specific cells by either viral vector systems or non-viral vector systems. The viral vector systems, including retrovirus, adenovirus, adeno-associated virus, herpes simplex virus and lentivirus, have been extensively investigated owing to their high transfection efficiency. However, their applications are limited by their complicated handling procedures for in vitro experiments and poor safety profiles for in vivo studies (1-2). Compared to the viral vector systems, the non-viral vector systems are easy to handle and have better safety profiles. Consequently, the development of effective non-viral gene delivery systems has become the the aim of many research laboratories (3-5).

Since lipids are the main components of cell membranes, most non-viral vectors are lipid-based so that the vectors can be effectively incorporated into the cell membranes and facilitate the delivery of genetic materials into specific cells. Among these non-viral vectors, cationic liposomes, which carry positive charge and electrostatically interact with negatively charged DNA to form complexes, are the most widely studied (6-11). However, the success of using cationic liposomes for gene transfection is partly

hampered by the cytotoxicity of the cationic lipids. Polymer-based non-viral vectors have also been widely investigated, including poly-L-lysine, polyethenimine, polyamidoamine dendrimer, and chitosan (12-20). One main disadvantage of these systems is the low efficiency of transfection. Recently, Kim et al. developed a new gene delivery system called Terplex system, which is based on a complex formed by natural low-density lipoprotein (LDL) and stearyl-poly-L-lysine (21-22). Through hydrophobic interaction, stearyl-poly-L-lysine can be incorporated into the LDL particles. The assembled complex possesses a positive charge and was able to carry negatively charged DNA and successfully deliver the DNA into vascular smooth muscle cells.

In this paper, we report the development and evaluation of a novel artificial lipoprotein delivery system that can carry DNA materials for effective in vitro gene transfection in tumor cells. Similar to the structure of natural lipoproteins, this artificial lipoprotein delivery system consists of nanoemulsion cores made of natural lipids and surface lipidized poly-L-lysine, which replaces the surface protein as in natural lipoproteins. With the proper weight ratio of poly-L-lysine to the lipids in the nanoemulsion, the artificial lipoprotein delivery system efficiently carried plasmid DNA containing β-galactosidase gene and transfected human SF-767 glioma tumor cells. Our experiments showed that because the lipids used in the system are all natural substances, the cytotoxicity of this delivery system could be significant lower than the commercial gene transfection systems using cationic liposomes. The benefit associated with the low cytotoxicity makes it especially useful as an alternative to Lipofectamine™ or other commercial gene transfection systems. Another advantage of this system is that it can be readily assembled using commercial available materials including phospholipids,

cholesterol and poly-L-lysine. The chemical composition, particle size and type of surface poly-peptide or surface protein can be controlled and optimized allowing widely-diversified gene or drug delivery applications.

MATERIALS AND METHODS

Materials

Triolein (99%), egg yolk phosphatidylcholine (99%), cholesterol (99%), poly-L-lysine hydrobromide (MW 57900 Dalton based on viscosity), chloroquine (99%), o-nitrophenyl-β-D-galactopyranoside (ONPG), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). L-α-lysophosphatidylcholine (99%) was purchased from Avanti (Alabaster, AL, USA). Cholesterol oleate (99%) was obtained from Acros (Pittsburgh, PA). 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) was from Life Technologies (Rockville, MD, USA). Electrophoretic grade agrose was purchased from FMC Bioproducts (Rockland, ME, USA). All other chemicals were of analytical grade obtained from Sigma or J.T. Baker (Phillipsburg, NJ, USA).

Preparation of the nanoemulsion

The oil phase of the emulsion was composed of triolein (70%), egg phosphatidylcholine (22.7%), lysophosphatidylcholine (2.3%), cholesterol oleate (3.0%) and cholesterol (2.0%). The lipid components were dissolved in chloroform individually and then mixed thoroughly. The chloroform was then removed completely by a stream of nitrogen gas. In each 100 mg of lipid mixture, 10 ml of 2.4 M NaCl solution was added. The mixture was sonicated under nitrogen flow for 30 min using Model 450 Sonifier® (Branson Ultrasonics Corporation, Danbury, CT) with a duty cycle dial setting of 90% at

output of 40 watts. The temperature of the mixture was maintained at 55ºC during sonication. The prepared emulsion was then passed to times through an Emulsiflex B3 device (Avestin, Ontario, Canada) at a pressure of 70 psi to reduce the particle size to nanometer level. The emulsion was dialyzed against phosphate-buffered saline (PBS) using Spectra/Por® 2 molecularporous membrane tubing with molecular weight cut-off of 6000-8000 dalton (Spectrum Medical Industries, Inc. Houston, Texas). The emulsion particle size distribution was measured by Submicron Particle Sizer Autodiluter Model 370 (NICOMP Particle Sizing Systems, San Barbara, CA). In addition, the nanoemulsion was stored at room temperature and the particle size distribution was measured in 2, 4, 8, 16 weeks, respectively, to examine the stability of the nanoemulsion particles.

Lipidization of poly-L-lysine

Lipidization of poly-L-lysine was performed as described by Kim et al. (21) with slight modification. Briefly, poly-L-lysine hydrobromide (30 mg) was dissolved in 2 ml DMSO in a 50 ml round-bottom flask. After triethylamine (10 µl) was added, palmitoyl chloride (20 mg) was added to the mixture to react with the amino group of the lysine residues in poly-L-lysine. The mixture was allowed to react at room temperature for 2 hrs and filtered. Acetone was added to the filtrate to precipitate the lipidized polymer, palmitoyl poly-L-lysine or abbreviated as p-PLL. The product was dissolved in methanol, re-precipitated by acetone, and dried under vacuum overnight. The modified polymer was characterized by proton NMR.

Incorporation of p-PLL into nanoemulsion particles

In each 1.5 ml microcentrifuge tube, nanoemulsion (50 µl) was diluted with 0.2 ml PBS solution and incubated with various amount of p-PLL at 37ºC based on the

weight ratio of p-PLL to triolein in nanoemulsion. The weight ratios of p-PLL to triolein in the mixture were 0.125:1, 0.25:1, 0.5:1, and 1:1, respectively. After incubation for 1 hour, the mobility of the nanoemulsion particles in electric field was examined by agarose gel electrophoresis using Nile Red as the fluorescent dye. Agarose gel (0.4%) was prepared in TAE buffer (40 mM Tris-acetic acid, 1 mM EDTA, pH 8.0). Five µl of Nile Red solution in acetone (100 µg/ml) was dried out in test tube and redissolved in 30 µl of the incubation mixture as described above. In each sample, 6 µl of glycerine was added to increase the density of the sample and the sample (30 µl) was loaded in each sample well of the agarose gel. Electrophoresis was conducted for 1 hr at 70 volts at room temperature using Horizontal Mini-gel System (CBS Scientific Company Inc. Del Mar, CA, USA). The mobility of the particles in an electric field was visualized by an Eagle Eye II Video System (Stratagene, CA, USA).

Amplification and purification of plasmid DNA

Plasmid DNA, pSV-β-Galactosidase Control Vector (Figure 2.1), was purchased from Promega (Madison, WI) and was introduced into Epicurian Coli® XL1-Blue MRF' (Stratagene, CA) by using standard transformation protocol. The transformed *E. coli* strain was maintained in Luria-Bertani (LB) medium containing 15% of glycerol at –80ºC. To amplify the plasmid DNA, the *E. coli* strain was cultured in LB medium containing 100 unit/ml of ampicillin at 37ºC overnight and the cells were harvested by centrifugation. Plasmid DNA in the cells was extracted and purified using Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). The purity of plasmid DNA was confirmed by determining the ratio of optical absorbance at 260 nm and 280 nm (>=1.8) and further by 0.6% agarose gel electrophoresis. The agarose gel was stained

with ethidium bromide (0.5 μg /ml) for 15 minutes and destained with deionized water for 10 minutes. DNA bands in the agarose gel were visualized by the Eagle Eye II Video System. The concentration of plasmid DNA was determined by spectrophotometer at wavelength of 260 nm (1 OD_{260} ≈ 50 μg/ml).

Assembly of the complex of nanoemulsion, p-PLL and plasmid DNA

Nanoemulsion (50 μl) in 0.2 ml PBS was mixed with various amounts of p-PLL in the same way as described above. The weight ratios of p-PLL to triolein in the nanoemulsion were 0.0625:1, 0.125:1, 0.25:1, 0.5:1 and 1:1, respectively. After incubation at 37ºC for 1 hour, DNA (2 μg) was added and incubated at room temperature for 15 minutes. Samples were then loaded into 0.4% agarose gel and the electrophoresis was performed as described above. Zeta potential and mobility of the assembled particles were measured by Submicron Particle Size Analyzer 90Plus (Brookhaven Instrument Corporation, Holtsville, NY, USA). Before they were measured for zeta potential, the samples were diluted with sodium nitrate solution (1mM, pH 7.4) until the count of particles in the sample reached 100-300 kilo-counts per second (KCPS). Water and solutions used in zeta potential measurement were filtered with 0.1 μm Supor Acrodisc (Gelman Sciences, Ann Arbor, MI, USA). The particle size, zeta potential and mobility was recorded by the built-in PC computer system.

Gene transfection experiment

Human glioma cell line SF-767 was obtained from the tissue bank of Brain Tumor Research Center (University of California-San Francisco, San Francisco, CA, USA) and used in our transfection experiment because of its characteristics of aggressive growth. The cells were grown at 37°C in 5% CO_2 with Eagle's Minimal Essential

Medium (EMEM) medium supplemented with 10% fetal bovine serum (BioCell Laboratories, Rancho Dominguez, CA, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin. Culture was passaged twice a week to maintain the cells in exponential growth. The transfection was conducted in 6-well (35-mm in diameter) culture plates. SF-767 cells were seeded with 3 x 10^5 cells in each well 24 hours before the transfection. During the day of transfection, nanoemulsion (50 μl) in 0.2 ml PBS was mixed with p-PLL in the ratios as described above, and incubated at 37ºC. After 1 hour of incubation, 2 μg of plasmid DNA was added and incubated at room temperature for 15 minutes to obtain the complex of nanoemulsion/p-PLL/DNA. Cells were washed with PBS buffer for three times and 1 ml of EMEM (without serum and antibiotics) was added to each well. The nanoemulsion/p-PLL/DNA complex was added to each well and mixed with the medium completely by swirling. The cells were then incubated at 37ºC in 5% CO_2 for 12 hours before 1 ml EMEM medium containing 20% fetal bovine serum was supplemented. The cells were incubated for additional 24 hours. Both the nanoemulsion/p-PLL complex and the naked DNA were used, respectively, as the negative controls. As the positive control, Lipofectamine™ reagent purchased from Invitrogen (Carlsbad, CA, USA) was incubated with the DNA and the transfection experiment was performed at the same condition.

Detection of β-galactosidase by X-Gal staining and enzymatic assay

After 24 hours of transfection incubation, the cells in each well were washed twice with PBS buffer and then fixed with 2 ml fixing solution (2% formaldehyde and 0.2% glutarldehyde in PBS buffer) for 15 minutes at room temperature. After the cells were washed for three times with PBS, 1.5 ml of staining solution (5 mM potassium

ferricyanide, 5 mM potassium ferrocyanide, 1 mM $MgSO_4$, 1 mg/ml X-Gal from 20 mg/ml stock in dimethyl formamide) was added. The cells were incubated at 37ºC in 5% CO_2 for 30 hours before the transfection was evaluated by light microscope. β-Galactosidase activity of cells was determined by β-Galactosidase Enzyme Assay System (Promega, Madison, WI, USA). In brief, the cells from each well of the plate were trypsinized and collected by centrifugation. They were disrupted by mixing with 200 µl of Lysis Buffer and incubating for 30 minutes at room temperature. The protein concentration of the cell lysate was determined by the Bradford method (23). Cell lysate (100 µl) was mixed with 100 µl of ONPG solution in 2X Assay Buffer (1.33 mg/ml) and incubated in water bath at 37ºC for 5 hours. The enzymatic reaction was terminated by adding 300 µl of 1 M sodium carbonate solution. After the reaction mixture was diluted, the absorbance at 420 nm was read in a spectrophotometer (Baush & Lomb Spectronic 2000, Rochester, NY, USA). The enzymatic activity unit was defined in a similar way as described by Kim et al. (21).

Effect of chloroquine on the transfection efficiency

In order to demonstrate the effect of chloroquine, which is a lysomotropic agent, on the transfection efficiency, chloroquine solution in PBS was added to the cell culture (80% confluent) with a final concentration of 100 µM. After 30 minutes of incubation at 37ºC and 5% CO_2, the culture medium was removed and the cells were washed with PBS for three times. Fresh medium was supplemented before the transfection experiment was started. The complex of nanoemulsion (50 µl in 0.2 ml PBS) with p-PLL (p-PLL:triolein = 0.25:1) was used as the carrier for 2 µg of plasmid DNA in the transfection experiment.

After transfection incubation, the cells were trypsinized and collected by centrifugation. The β-galactosidase activity was measured by the method as described above.

Cytotoxicity comparison of nanoemulsion/p-PLL complex and Lipofectamine™

Cellular toxicity of the nanoemulsion system was tested according to the MTT method reported by Mosmann (24). A complex of nanoemulsion (50 µl in 0.2 ml PBS) with p-PLL (p-PLL:triolein = 0.25:1) was prepared in similar way as described above. In a 96-well microplate, SF-767 cells were seeded at 2 x 10^4 cells in each well containing 0.1 ml of EMEM medium. After 24 hours of incubation at 37ºC and in 5% CO_2, the nanoemulsion/p-PLL complex or Lipofectamine™ reagent was added to the cell culture. The amount of nanoemulsion/p-PLL complex or Lipofectamine™ reagent added to the cell culture was determined such that similar transfection efficiency could be obtained based on the transfection experiments. The cell culture grown on EMEM medium without nanoemulsion/p-PLL complex or Lipofectamine reagent was used as the control. Since only living cells are able to cleave the tetrazolium ring to produce dark blue crystals, which can be measured colorimetrically, the viability of cells after additional 1, 2, 3, and 4 days of growth was determined by measuring the ability of the cells to degrade tetrazolium salt MTT. Briefly, 25 µl of MTT solution in PBS buffer (0.5 mg/ml) was added to each well of culture and incubated at the same condition for additional 4 hours. The medium was then removed and 150 µl of DMSO was added to each well and mixed thoroughly until all the dark blue crystals were dissolved. The plate was read on an OPTImax Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at wavelength of 550 nm. The cell viability was calculated and expressed as (OD_{trt}/OD_{ctrl}) x100%, where OD_{trt} is the optical absorbance from the culture treated with

either nanoemulsion/p-PLL complex or Lipofectamine™ reagent, and OD_{ctrl} is the optical absorbance from the culture control.

RESULTS

Preparation of the nanoemulsion and its size distribution

With the proper control of temperature (55ºC) during sonication, the obtained emulsion appeared to be homogeneous. The number weighted mean size of the emulsion particle was 110.2 ± 42.9 nm. Since the emulsification was conduced by metal probe sonication, chelating agent (0.1 mM EDTA) was added to remove the free iron ion before it is dialyzed against PBS overnight (PBS changed every 6 hours). Followed by 10 cycles of size reduction by Emusiflex B3 device (Avestin, Ottawa, Canada), the number weighted mean particle size was reduced to 48.9 ± 19.8 nm. The particle size distribution of the emulsion is shown in Figure 2.2.

The physical stability of the nanoemulsion can be investigated by measuring the change in size distribution of the particles. The size distribution of the nanoemulsion particles was measured at 0, 2, 4, 8, 16 weeks after the preparation and results are shown in Figure 2.3. The size distribution did not change significantly upon storage at room temperature. After 16 weeks of storage at room temperature, the mean size of the emulsion particles was 78.9 ± 14.6 nm, indicating that the nanoemulsion particles are rather stable.

Incorporation of p-PLL into nanoemulsion particles

Since p-PLL is positively charged, the incorporation of p-PLL molecules into nanoemulsion particles will result in a change in surface charge of the particles. The change can be clearly seen in the picture of agarose electrophoresis (Figure 2.4).

Nanoemulsion particles moved to anode since they were negatively charged (Lane 1). The negative surface charge of the nanoemulsion particles was also confirmed by the zeta potential and mobility, which was – 42.28 ± 2.3 mV and –3 ± 0.06 (m/s)/(V/cm), respectively. The incorporation of p-PLL neutralized the surface charge (Lane 2 to Lane 4) and resulted in the retardation of movement in the electric field. When they were incubated with sufficient amount of p-PLL, the surface charge of particles was reversed to be positive and moved towards opposite direction in the electric field (Lane 5). The results indicated that p-PLL could be incorporated into the nanoemulsion particles.

Interaction of p-PLL associated nanoemulsion with DNA

After incorporation of a sufficient amount of p-PLL molecules into the nanoemulsion particles, the complex carried a positive charge and could electrostatically interact with negatively charged DNA molecules. As indicated in Figure 2.5, plasmid DNA (Lane 1) migrated towards the positive anode. When DNA plasmid was incubated with p-PLL (Lane 2), no DNA migration was observed, possibly because DNA molecules were bound by p-PLL and thus the ethidium bromide molecules could not intercalate into the DNA molecules resulting in no fluorescence emission. Lane 3 to Lane 7 showed the change in DNA carrying capability of the complex resulted from different ratios of p-PLL to nanoemulsion (i.e. the p-PLL to triolein ratio). At a high ratio of p-PLL to nanoemulsion, DNA was tightly held by the complex and thus no DNA migration band appeared (Lane 3 to Lane 6). When the ratio of p-PLL to nanoemulsion became sufficiently low (0.0625:1 as the p-PLL to triolein ratio), plasmid DNA started to escape from the complex and a free DNA band (Lane 7) appeared in the agarose gel.

Since the surface charge of the nanoemulsion/p-PLL/DNA complex is very important to transfection, the zeta potential and mobility of these complexes were measured and the results are shown in Figure 2.6 and Figure 2.7. With fixed amount of plasmid DNA (2 μg), the increased amount of p-PLL led to an increase in zeta potential of the particles.

Transfection of glioma cell line SF-767 by the complex of nanoemulsion/p-PLL/DNA

Most of the positively charged nanoemulsion/p-PLL/DNA complexes (with varying ratios of nanoemulsion/p-PLL/DNA) used in the experiments were found to transfect the glioma SF-767 cells, but with different transfection efficiency. The complex containing nanoemulsion and p-PLL (p-PLL:triolein = 0.25:1) and 2 μg DNA, which had a zeta potential of 8.47 ± 1.85 mV and a loading capacity of 1 μg DNA per 0.25 mg of lipid (or per 50 μl of formulation), had the highest transfection efficiency. Its efficiency was comparable to that by Lipofectamine™ reagent (Figure 2.8). Under the microscope, those cells that expressed active β-galactosidase appeared to be blue-green (as dark spots in Figure 2.8) by X-Gal staining and the extent of transfection appeared to be comparable for the nanoemulsion complex (Figure 2.8, C) to that by Lipofectamine™ reagent (Figure 2.8, A). However, Lipofectamine™ reagent appeared to be much more toxic than the nanoemulsion complex, as indicated by the significant difference in cell counts.

Effect of chloroquine on the transfection

Cellular uptake of particles via endocytosis will result in the particles being processed by endosomal-lysosomal pathway. This pathway will lead to the degradation of the carried plasmid DNA and greatly lower the transfection efficiency. Chloroquine is a weakly basic, lysomotropic drug, which will interfere with endosomal acidification and

cause the bursting of endosomes. In order to examine whether the nanoemulsion vector is delivered via endosomal-lysosomal pathway, cells were treated with 200 μM of chloroquine at 37ºC for 30 minutes before the transfection procedures was conducted. The β-galactosidase activity of the cells with or without the treatment of chloroquine was shown in Figure 2.9, based on two quantities of the nanoemulsion/p-PLL/DNA complexes. The treatment of the cells by chloroquine solution greatly increased the transfection efficiency and the effect was obvious at both complex quantities. This result suggested that endocytosis be the main mechanism of the cellular uptake of the nanoemulsion/p-PLL/DNA complex by glioma cells.

Cellular toxicity evaluation of nanoemulsion/p-PLL complex

Cellular toxicity is one of the main concerns in the development of gene delivery system. It has been commonly shown that positively charged gene delivery systems, such as cationic liposomes, are cytotoxic. Other cationic polymers, e.g. poly-L-lysine, hydrophobized poly-L-lysine, were also reported to be cytotoxic (21, 25). Since the nanoemulsion particles in this research were negatively charged, the incorporation of positively charged and lipidized poly-L-lysine resulted in a neutralization of the charges on the surface. The cytotoxicity of the nanoemulsion/p-PLL complex, in comparison with that of the commercial Lipofectamine™ reagent, was shown in Figure 2.10. The cytotoxicity was evaluated based on the relative viability of cells grown on the EMEM medium with and without the delivery systems. Four days after transfection, cell culture supplemented with the nanoemulsion/p-PLL complex had 75% cellular viability while that supplemented by Lipofectamine™ reagent (which is cationic liposome) had only 24% cell viability.

DISCUSSION

Effective gene transfection depends on the ability of the carrier system to deliver gene to and transfect in specific cells with high transfection efficiency and low cytotoxicity. Many synthetic carrier systems have been investigated with certain success and most of them belong to the category of cationic liposomes. However, DNA/liposome complex is, in general, cytotoxic and therefore, less cytotoxic but efficient gene carriers have been investigated. One such approach is to develop gene carriers that somewhat mimic the natural carriers in human body. Kim et al. (21, 22) developed a novel terplex system based on the natural low-density lipoprotein associated with hydrophobized poly-L-lysine. The system is capable of condensing DNA and subsequently transfecting cells. Hara et al. (26) described the use of reconstituted chylomicrons remnants (RCR) as non-viral vectors for gene delivery. DNA was complexed with cationic lipid and solubilized in the core of these RCR particles. Both of these lipoprotein-based systems appeared to offer advantages over the conventional cationic liposome systems. Recently, our laboratory has attempted to develop an artificial lipoprotein system for controlled drug delivery. The artificial lipoprotein, similar to natural lipoprotein, consists of phospholipid nanoemulsion particles with functional proteins attached on the particle surfaces. A schematic drawing of the artificial lipoprotein system can be seen in Figure 2.11, in comparison with natural human lipoproteins. Based on our earlier work in lipoprotein-resembling nanoemulsion for controlled delivery of an anti-tumor cholesteryl carborane compound (27), this paper describes a new attempt to develop an artificial lipoprotein system that has poly-L-lysine attached on the particle surfaces for the purpose of gene

delivery. The cytotoxicity of such a system has been specifically investigated in comparison with commercial Lipofectamine™ reagent.

The lipoprotein-resembling particles were made of commercially available lipids and lipidized poly-L-lysine. The lipidization of poly-L-lysine was achieved through N-alkylation of the free ε-amino groups with palmitoyl chloride and confirmed via proton NMR (data not shown). The reaction condition was controlled to only lipidize about 25% of the lysine residues preserving sufficient amount of free ε-amino groups for maintaining the ability of poly-L-lysine to condense DNA. The nanoemulsion particles carried negative surface charge as shown by agarose gel electrophoresis in Figure 2.4. When nanoemulsion particles are combined with unlipidized poly-L-lysine, immediate precipitation was observed indicating the formation of large aggregates due to charge neutralization. When appropriate amount of p-PLL was incubated with the particles, no precipitation or change in the turbidity was observed. These results indicate that with lipidized poly-L-lysine the interaction was not merely through electrostatic interaction, but also through hydrophobic interaction between the palmitoyl chains of p-PLL and the phospholipid of nanoemulsion particles. The charge of the nanoemulsion particles became reversed when sufficient amount of p-PLL was added (Figure 2.4).

The surface charge of the complex formed by nanoemulstion and p-PLL depends on their relative ratio. In order to carry DNA molecules, which are negatively charged, the carrier needs to be positive. On the other hand, the surface charge of the complex after DNA is incorporated is also critical. Since the cell surface is negatively charged, a positively charged nanoemulsion/p-PLL/DNA complex is essential for successful transfection. The surface charge of these particles can be monitored by agarose gel

electrophoresis qualitatively or by zeta potential and mobility measurement quantitatively (Figure 2.5, 2.6 and 2.7). Our studies showed that most of the positively charged complexes formed by varying the ratios of nanoemulsion, p-PLL and DNA can transfect the glioma cells to a certain extent. The complex containing nanoemulsion, p-PLL and DNA with a zeta potential of 8.47 ± 1.85 mV achieved the highest transfection efficiency indicating the proper charge balance among these components was important for transfection. This observation is consistent with that reported by Kim et al. (21) when the terplex carrier system involving natural LDL was employed for gene delivery.

Chloroquine has been widely used to investigate the cellular uptake mechanism (28-29). It will interact with endosome inside the cell. A positive correlation of chloroquine level with the transfection efficiency indicates that the DNA is taken up through endocytosis and, furthermore, the endosomal-lysosomal pathway. Through chloroquine treatment, we have shown that the endocytosis appears the major cellular uptake pathway for the lipoprotein-resembling gene carrier.

Cytotoxicity is an important consideration for developing novel gene delivery systems. Using new gene carriers that mimic the nature substance such as human lipoproteins, we can significantly reduce the cytotoxicity associated with the delivery systems. It is known that poly-L-lysine is very toxic to cells (Morgan, 1989). Its complex with the phospholipid nanoemulsion particles and plasmid DNA, however, has low cytotoxicity as indicated by our experiments in comparison with the Lipofectamine™ system. The nature of phospholipids, the neutralization of the positive charge of poly-L-lysine and the proper balance among nanoemulsion, p-PLL and DNA apparently contribute to the reduced cytotoxicity for this new gene delivery system.

In conclusion, a novel artificial lipoprotein system has been developed for in vitro gene transfection to tumor cells. The system mimics natural lipoprotein in composition but contains lipidized poly-L-lysine (instead of surface protein) to carry genetic materials. Such a system can be conveniently formulated from natural lipids, with the ability to control the size and surface charge. With proper ratios among its components, the new gene delivery system shows similar transfection efficiency but a lower cytotoxicity compared with the commercial Lipofectamine™ gene transfection system, making it especially useful as an alternative to these commercial gene transfection systems.

FIGURE LEGENDS:

Figure 2.1. The diagram of pSV-b-galactosidase vector

Figure 2.2. Size distribution of nanoemulsion particles

Figure 2.3. The mean size change of nanoemulsion particles upon storage at room temperature

Figure 2.4. Agarose gel electrophoresis of nanoemulsion particles and their complexes with p-PLL stained with Nile Red (Lane 1: nanoemulsion; Lane2 to Lane 5 were the complex of nanoemulsion and p-PLL with the ratio of p-PLL to triolein to be 0.125:1, 0.25:1, 0.5:1 and 1:1, respectively)

Figure 2.5. Agarose gel electrophoresis of the complex of nanoemulsion and p-PLL with plasmid DNA stained with ethidium bromide (Lane 1 and Lane 8: Pure DNA; Lane 2: DNA/p-PLL; Lane 3 to Lane 7 were complexes of nanoemulsion with different amount of p-PLL and DNA. The ratio of p-PLL to triolein was 1:1, 1:0.5, 1:0.25, 1:0.125, and 1:0.0625, respectively).

Figure 2.6. Zeta potential of the nanoemulsion particles and their complexes with different amount of p-PLL and DNA (2 μg)

Figure 2.7. The mobility of the nanoemulsion particles and their complexes with different amounts of p-PLL and DNA (2 μg)

Figure 2.8. X-Gal staining of glioma cells (A: Cells transfected using Lipofectamine™ reagent; B: Control; C: Cells transfected using nanoemulsion/p-PLL/DNA complex)

Figure 2.9. The effect of chloroquine on transfection by the nanoemulsion gene delivery system (white bar – untreated with chloroquine; gray bar – treated with chloroquine)

Figure 2.10. Cytotoxicity comparison between nanoemulsion/p-PLL and Lipofectamine: control (♦, top), nanoemulsion/p-PLL (■, middle) and Lipofectamine (▲, bottom).

Figure 2.11. (A) Artificial lipoproteins (20-100 nm) and (B) natural human lipoproteins (those in 20-100 nm range)

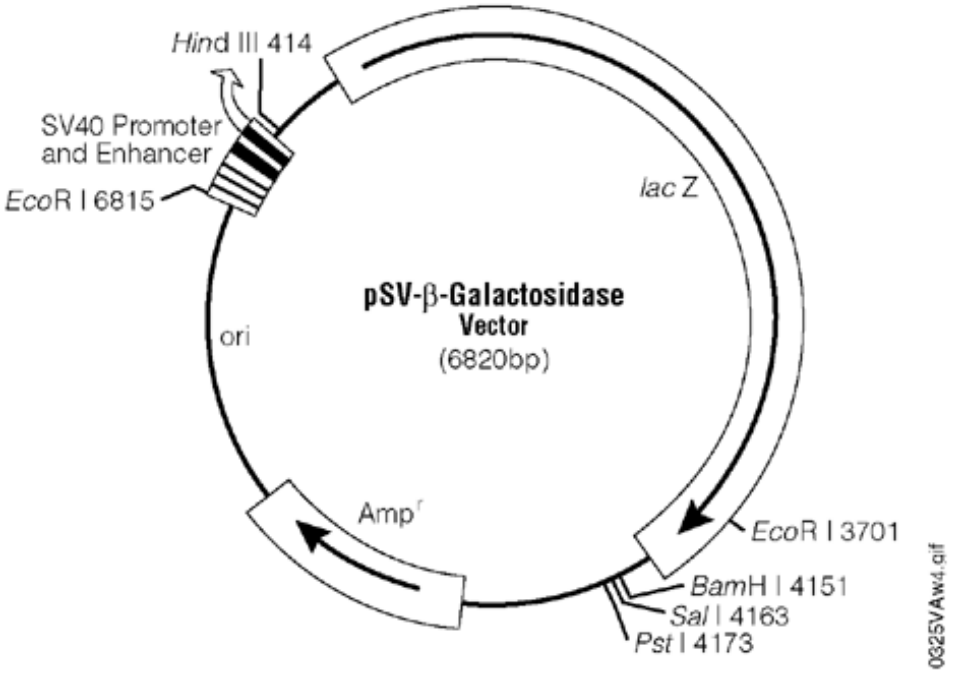
Hind III 414
SV40 Promoter
and Enhancer
EcoR I 6815
lac Z
pSV-β-Galactosidase
Vector
(6820bp)
ori
Amp^r
EcoR I 3701
BamH I 4151
Sal I 4163
Pst I 4173
0325VAw4.gif

Figure 2.1

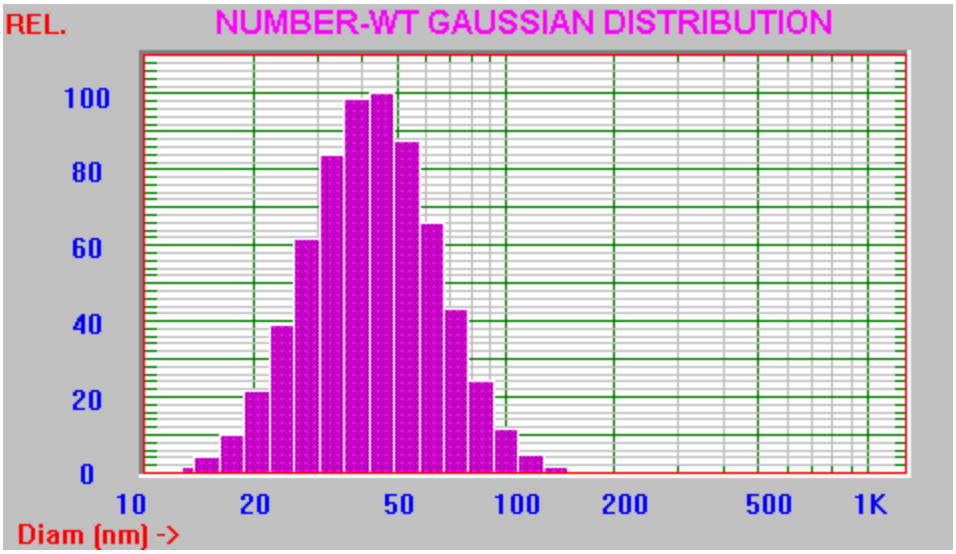
REL.
NUMBER-WT GAUSSIAN DISTRIBUTION
100
80
60
40
20
0
10
20
50
100
200
500
1K
Diam (nm) ->

Figure 2.2

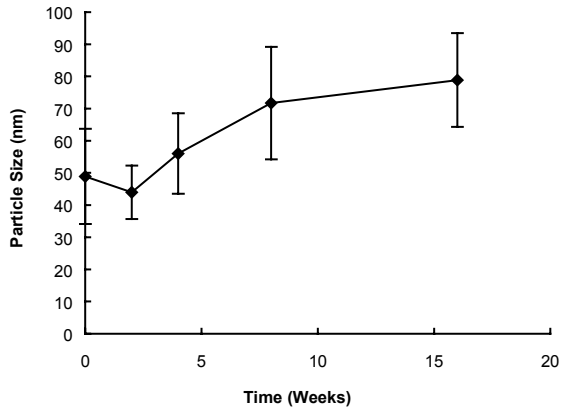
100
90
80
70
60
50
40
30
20
10
0
Particle Size (nm)
0
5
10
15
20
Time (Weeks)

Figure 2.3

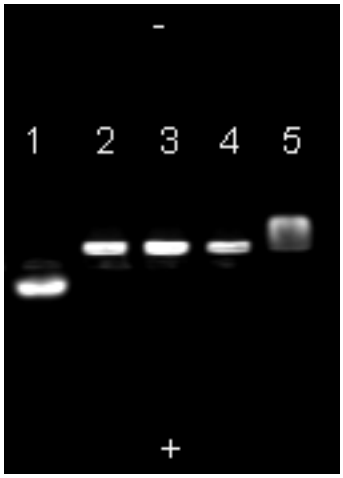

Figure 2.4

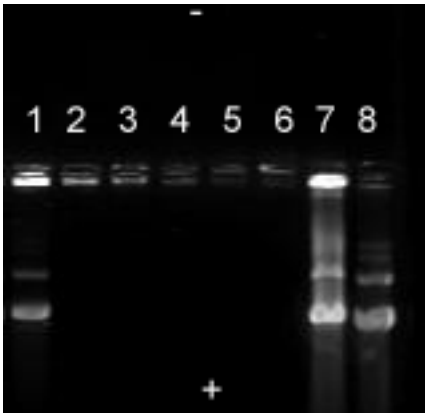

Figure 2.5

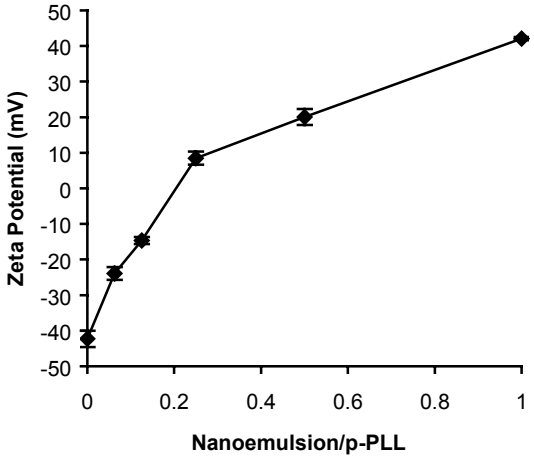
Zeta Potential (mV)
50
40
30
20
10
0
-10
-20
-30
-40
-50
0
0.2
0.4
0.6
0.8
1
Nanoemulsion/p-PLL

Figure 2.6

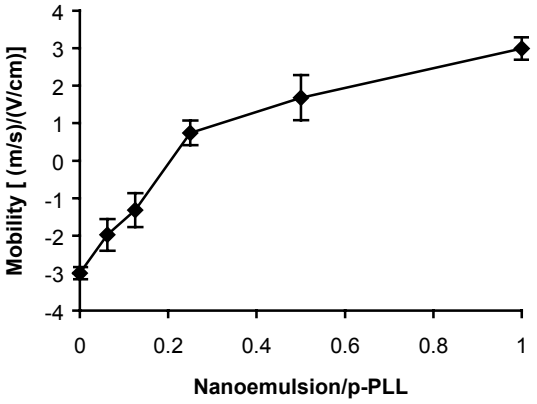

Figure 2.7

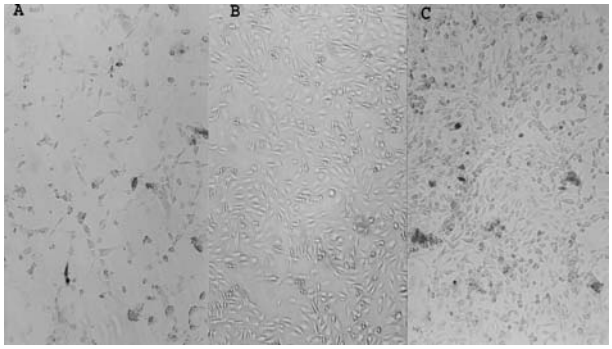

Figure 2.8

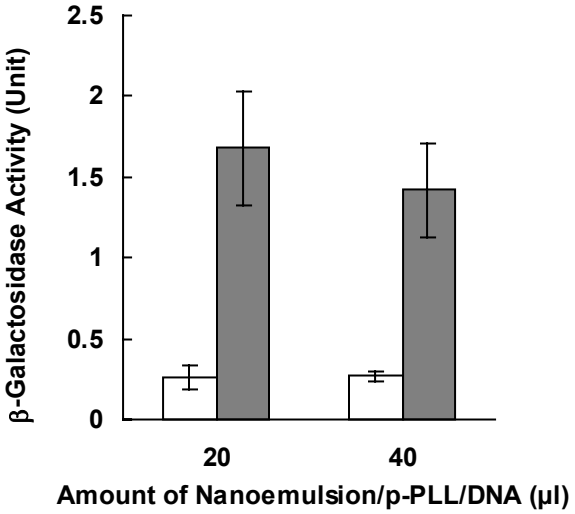
2.5
2
1.5
1
0.5
0
β-Galactosidase Activity (Unit)
20
40
Amount of Nanoemulsion/p-PLL/DNA (µl)

Figure 2.9

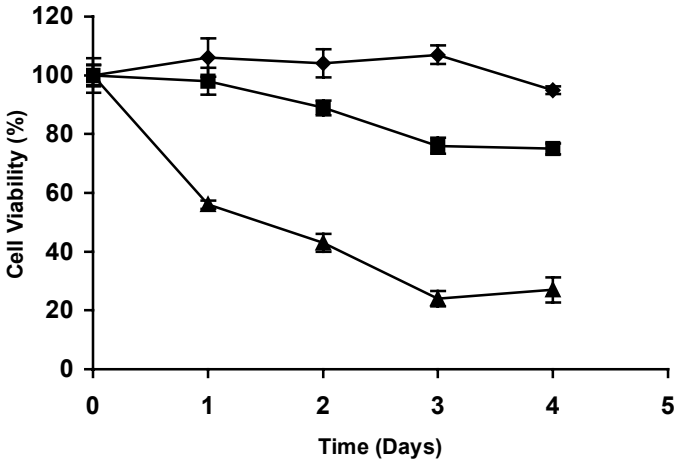

Figure 2.10

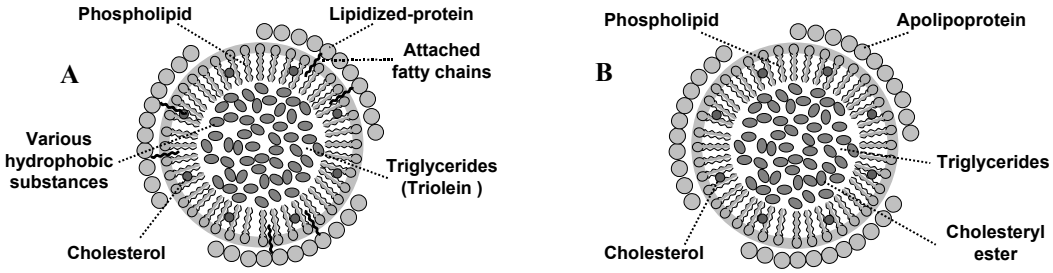
A
Phospholipid
Lipidized-protein
Attached
fatty chains
Various
hydrophobic
substances
Triglycerides
(Triolein)
Cholesterol
B
Phospholipid
Apolipoprotein
Triglycerides
Cholesteryl
ester
Cholesterol

Figure 2.11

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CHAPTER 3

IN VITRO CELLULAR UPTAKE OF A NEW CHOLESTERYL CARBORANE ESTER COMPOUND BY HUMAN NORMAL NEURON CELLS AND GLIOMA CELLS[1]

[1]Guangliang Pan, Svein Øie, D. Robert Lu. Submitted to Journal of Pharmaceutical Sciences (2003).

ABSTRACT

The cellular uptake of a new cholesteryl carborane ester compound, cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH), by one human normal neuron cell line, HCN-1a, and two human glioma cell lines, glioblastoma multiforme SF-763 and SF-767, was evaluated. BCH, which is an extremely hydrophobic compound, was formulated into liposomes and incubated with the cells at 37 ^{o}C and 5% CO_2 for 16 hours. The amount of BCH uptake by the cells was measured by high performance liquid chromatography (HPLC). In addition, BCH uptake by tumor cells was examined in the presence and absence of lipoprotein in the culture medium. It was found that the amount of BCH taken in by the glioma cell lines was much more (up to 14 times) than that by the normal neuron cell line. The cellular uptake of BCH by SF-763 and SF-767 cells after 16 hours of incubation was 283.3 ± 38.9 μg boron/g cells and 264.0 ± 36.5 μg boron/g cells, respectively. When the normal serum was replaced by lipoprotein deficient serum, the cellular uptake of BCH by the tumor cells was reduced. In conclusion, the cellular uptake of BCH by glioma cells was about 14 times higher than by normal neuron cells. The uptake in glioma cells was up to 10 times higher than that required for successful cancer treatment. Lipoprotein appeared to play an important role in the BCH uptake by glioma cells.

KEYWORDS: Cholesteryl conjugate; liposomes; boron; tumor; cellular uptake; lipoprotein

INTRODUCTION

Glioblastoma multiforme brain tumors, similar to many other type of malignant tumors, are characterized by very aggressive cell growth and tend to be resistant to conventional methods of treatment, including surgical resection of tumors, radiation therapy, chemotherapy and combinations of these treatments (1). Among several approaches that may enhance the efficacy of chemotherapy, targeted drug delivery that produces a high concentration of therapeutic compounds in tumor cells and relatively low concentrations in neighboring normal cells remains attractive. Recently, Maletinska, et al. reported that seven human glioblastoma multiforme cell lines had very high numbers of low-density lipoprotein (LDL) receptors per cell, indicating that the LDL pathway may provide a method for selectively targeting compounds to glioblastoma multiforme cells (2).

Rapidly dividing cells, such as malignant tumor cells, have high cholesterol requirements because cholesterol is utilized to form the new cell membrane. Cells can obtain cholesterol either by taking up plasma LDL (containing about 1500 cholesteryl esters per LDL particle) via receptor-mediated endocytosis or by *de novo* synthesis. However, it is known that up to 90% of cholesterol is obtained from receptor-mediated endocytosis involving the degradation of LDL (3, 4). Many types of cancer cells have been reported to take up more LDL than the corresponding normal cells in order to absorb more cholesterol (5-10). These literature data suggest the potential of utilizing cholesterol-drug conjugates to interact with lipoproteins and to follow through the LDL pathway for targeted drug delivery.

Recently, our laboratory has designed and synthesized a cholesteryl carborane ester compound mimicking native cholesteryl esters for targeted drug delivery to tumor cells expressing a high degree of LDL receptors. The compound, cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH), is a mimic of native cholesteryl ester containing carborane as an anti-tumor unit for boron neutron capture therapy (BNCT). Unlike the utilization of other prodrugs, BNCT does not require the release of the antitumor unit in cells. The boron-rich carborane unit, however, must be delivered to the tumor cell preferentially, compared to surrounding normal cells (11). Similar to the native cholesteryl esters, BCH is extremely hydrophobic requiring a suitable pharmaceutical carrier. Since liposomes have been widely studied for the delivery of various boron compounds (12, 13), we have formulated the compound in liposomes for cell culture studies.

The objectives of the present cell culture study were to determine: 1) whether the tumor cells could take up a sufficient amount of BCH; 2) whether the tumor cells took up more BCH than normal brain cells; 3) whether the uptake process is related to the presence of lipoproteins in culture medium. Correspondingly, the cellular uptake of BCH in two human glioblastoma multiforme cell lines (SF-763 and SF-767) was evaluated. These two tumor cell lines were known to have elevated level of LDL receptors on the cell surface (2). A human normal brain neuron cell line (HCN-1a) was also examined to compare its uptake with that by the tumor cell lines. In addition, the effect on BCH uptake by tumor cells with a depletion of lipoprotein in culture medium was examined.

MATERIALS AND METHODS

Materials

BCH (Figure 3.1) was synthesized in our laboratory as described earlier (14). The phospholipid DL-α-dipalmitoyl phosphatidylcholine (DPPC) and cholesterol were purchased from Sigma Chemicals (St. Louis, MO). Human glioblastoma multiforme (glioma) 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). Human neuron cell line HCN-1a was purchased from American Type Culture Collection (Manassas, VA). Eagle's Minimum Essential Media (EMEM), Dulbecco's phosphate-buffered saline (PBS), trypsin-EDTA, and gentamicin solution were obtained from Fisher Scientific Products (Suwanee, GA). Fetal bovine serum (FBS) and lipoprotein deficient serum (LPDS) were obtained from BioCell Laboratories (Rancho Dominguez, CA). Analytical grade chloroform, methanol, isopropanol, and other chemicals were obtained from J.T. Baker (Phillipsburg, NJ).

Preparation of BCH in liposome formulation

The BCH liposomal formulation was prepared by the solvent evaporation method. Lipids and BCH were mixed in organic solvent (methanol/chloroform in a ratio of 2:1) in a round bottom flask and evaporated to a thin film. Liposomes were prepared by hydration of the lipid film with phosphate buffered saline (PBS) solution at 55°C in a water bath shaken at 120 rpm. Specifically, 52 mg DPPC, 10 mg cholesterol and 2.5 mg BCH were dissolved in approximately 6 ml organic solvent in a round bottom flask. The solvent was evaporated under vacuum in a rotary evaporator (Buchi Rotavap, RE121, Brinkmann Instruments, NY). The dried lipids on the flask wall were hydrated with 10 ml

PBS that had been heated to 55°C. The flask was shaken mechanically for 5 hours at 55°C. Size reduction of the multilamellar vesicles was carried out using the Emulsiflex B3 device (Avestin, Ontario, Canada) and running the vesicles for 10 cycles. The size distribution of the resulting small liposomes was measured using a Nicomp submicron particle sizer (Model 370, Nicomp, CA).

Cell culture studies

Human glioma cells (SF-763 and SF-767) were grown in 150 cm^2 plastic cell culture flasks containing 26 ml EMEM with 10% FBS, 50 U/ml penicillin, and 50 μg/ml gentamicin. After three passages from the stock culture, the flasks were seeded with approximately 2 x 10^6 cells placed in a humidified 5% CO_2 incubator at 37°C for 48 hours prior to incubation with BCH liposomal formulation. Human neuron cells (HCN-1a) were grown in the same condition except that one month was required because they grew more slowly. Each flask of cells was then incubated with a specified quantity of BCH in the liposome formulation. After a pre-determined incubation period, the culture medium was removed; the cells were rinsed 3 times with PBS, then harvested with trypsin-EDTA and counted under a microscope. The cells were centrifuged at 3500 rpm for 10 minutes. The cell pellets were weighed and stored at –20 °C until analysis.

Cellular uptake of BCH in liposome formulation by HCN-1a, SF-763 and SF-767 cell lines

To compare the BCH uptake by the normal cell line with the tumor cell lines, HCN-1a, SF-763 and SF-767 cells were prepared and grown as described above. One ml of BCH liposomal formulation (154.5 μg BCH/ml, equal to 30 μg boron/ml) was added

and incubated with each cell line for 16 hours. Cells were harvested and the BCH content in the cells was determined by HPLC.

The effect of lipoprotein on cellular uptake of BCH in liposome formulation by SF-763 and SF-767 cell lines

To determine the effect of lipoprotein on BCH uptake, 5%, 7%, 8%, 9%, and 10% of delipidized human serum was supplemented in the culture medium to replace part of the human normal serum to make up a total of 10 % serum. Cells were grown in 150 cm^2 flasks at the same condition and the amount of BCH uptake by the tumor cell lines was measured by HPLC.

Sample analyses

All the samples were analyzed by a high-performance liquid chromatography (HPLC) method which was specific for BCH and the details of the HPLC method are described elsewhere. Briefly, samples were prepared by sonication, evaporation to dryness and reconstitution with 1 ml mobile phase (50:50 methanol-isopropanol). Cholesteryl heptadecanoate was used as the internal standard. The analysis was performed using a Waters model 2690 separations module equipped with a column heater and Hewlett-Packard ZORBAX Stable Bond C-18 (5μm, 150 x 4.6mm) analytical column. Since the cell division rates between normal neuron cells and the glioma cells were different, the cellular BCH uptake was calculated with the consideration of cell number counts and expressed as μg boron/g cells.

RESULTS

Characterization of BCH liposomal formulation

After size reduction using Emulsiflex, the size distribution of the resulting liposomes was examined by photon correlation spectroscopy and a number-weighted bimodal distribution was observed. Approximately 90 % of the vesicles had a mean diameter of approximately 51 nm ± 7.3 nm and the remaining 10% of the vesicles had a mean diameter of 165.1 ± 25.8 nm. Based on the HPLC analysis, the average BCH content was 154.5 μg/ml (equal to 30 μg boron/ml) and entrapment efficiency was 61.8 %.

BCH uptake by three different cell lines

The addition of 1 ml BCH liposomal formulation to 26 ml culture medium resulted in a final concentration of 1.2 μg boron/ml in each medium. After 16 hours of incubation, the amount of BCH uptake by HCN-1a, SF-763, and SF-767 cells is shown in Table I. The amount of BCH uptake by the two tumor cell lines was much more (up to 14 times more) than that by the normal neuron cell line ($p<0.05$). It should be noted that there was a small difference in total surface area per gram of cells between the tumor cells and the normal cells. The ratio of the total surface area per gram of tumor cells over that of normal cells was equal to 1.32. Since the cellular BCH uptake of tumor cells and normal cells was significantly distinct, the small difference in surface area appeared not to be a major consideration.

Effect of lipoprotein on the cellular uptake of BCH in liposome formulation by the glioma cell lines

Although the inhibition of cellular uptake BCH in liposome formulation by the presence of anti-LDL receptor antibody suggested the involvement of LDL receptor-mediated pathway, we were not clear yet how the BCH in liposome formulation interacted with this pathway. One possible interaction might be the transfer of the BCH in the liposome formulation to the LDL particles by contact or fusion. This interaction could be examined by the depletion of lipoprotein from the serum, which was supplemented to the cell culture medium. During the uptake experiment of BCH in liposome formulation, a combination of normal human serum and lipoprotein deficient serum was supplemented in the culture medium, with a total serum to be 10% in the medium. The uptake of BCH in liposome formulation by the glioma cells was determined and the result is shown in Figure 3.2. The more the lipoproteins depleted from the culture medium, the less BCH was taken up the cells. It appeared that lipoprotein is a very important factor for the cellular uptake of BCH in the liposome formulation.

DISCUSSION

Effective chemotherapy for most malignant tumors requires preferential accumulation and retention of the therapeutic compounds in tumor cells. Similarly, BNCT requires preferential accumulation and retention of boron compounds in tumor cells prior to the neutron irradiation. Laster et al. constructed boronated LDL by replacing the cholesterol ester core with carborane carboxylic acid esters of fatty alcohols (4). Their studies in cell culture indicated that resulting boron concentrations in tumor cells was 10 times higher than that required for BNCT and the amount of uptake was consistent with a

receptor-mediated binding mechanism. The boron concentration in tumor cells, about 240 μg boron/g cells, was significantly higher than that obtained with any other boron compound previously evaluated for clinical applications (4).

To develop a cholesterol-based drug targeting approach by synthesizing new cholesteryl carborane ester compounds to mimic the native cholesteryl esters present in the hydrophobic core of LDL, our laboratory has recently synthesized a cholesteryl carborane ester, cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH). Our results from the present experiments indicate that the cholesteryl carborane ester, carried by liposomes, resulted in more than adequate cellular uptake of BCH by the tumor cell lines. The uptake by the normal neuron cell line was, however, much lower (Table I) indicating the cellular uptake appeared to be related to the rapidly growing rate of the tumor cells. For the tumor cells, the boron uptake was significantly higher than that required for successful BNCT and was similar to that reported by Laster et al. when carborane carboxylic acid esters of fatty alcohols were used to boronate LDL and CHO and V-79 Chinese hamster cells were used to examine the boron uptake (4). It can be seen in Table 3.1, for the SF-763 cells, the boron uptake reached up to 283.3 μg boron/g cells, about 11 times higher than the required boron level (≥ 20-25 μg boron per gram cells) for successful BNCT. For SF-767 cells, the boron uptake reached 264 μg boron/g cells, about 10 times higher than the required boron level. It has been noticed that the degree of cell confluence affected BCH cellular uptake in tumor cells. Since it was very difficult to precisely control the cell confluence, the batch-to-batch difference in the amount of BCH uptake can be seen throughout our experiments. However, high BCH uptake in the tumor cells was consistently observed with a reasonable variation.

The uptake, in both tumor cell lines, was dependent on the BCH concentration in the incubation medium and the relationship between concentration and uptake appeared to be linear within this concentration range. The studies, however, were limited as to the maximum concentration of BCH in the culture medium because of the low aqueous solubility of BCH and the potential toxicity from the high concentration of lipids with a larger amount of the liposomal formulation. A high concentration of lipids in the incubation medium may result in toxic effects as indicated in our earlier rat cell culture studies (15). Our result also showed that the BCH uptake by SF-767 tumor cells in the presence of lipoprotein was about 4 times higher than that in the absence of lipoprotein. This suggested that the BCH uptake process involved lipoprotein. The amount of BCH taken up by the human glioma cells was about 6-7 % of the total amount, indicating a significant amount of BCH (in liposomal formulation) remained in the culture medium and was washed out before the cells were collected.

In our earlier study involving 9L rat glioma cell lines, it was observed that BCH, formulated in conventional and PEG liposomes, produced marginally sufficient levels of boron in the rat tumor cells (about 50 μg boron/g of cells) (15). When the cellular BCH uptake in the two human glioma cell lines was compared with the cellular uptake from our previous studies conducted on the 9L rat glioma cell line with the same experimental conditions, the cellular uptake in the two human glioma cell lines was much higher. This disparity in uptake is obviously due to the differences between the rat glioma cell line and the human glioma cell lines, possibly in the expression and activity of the LDL receptors.

When each glioma cell line was exposed to the BCH liposomal formulation for different incubation times the uptake at 2 hours was equal to approximately 40% of the

uptake at 16 hour for the SF-763 cells and approximately 30 % of the uptake at 16 hour for the SF-767 cells. The results indicated that the uptake for both cell lines was related to the incubation time. The uptake at 48 hours of incubation was virtually the same as at 16 hours indicating that 16 hours of incubation would be a suitable experimental condition for the subsequent cell culture studies.

In addition to the requirement for the cells to obtain adequate amount of BCH, it is also essential for BCH to remain in the cells for a sufficient time period to allow the subsequent neutron radiation. The results from our retention experiment indicated that a significant amount of BCH taken up in the human glioma cells was retained in the cells after the subsequent 24-hour incubation without the presence of BCH formulation.

In conclusion, BCH formulated in liposomes appears to be effectively taken up by and retained in SF-763 and SF-767 human glioma cells. The cellular concentration was about 10 to 11 times higher than that required for successful cancer treatment. The BCH uptake in human normal neuron cells, however, was significantly lower indicating the cellular uptake was related to the rapidly-growing rate of the tumor cells. The uptake process also appeared related to the lipoprotein presence in the culture medium. At present, the mechanism of cellular uptake of this type of cholesteryl carborane ester compounds in glioma tumor cells is not clear. Further studies are needed to provide detailed evidence to illustrate the uptake mechanism.

Table 3.1. Cellular uptake of BCH by different cell lines

Cell line	Cellular BCH uptake (μg boron/g cell)
Normal neuron cell, HCN-1a	19.9 ± 2.9
Glioma tumor cell, SF-763	240.1 ± 22.3
Glioma tumor cell, SF-767	283.4 ± 48.1

FIGURE LEGEND

Figure 3.1. The chemical structure of cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH).

Figure 3.2. The effect of lipoprotein on the cellular uptake of BCH in liposome formulation by glioma cell line SF-763 and SF-767 (Gray bar – with FBS; White bar – without LPDS).

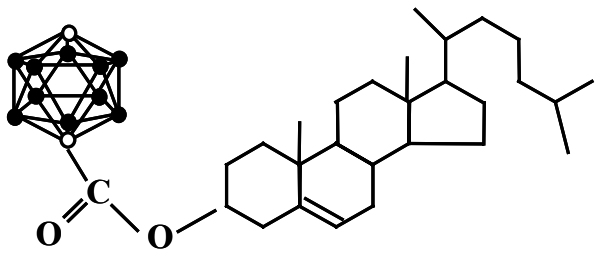

Figure 3.1

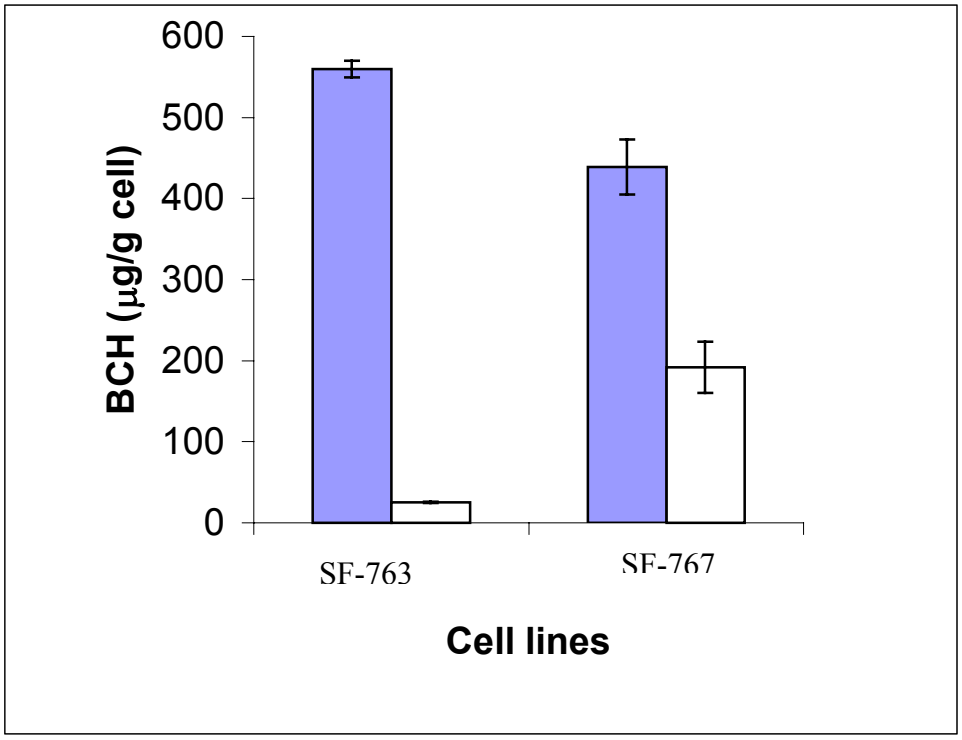

Figure 3.2

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CHAPTER 4

UPTAKE OF CARBORANE DERIVATIVE OF CHOLESTERYL ESTER BY GLIOMA CANCER CELLS IS MEDIATED THROUGH LDL RECEPTORS[1]

[1]Guangliang Pan, Svein Øie, D. Robert Lu. To be submitted to Biochimica et Biophysica Acta.

ABSTRACT

Purpose. To elucidate the mechanism of cellular uptake of BCH in a liposomal formulation by the glioma cell lines SF-763 and SF-767. **Methods**. BCH, an extremely hydrophobic cholesteryl carborane ester, was synthesized in our laboratory. It was formulated into liposomes by solvent evaporation and hydration method. After the cells were grown to about 80% confluence, the BCH formulation was added to the culture in the presence of different amount of monoclonal anti-LDL receptor antibody. The cells were incubated at 37 ^{o}C and 5% CO_2 for 4 hours. After repeated wash with PBS, the cells were trypsinized and harvested by centrifugation. The amount of BCH in the cells was measured by high performance liquid chromatography (HPLC). At the same time, the effect of lipoprotein deficient serum (LPDS) on the cellular uptake of BCH in liposome formulation was examined by substituting different amount of human normal serum in the medium with LPDS. The effect of divalent calcium ion and low temperature on uptake of BCH in liposome formulation was also investigated. **Results**. The cellular uptake of BCH in liposome formulation by glioma cell lines SF-763 and SF-767 was inhibited by monoclonal anti-LDL receptor antibody. The inhibition effect reached the maximum when the concentration of monoclonal anti-LDL receptor antibody was 0.1 mg/ml. The BCH uptake by both cell lines was positively correlated with the amount of normal human serum. The BCH uptake by glioma cells was significantly reduced by the complete substitution of normal human serum by LPDS. The cellular uptake of BCH in liposome formulation in the presence of calcium ion is about 2.7 times as much as that in the absence of calcium ion for SF-763. For the SF-767, the uptake of BCH in liposome formulation in the presence of calcium ion is about 1.5 times as much as that in the

absence of calcium ion. At 4^{o}C, only small amount of BCH was taken up by both glioma cell lines. **Conclusions**. The cellular uptake of BCH in liposomal formulation by both glioma cell lines was inhibited by monoclonal anti-LDL receptor antibody and reduced by the substitution of normal human serum with lipoprotein deficient serum. Calcium ion had a positive effect on the uptake of BCH in liposome formulation by both glioma cell lines. At low temperature, no significant BCH uptake occurred. These results suggested that LDL receptors play a very important role in the cellular uptake of BCH in liposome formulation by the glioma cell lines SF-763 and SF-767.

Keywords: BCH, cellular uptake, glioblastoma, LDL receptor, monoclonal anti-LDL receptor antibody, LPDS, calcium ion, low temperature binding

Abbreviations: BCH, cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate; FBS, fetal bovine serum; ICP, inductively coupled plasma; LPDS, lipoprotein deficient serum; BNCT, boron neutron capture therapy; DPPC, DL-α-dipalmitoyl phosphatidylcholine; EMEM, Eagle's Minimum Essential Medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel

INTRODUCTION

Cellular targeting requires site-specific drug deliveries that targets to a specific cell type within a tissue. Two important steps that are involved in this process are the recognition and interaction of drugs or drug carriers with specific target cells and delivery of the therapeutics into the target cells with reduced uptake by the non-target cells (Poste, 1983). Such a process is either cell-surface receptor mediated by ligand-receptor interaction or cell-surface epitope mediated by antigen-antibody interaction. The cellular targeting is thus largely dependent on the specificity of the target cell surface proteins. It is known that many cancer cells over-express certain types of cell surface receptors, including transferrin receptor (Plant et. al. 1989; Huwyler et. al. 1996), folate receptor (Garin-Chesa et. al. 1993; Ross et. al. 1994; Reddy et. al. 1998), and LDL receptor (Vitols et. al, 1984, 1985, 1992; Rudling et. al. 1990; Jung-Testas et. al, 1992; Maletinska et. al., 2000), to meet the increased cell proliferation and growth requirement. One strategy to develop targeted drug delivery technique for cancer therapy is to take advantage of these over-expressed cell surface receptors. By incorporating the corresponding ligands for the cancer cells surface receptors, anticancer drugs can be specifically delivered to the cancer site.

Among various ligands for the over-expressed receptors in cancer cells, low-density lipoprotein (LDL) is unique. It is an endogenous lipoprotein complex, which has an approximate size of 22-25 nm in diameter and serves as a natural carrier of cholesterol and cholesterol ester in blood circulation. Composed of about 1500 molecules of cholesteryl esters, the core of one LDL particle is surrounded by about 500 molecules of cholesterol and 800 molecules of phospholipids in its polar shell. Apolipoprotein,

which contains unique binding site for LDL receptor, is incorporated into the particles by hydrophobic interaction (Havel et al., 1980). For normal cell growth, the cholesterol level is balanced by LDL receptor-mediated uptake and subcellular degradation of LDL. However, because of the increased growth and proliferation of cancer cells, high turnover of cellular cholesterol for membrane growth and metabolism is required. As a result, over-expressed LDL receptor level has been found in many cancer cells (Vitols et. al, 1984, 1985, 1992; Rudling et. al. 1990; Jung-Testas et. al, 1992; Maletinska et. al., 2000).

Owing to its natural carrier capability and targeting specificity, LDL has been extensively studied as anti-cancer carriers for hydrophobic drugs or prodrugs (Firestone, 1994; Kader and Pater, 2002, Sarkar and Lu, 2002). Recently, our laboratory has designed and synthesized an anti-cancer cholesterol-carborane conjugate, cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH), to mimic the structure of cholesteryl esters located in the hydrophobic core of LDL (Ji and Lu, 2002). Because these anti-cancer cholesteryl ester mimics have similar structures of natural cholesteryl esters, they have the potential to interact with lipoproteins and to follow through the LDL pathway for targeted drug delivery. The preformulation of BCH and its in vitro cellular uptake by two cancer cell lines, Glioblastoma multiforme SF-763 and SF-767, have been previously studied (Shawer et al., 2002; Peacock et al., 2003). The results indicated that the cellular uptake of BCH by glioma cells was about 14 times higher than by normal neuron cells and the uptake in glioma cells was up to 10 times higher than that required for successful cancer treatment. However, the mechanism of BCH uptake in cancer cells and the involvement of LDL receptor-mediated process are not clear.

In this research, we seek to elucidate the mechanism of BCH uptake by glioma cells. In order to determine whether the BCH uptake is LDL receptor mediated, BCH uptake by the glioma cells in the presence of monoclonal anti-LDL receptor antibody was measured. The involvement of LDL during the uptake process was studied by uptake measurement while supplementing the culture medium with a series of combinations of lipoprotein deficient serum and normal serum and by electrophoresis for the interactions between BCH and lipoproteins. In addition, the effect of calcium iron, a required divalent metal ion for LDL receptor-mediated endocytosis, and temperature on the BCH uptake was also investigated.

MATERIALS AND METHODS

BCH was synthesized in our laboratory as described previously (Ji and Lu, 2002). DL-α-dipalmitoyl phosphatidylcholine (DPPC) and cholesterol were purchased from Sigma Chemicals (St. Louis, MO). Human Glioblastoma multiforme (glioma) 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). Eagle's Minimum Essential Media (EMEM), Dulbecco's phosphate-buffered saline (PBS), trypsin-EDTA, and gentamicin solution were obtained from Fisher Scientific Products, Inc. (Suwanee, GA). Fetal bovine serum (FBS), human normal serum, human lipoprotein deficient serum (LPDS) was obtained from BioCell Laboratories, Inc. (Rancho Dominguez, CA). Analytical grade chloroform, methanol, isopropanol, and other chemicals were obtained from J.T. Baker (Phillipsburg, NJ).

Preparation of BCH liposomal formulation

Similar to the native cholesteryl esters, BCH is extremely hydrophobic and thus it was formulated in liposomes as used in the previous studies (Peacock et al., 2003). The BCH liposomal formulation was prepared by the solvent evaporation method. In brief, 52 mg DPPC, 10 mg cholesterol, and 2.5 mg BCH were dissolved in approximately 6 ml chloroform in a round bottom flask. The chloroform was evaporated under vacuum in a rotary evaporator (Buchi Rotavap, RE121, Brinkmann Instruments, NY). The dried lipid film formed on the flask wall was hydrated with 10 ml PBS that had been pre-heated to 55°C. The flask was shaken in water bath at 55°C and 120 rpm for 5 hours. Size reduction of the resulting multilamellar vesicles was carried out using Emulsiflex B3 device (Avestin, Ontario, Canada) for 10 cycles. The size distribution of the resulting small liposomes was measured using a Nicomp Submicron Particle Sizer (Model 370, Nicomp, CA). The liposome was stored at room temperature for short term before use.

Cell culture

Human Glioblastoma multiforme SF-763 and SF-767 cells were routinely grown in 75 cm^2 plastic cell culture flasks (Corning Inc., Corning, NY, USA) containing 10 ml EMEM supplemented with 10% FBS and 50 unit/ml penicillin and 50 μg/ml streptomycin. The flasks were seeded with approximately 1 x 10^6 cells and placed in a humidified 5% CO_2 incubator at 37°C. The cultures were passaged twice a week to maintain the cells in exponential growth phase.

Determination of BCH in cell samples by high performance liquid chromatography

Cells containing BCH compound were dried by a freeze dry system (Labconco Corporation, Kansas City, MO). BCH in the cells was extracted by a solvent mixture of

methanol and isopropanol (50:50 % v/v) and concentrated by Savant Speed Vac Concentrator System (Albertville, Minnesota). A Zorbax Stable Bond C-18 column (4.6 x 150 mm; particle size, 5 μm) coupled to a Waters 2690 HPLC system was used to analyze the BCH in samples. The column was equilibrated with the mobile phase consisting of 50 % methanol and 50 % isopropanol at a flow rate of 0.5 ml/min. The bound BCH was eluted isocraticly by the mobile phase and measured at 220 nm.

Monoclonal anti-LDL receptor antibody production and purification

Cell line ATCC CRL-1691 (C7, Mouse hybridoma) was grown in Dulbecco's Modified Eagle's Medium supplemented with 4mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 10 % fetal bovine serum (FBS) at 37^{o}C and 5% CO2. Two liters of culture supernatant was harvested by high-speed centrifugation at 4^{o}C. The antibody-containing supernatant was mixed with a saturated ammonium sulfate solution at 50% v/v ratio. Precipitation of antibody was conducted at 4^{o}C and the precipitate was harvested by high-speed centrifugation. The precipitate was resuspended in PBS and dialyzed against PBS at 4 ^{o}C overnight. The antibody was further purified by affinity liquid chromatography using Affi-Gel® Protein A MAPS® II Kit (Bio-Rad Laboratories, Hercules, CA). Briefly, 1 ml of Affi-Gel Protein A agarose was packed into a 1 x 10 cm Econo-Column chromatography column and equilibrated with 5 bed volumes of binding buffer so that the effluent pH equals to the pH of the binding buffer (pH 9.0). Antibody sample was loaded to the column and then washed with 15 bed volume of binding buffer. The antibody was eluted with 5 bed volume of elution buffer. The column was regenerated with 5 bed volume of regeneration buffer. The purity of the antibody was

confirmed by SDS-PAGE containing 12 % acrylamide. Protein concentration was measured by the Bradford method (Bradford, 1976).

Study of monoclonal anti-LDL receptor antibody on the BCH uptake by Glioblastoma multiforme SF763 and SF767 cells

Glioblastoma multiforme SF763 and SF767 cells were grown in EMEM medium supplemented with 10 % human normal serum and 2 mM of calcium ion. After the cells were grown to be about 80% confluent, 1 ml liposomal formulation of BCH was added to the cell culture and mixed completely. A different amount of monoclonal anti-LDL receptor antibody was added to each cell culture to achieve final concentrations of 0.05, 0.1, 0.15, 0.2 and 0.25 mM, respectively. The culture was incubated at the same condition for 4 hours before the cells were trypsinized and harvested by centrifugation. The amount of BCH in cells was determined by HPLC.

Study of lipoprotein on the BCH uptake by Glioblastoma multiforme SF763 and SF767 cells

To determine the effect of lipoprotein on BCH uptake, 5%, 7%, 8%, 9%, and 10% of delipidized human serum was supplemented in the culture medium to replace part of the human normal serum to make up a total of 10 % serum amount in the medium. Cells were grown in 150 cm^2 flasks at the same condition and the amount of BCH uptake by the tumor cell lines was measured by HPLC.

Study of calcium ion on the BCH uptake by the glioma cells

The binding of LDL to LDL receptor requires Ca^{2+} (Goldstein and Brown, 1977). In order to further demonstrate whether LDL-receptor-mediated pathway was involved in the BCH uptake, Ca^{2+} was depleted from the medium during the BCH uptake experiment.

The medium containing 2 mM Ca^{2+} was used as a control. After 24 hrs of incubation of the cell culture with BCH liposomal formulation, cells were harvested. Since inductively coupled plasma (ICP) is very sensitive method for element assay, it is used to determine the boron content in the cells. Briefly, 200 μl of concentrated (70%) nitric acid was added to each harvested cells and vortexed thoroughly. After 2 hours, the cells were completely disrupted and appeared to be clear solution. 800 μl deionized water was then added and vortexed prior to analysis by ICP.

Study of low temperature on the BCH uptake by the glioma cells

At 4°C, the binding of LDL and LDL receptor reaches equilibrium after 3 to 4 hours incubation, and no significant internalization occurs (Innerarity et al., 1986). Thus if LDL receptor-mediated pathway is involved in BCH uptake, the content of BCH taken by the cells at 4°C should be much lower than that taken by the cells at 37°C. Both glioma cell lines SF-763 and SF-767 were cultivated in the same conditions as before. The BCH uptake experiment was conducted at 4°C and 37°C, respectively. After 4 hours incubation, the cells were harvested and cellular content of BCH was determined by ICP.

RESULTS

Purification of monoclonal anti-LDL receptor antibody

Purification of monoclonal antibody generally follows two steps, ammonium sulfate precipitation and affinity chromatography. By using 45-50% saturated ammonium sulfate, most of the serum proteins contained in the culture medium can be eliminated. The subsequent affinity chromatography can further purify the monoclonal antibody. After these two steps, the anti-LDL receptor antibody was purified and their purity was examined by SDS-PAGE. The result is shown in Figure 4.1. Monoclonal antibody

molecules have two heavy chains and two light chains, which are linked by disulfide bridges. In SDS-PAGE, the disulfide bridges were broken and thus two bands, which have approximate molecular weights of 50,000 Da and 25,000 Da, respectively, appeared. Figure 4.1 shows that the monoclonal anti-LDL receptor antibody was pure and ready to be used for further research.

Effect of monoclonal anti-LDL receptor antibody on the BCH uptake

If the LDL receptor-mediated pathway is involved in the BCH uptake, the uptake will be affected by the presence of the antibody specific to LDL receptor. In order to examine the nature of the process, the BCH uptake experiment was conducted in the presence of different concentrations of the purified anti-LDL receptor antibody. The result is shown in Figure 4.2. In the presence of anti-LDL antibody, the uptake of BCH in liposomal formulation by the glioma cells was significantly reduced. The inhibition of the BCH uptake by glioma cells is positively correlated with the amount of antibody in the culture medium. The reduction of BCH uptake by antibody reached the maximum effect in the presence of 0.1mg/ml antibody. This result suggested that LDL receptor-mediated pathway play an important role in the BCH uptake by glioma cell lines.

Effect of lipoprotein concentration on the BCH uptake

The possible involvement of lipoprotein in the BCH uptake process was examined by the depletion of lipoprotein from the serum, which was supplemented to the cell culture medium. During the BCH uptake experiment, a combination of normal human serum and lipoprotein deficient serum was supplemented in the culture medium, to achieve a total serum amount of 10% in the medium. The BCH uptake by glioma cells was determined and the result is shown in Figure 4.3. The more the lipoproteins depleted

from the culture medium, the less BCH was taken up by the cells. It appeared that lipoprotein is a very important factor for the BCH uptake.

Effect of Ca^{2+} on the BCH uptake

To further illustrate the involvement of LDL receptor-mediated pathway in the BCH uptake, the effect of Ca^{2+} on the BCH uptake was determined by depleting the Ca^{2+} completely from the culture medium. The uptake of BCH in the liposomal formulation in the presence and absence of Ca^{2+} in culture medium is shown in Figure 4.4. The BCH uptake in the presence of calcium ion was about 2.7 times as much as that in the absence of calcium ion for SF-763 cells. For SF-767 cells, the BCH uptake in the presence of calcium ion is about 1.5 times as much as that in the absence of calcium ion. Ca^{2+}, as a mediator for the LDL receptor-mediated pathway, appeared indispensable for the BCH uptake by glioma cells.

BCH uptake by glioma cells at 4^oC

At 4^oC, LDL receptor-mediated internalization of LDL is not significant (Thomas et al., 1986). Therefore, the uptake of BCH in liposomal formulation by the glioma cells at this temperature should also be insignificant if LDL receptor-mediated pathway play a major role in BCH uptake. The BCH uptake experiment was conducted at 4^oC and 37^oC, respectively, with the same conditions described above. The result is demonstrated in Figure 4.5. It can be seen that very limited amount of BCH was taken by the glioma cells at 4^oC as compared to that in 37^oC. This result further confirmed that a receptor-mediated mechanism was involved in BCH uptake by glioma cells.

DISCUSSION

Cellular uptake of drugs is affected by many factors such as cell types, growth stage of the cells and surrounding conditions. In our previous studies, the BCH uptake in brain cancerous cells, SF-763, SF-767 cells, and brain normal cells, HCN-1a cells, were compared. The cell culture was scheduled in an appropriate way such that all three cell lines were in a similar stage before the BCH in liposomal formulation was added to the culture medium. After 16 hours incubation with the BCH liposomal formulation, the cells were harvested by trypsinization and centrifugation. The amount of boron uptake by cell lines HCN-1a, SF-767, and SF-763 was determined by HPLC and the results are shown in Table I. The amount of BCH uptake by the tumor cell lines was up to 14 times as high as that by the normal human neuron cell line. The amount of BCH accumulated in the glioma tumor cells was also much higher than the required amount of boron for successful boron neutron capture therapy (BNCT). BCH is a carborane derivative of cholesteryl esters, which is naturally located in the oily core of lipoproteins. The uptake is thus potentially mediated by the lipoprotein pathway and possibly through the LDL receptors. To address this question, the present research focuses on the elucidation of the involvement of the LDL receptors during the uptake process.

If the cellular uptake of BCH is LDL receptor-mediated, the difference of BCH uptake between the glioma cells and normal neuron cells becomes self-explanatory, because glioma cell lines SF-763 and SF-767 overexpress LDL receptors on the cell surface as compared with the normal cells (Maletinska et al., 2000). Since the internalization of natural LDL by cells is LDL receptor-mediated (Brown and Goldstein, 1984; 1986), the binding of the LDL receptors by anti-LDL receptor antibodies could

significantly reduce LDL particle uptake by the cells. Our results have demonstrated the reduction of BCH content in the cells when monoclonal anti-LDL receptor antibody was present in the culture medium. This suggested the possibility of the involvement of the LDL and LDL receptor in the uptake of BCH in liposome formulation by the glioma cells.

On the other hand, if LDL and LDL receptor are involved in the cellular uptake of BCH in liposome formulation, the depletion of LDL from the culture medium would lead to the reduction of cellular uptake of BCH. Our result showed that the cellular uptake of BCH in the liposome formulation by glioma cells was positively correlated with the amount of LDL present in the culture medium. This result further inferred that LDL functioned as a carrier of BCH during the cellular uptake of BCH in liposome formulation by the glioma cells.

The specific binding of LDL and LDL receptor, which is the first step of receptor-mediated pathway, requires divalent calcium ion (Innerarity et al., 1986). By depleting the calcium ion in the culture medium, cellular uptake of BCH in liposome formulation was reduced. Furthermore, the receptor-mediated endocytosis is temperature dependent, and at 4^{o}C, LDL is only bound to the cell surface and the internalization is not significant (Innerarity et al., 1986). Therefore, the determination of BCH uptake at 4^{o}C and 37^{o}C would help to confirm whether a receptor-mediated mechanism was involved. When the cellular uptake experiment was conducted at 4^{o}C, it was found that much smaller amount of BCH was detected in the cells as compared to that at 37^{o}C.

In conclusion, the inhibition of cellular uptake of BCH in liposome formulation by the presence of anti-LDL receptor antibody and the positive correlation of uptake of

BCH in liposome formulation with the amount of LDL in the culture medium, together with the effect of calcium ion and temperature on the uptake of BCH in liposome formulation, have strongly suggested the involvement of an LDL receptor-mediated pathway in the uptake of BCH in liposome formulation. However, we were not clear yet how the BCH in liposome formulation interacted with this pathway. One possible interaction might be the transfer of the BCH in liposome formulation to the LDL particles by contact or fusion. However, this need to be further clarified in future experiments.

Table 4.1. Cellular uptake of BCH by different cell lines and the effect of lipoprotein on BCH uptake

Cell line	Cellular BCH uptake (μg boron/g cell)
Normal neuron cell, HCN-1a	19.9 ± 2.9
Glioma tumor cell, SF-763	240.1 ± 22.3
Glioma tumor cell, SF-767	283.4 ± 48.1

Figure Legends:

Figure 4.1. SDS-PAGE (12% acrylamide) of the purified monoclonal anti-LDL receptor antibody

Figure 4.2. The effect of monoclonal anti-LDL receptor antibody on the uptake of BCH in liposome formulation by glioma cell lines SF-763 and SF-767

Figure 4.3. The effect of serum on the uptake of BCH in liposome formulation by glioma cell lines SF-763 and SF-767

Figure 4.4. The effect of Ca^{2+} on the uptake of BCH in liposome formulation by glioma cell lines SF-763 and SF-767 (white bar- with 2 mM Ca^{2+}; gray bar-without Ca^{2+})

Figure 4.5. The effect of temperature on the uptake of BCH in liposome formulation by glioma cell lines SF-763 and SF-767 (white bar-4^{o}C; gray bar-37^{o}C)

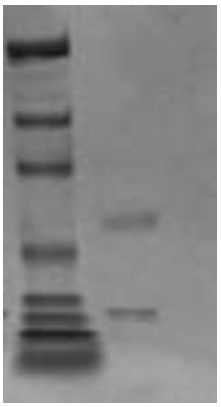

Figure 4.1

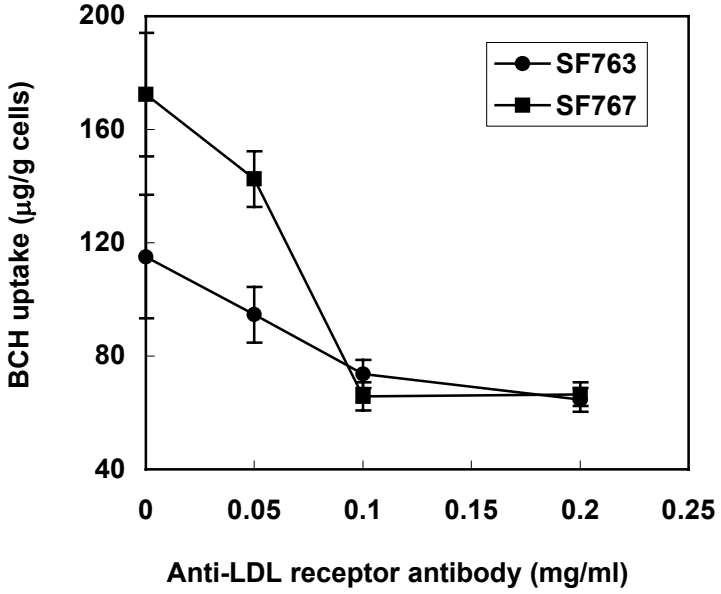
200
160
120
80
40
BCH uptake (µg/g cells)
SF763
SF767
0
0.05
0.1
0.15
0.2
0.25
Anti-LDL receptor antibody (mg/ml)

Figure 4.2

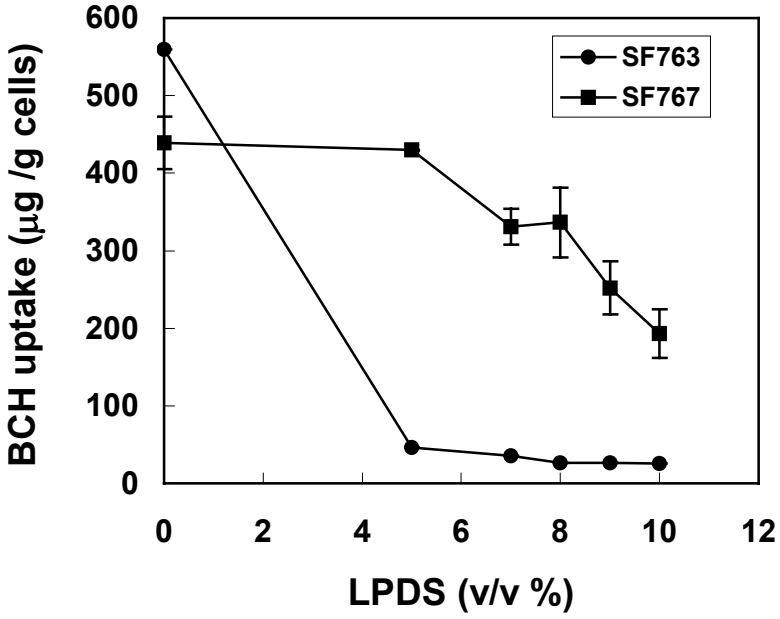
BCH uptake (μg /g cells)
600
500
400
300
200
100
0
0
2
4
6
8
10
12
LPDS (v/v %)
SF763
SF767

Figure 4.3

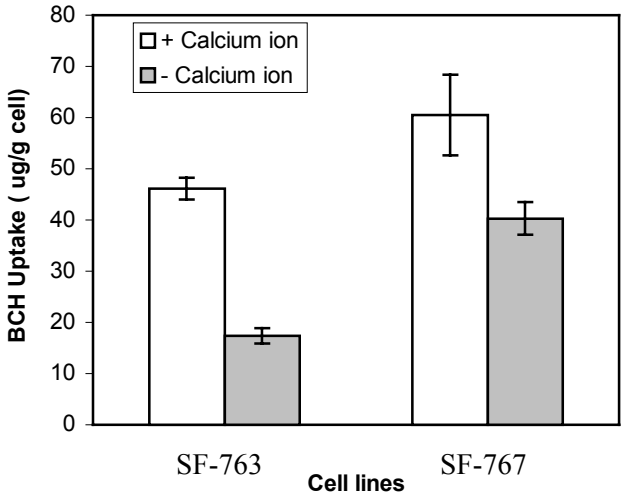
80
70
60
50
40
30
20
10
0
+ Calcium ion
- Calcium ion
BCH Uptake (ug/g cell)
SF-763
SF-767
Cell lines

Figure 4.4

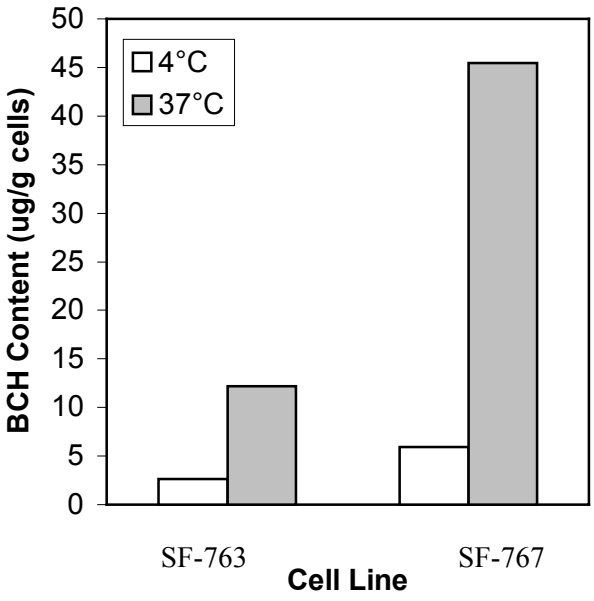
50
45
40
35
30
25
20
15
10
5
0
4°C
37°C
BCH Content (ug/g cells)
SF-763
SF-767
Cell Line

Figure 4.5

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CHAPTER 5

IN VITRO EVALUATION OF THE ANTICANCER EFFECT OF A METHOTREXATE-CHOLESTEROL CONJUGATE ON GLIOMA CELL LINE SF-767[1]

[1]Guangliang Pan, Hengguang Li, Svein Øie, D. Robert Lu. To be submitted to International Journal of Pharmaceutics (2003).

ABSTRACT

Objective. To evaluate and compare the *in vitro* cytotoxicity of anticancer drug methotrexate and its derivative methotrexate-cholesterol conjugate to Glioblastoma multiforme cell line SF-767. **Methods**. Methotrexate-cholesterol was synthesized by a three step chemical reaction. The cytotoxicity of methotrexate and methotrexate-cholesterol on Glioblastoma multiforme cell line SF-767 was evaluated by determining the cell viability using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) after the incubation of the cells with the compounds. The effect of the concentration of methotrexate and methotrexate-cholesterol conjugate on the cytotoxicity to SF-767 was examined. In addition, the cell viability after 24, 48, and 72 hours of incubation with methotrexate-cholesterol at a concentration of 0.125 mM were determined. **Results.** The cytotoxicity of methotrexate-cholesterol to glioma cell SF-767 was higher than that of methotrexate. After 24 hours of incubation with the drug at a concentration of 0.25 mM, only 2.6 % of glioma cells were viable when methotrexate-cholesterol was added, while 15.4% of glioma cells were viable when methotrexate was added. The cytotoxicity of methotrexate-cholesterol was concentration and time dependent. When the glioma cells were treated with 0.125 mM methotrexate-cholesterol, only 2.4 % cells were viable after three days. The cytotoxicity of the compounds to glioma cell SF-767 at different concentrations and times was further demonstrated by the microscopic pictures of the cells. **Conclusion**. The cholesteryl ester derivative of methotrexate demonstrated increased cytotoxicity as compared methotrexate. Further studies are needed to elucidate the reason of such increase in cytotoxicity.

Keywords Methotrexate; Methotrexate-cholesterol conjugate; Anticancer; Cytotoxicity; Cell viability; MTT; Cellular uptake:

INTRODUCTION

The growth of human cells requires cholesterol for the bioconstruction of plasma membrane. Cells obtain cholesterol either by *de novo* synthesis from acetyl-CoA or by taking up plasma LDL which contains about 1500 cholesteryl esters per LDL particle. The rate of *de novo* synthesis of cholesterol is relatively low and about 90% of cholesterol in the cell is obtained from LDL receptor-mediated endocytosis of LDL particles (Ho et al., 1978; Laster et al., 1991). However, there is a significant difference in LDL consumption between human normal cells and cancer cells (Firestone, 1994; Rudling et al., 1983,1990; Vitols et al., 1984,1987,1990). Most cancer cells are rapidly dividing cells and thus have a higher cholesterol requirement for new cell membranes. Correspondingly, these cells express significantly high numbers of cell surface LDL receptors (Jung-Testas et al., 1992; Maletinska et al., 2000; Vitols, 1984, 1987, 1990). This difference between the normal cells and cancer cells provides a fundamental drug targeting strategy of utilizing cholesterol-drug conjugates to interact with lipoproteins and to follow through the LDL pathway to selectively deliver a higher amount of anticancer agents to cancer cells.

In our laboratory, a series of cholesterol-drug conjugates aimed at targeted drug delivery to cancer cells have been designed and synthesized (Ji et al. 2002; Alanzi et al., 2003a, 2003b). As an example, cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH), is a mimic of natural cholesteryl ester but contains carborane as an anti-cancer unit for boron neutron capture therapy (BNCT) (Gutman et al., 2000; Ji et al., 2002;

Peacock, 2003). Cell culture studies in our laboratory indicated that BCH was taken up extensively by human glioma cancer cells and the uptake was significantly higher in the glioma cells than that in normal neuron cells (Peacock et al., 2003). However, the anticancer activity of these boron-containing cholesterol-drug conjugates can not be demonstrated because of the unavailability of the neutron radiation facility required by BNCT.

In order to further demonstrate whether this type of compounds can be beneficial for drug targeting and therapeutic effectiveness, cholesterol conjugates to other chemotherapeutic agents was studied. Methotrexate is an antimetabolite used in the treatment of certain neoplastic diseases, severe psoriasis, and adult rheumatoid arthritis by inhibiting dihydrofolate reductase (Freeman et al., 1983; Weinblatt et al., 1985; Roenigk et al., 1988; Jolivet et al., 1983). Actively proliferating tissues such as malignant cells, bone marrow, fetal cells, buccal and intestinal mucosa are more sensitive to methotrexate. Since cellular growth and proliferation in malignant tissues is greater than in most normal tissues, methotrexate may impair malignant cell growth without significant irreversible damage to normal tissues. Since the methotrexate contains a carbonyl group, it can form an ester bond with the hydroxyl group in the cholesterol molecule. In this research, methotrexate-cholesterol conjugate was synthesized by a three step chemical reaction. The cytotoxicity of the methotrexate-cholesterol conjugate to glioma cell line SF-767 was evaluated and compared with that of methotrexate.

MATERIALS AND METHODS

Materials

Human glioblastoma multiforme (glioma) SF-767 cell line was obtained from the tissue bank of the Brain Tumor Research Center (University of California-San Francisco, San Francisco, CA). Eagle's Minimum Essential Media (EMEM), Dulbecco's phosphate-buffered saline (PBS) and trypsin-EDTA were obtained from Fisher Scientific Products (Suwanee, GA). Fetal bovine serum (FBS) was obtained from BioCell Laboratories (Rancho Dominguez, CA). Methotrexate, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and penicillin/gentamycin solution was purchased from Sigma (St. Louis, MO). N, N'-dicyclohexylcarbodiimide (DCC) was ordered from Fisher Scientific (Pittsburgh, PA). Carbonyldiimidazole (CDI) was purchased from Lancaster Synthesis (Windham, NH). Analytical grade solvents and other chemicals were obtained from J.T. Baker (Phillipsburg, NJ).

Synthesis of Methotrexate-Cholesterol Conjugate

The chemical structure of methotrexate-cholesterol and its chemical synthesis are shown in Figure 5.1 and Figure 5.2. Methotrexate (0.01 mmol) was first dissolved in 2 ml of water and 5 drops of triethylamine at room temperature. To the solution, 3.3 mg (0.015 mmol) of Di-tert-butyl dicarbonate was added and the mixture was stirred for 6 hours at room temperature. The reaction mixture was then condensed under vacuum to get product 1. In 3 ml dimethyl formamide (DMF), 6.65 mg of product 1 and 3.86 mg (0.01 mmol) cholesterol were dissolved. To the mixture, 1.58 mg (0.01 mmol) of carbonyldiimidazole was added with stirring at room temperature. After 5 minutes, 3.86 mg of cholesterol (0.01 mmol) and 2.0 mg DCC (0.01 mmol) were added and the mixture

was stirred for 6 hours. The reaction mixture was filtered and purified via silica column chromatography eluted with petroleum ether and ethyl alcohol to get product 2. The product 2 was then dissolved in 10 ml of dichloromethene. To the solution, three drops of trifluoroacetic acid was added and stirred at room temperature for 2 hours. The mixture was condensed under vacuum and the residue was further purified by silica column chromatography eluted with ethyl alcohol to get product 3, which was methotrexate-cholesterol conjugate.

Cell culture studies

Human glioma SF-767 cell line was grown and maintained in 75 cm^2 culture flask containing 10 ml of EMEM with 10% FBS, 50 μg/ml gentamycin and 50 U/ml penicillin. After three passages from the stock culture, the cells were sub-cultured to 96-well cell culture plate with approximately 8 x 10^3 cells in 0.1 ml culture medium in each well. The plates were placed in a humidified 5% CO_2 incubator at 37°C for 24 hours prior to incubation with the compunds. The stock solutions of methotrexate and methotrexate-cholesterol were prepared by dissolving them in DMSO and were stored in a refrigerator before use. When the cell culture was ready, different amount of methotrexate and methotrexate-cholesterol (0.125, 0.25, 0.375 mM) were added to each well and mixed. The culture was incubated at the same condition for 24 hours and the cell viability was determined. In order to examine the cytotoxicity of methotrexate-cholesterol after different time of incubation, 0.125 mM of methotrexate-cholesterol was incubated with SF-767 cells for 24, 48, and 72 hours and the cell viability was determined. Microscopic pictures of cell cultures were taken at various stage of the incubation with the compunds.

Cell viability determination after the incubation with methotrexate and methotrexate-cholesterol

Cell viability was examined after incubation of the cells with different concentration of the test compounds by the standard MTT method which measures the ability of the cells to degrade tetrazolium salt MTT. The cells incubated with DMSO only was used as the control. After the incubation, 20 μl of MTT solution in PBS buffer (0.5 mg/ml) was added to each well of culture and incubated at the same condition for an additional 4 hours. The medium was then removed and 150 μl of DMSO was added to each well and mixed thoroughly until all the dark blue crystals were dissolved. The plate was read on an OPTImax Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at wavelength of 550 nm. The cell viability was calculated and expressed as (OD_{trt}/OD_{ctrl}) x100%, where OD_{trt} is the optical absorbance from the culture treated with the tested compounds, and OD_{ctrl} is the optical absorbance from the culture incubated with DMSO only.

RESULTS

Comparison of the cytotoxicity of methotrexate and methotrexate-cholesterol conjugate

The chemical linkage between methotrexate and cholesterol was successfully obtained through the reactions shown in Fig. 2. The introduction of the cholesterol unit greatly increased the hydrophobicity of the whole molecule. DMSO was used as the solvent for both methotrexate and methotrexate-cholesterol. The anticancer activity of these two compounds was then examined through the comparison of the cytotoxicity of methotrexate and methotrexate-cholesterol conjugate.

Figure 5.3 showed that the cytotoxicity of methotrexate-cholesterol was higher than methotrexate. At low concentration (0.125 mM), although methotrexate-cholesterol had higher cytotoxicity than methotrexate, the difference in cytotoxicity was not significant. However, when the concentrations of the test compunds were increased to 0.25 mM, only 2.6 % of glioma cells were viable when methotrexate-cholesterol was added while 15.4% of glioma cells were viable when methotrexate was added. The cytotoxicity of both compounds to glioma cells was also demonstrated by the cell culture pictures (Figure 5.4). In the presence of either compunds, cells lost the capability to attach to the wall of the flask and started to float in the medium. When methotrexate-cholesterol was applied, no viable cells could be observed when the drug concentration reached to 0.375 mM. For both compounds, as the concentration in the cell culture increased, the cell viability decreased.

Dependence of the cytotoxicity on incubation time

In order to examine the effect of incubation time on cell death caused by methotrexate-cholesterol, SF-767 cells were incubated with methotrexate-cholesterol at concentration of 0.125 mM for 24, 48, and 72 hours. The low concentration of the test compound was used because our previous result showed that the dependence of cytotoxicity on the drug concentration was linear at low concentration. Figure 5.5 shows that the cell viability was continuously reduced as the incubation time was prolonged. Within the first 24 hours, about 75% of the cells died. After 72 hours incubation, only 2.4% of glioma cells were viable. The death of glioma cells were also reflected by the changes on the cell culture picture (Figure 5.6). After 24 hours, most cells showed a round shape as they lost the ability to attach to the plastic surface of the culture plate.

After 48 and 72 hours, most cells floated in the culture and aggregated to form clumps as shown in picture 3 and picture 4.

DISCUSSION

Effective chemotherapy for most malignant cancers requires preferential accumulation and retention of the therapeutic compounds in cancer cells. Since many cancer cells overexpress surface receptors, such as transferrin receptor (Plant et. al. 1989; Huwyler et. al. 1996), folate receptor (Ross et. al. 1994; Reddy et. al. 1998), and LDL receptor (Vitols et. al, 1984, 1985, 1992; Rudling et. al. 1990; Maletinska et. al., 2000), targeted drug delivery systems can be designed to utilize the overexpression for effective cancer treatment. Among the different overexpressed receptors, LDL receptor is unique because its ligand, LDL, is a natural carrier in blood circulation containing cholesterol and cholesteryl esters. Therefore, it is possible to develop drug-conjugated cholesteryl esters that can follow the LDL pathway for targeted drug delivery to cancer cells. Experiments in our laboratory has shown that a carborane-conjugated cholesteryl ester, BCH, can be taken up by glioma cancer cells in significantly higher amount than that by normal nuron cells (Peacock et al., 2003). The mechanistic study involving anti-LDL receptor antibody and lipoprotein deficient serum indicated that the uptake was through the LDL receptors (Chapter 4).

In order to test whether such a strategy could be applied to other chemotherapeutic units, our laboratory has recently synthesized a methotrexate-conjugated cholesteryl ester. Methotrexate and its analogs are potent inhibitors of hydrofolate reductase, which is required for the reduction of dihydrofolates to tetrahydrofolate (THF). THF is essential carrier for one-carbon groups in the biosynthesis

of methionine, purines, and the pyrimidine thymine. Since growing cells, especially those cells with rapid growth and proliferation have a high requirement for purine and thymine, methotrexate and its analogs have been used as effective blockers of cancer growth. Since the methotrexate molecule contains carbonyl groups, it can form an ester bond with the hydroxyl group in the cholesterol molecule. Thus it was chosen as the model chemotherapeutic agent for cholesteryl conjugate synthesis.

By comparing the cytotoxicity of methotrexate and methotrexate-cholesterol conjugate, our results has shown that the cytotoxicity of cholesteryl derived methotrexate increased significantly as compared to methotrexate. Methotrexate, the pharmacologically effective part, was not changed signicantly after conjugating with the cholesterol molecule. So it may still keep the binding ability to the dihydrofolate reductase and interfere with the nornmal metabolism of the cancer cells. The increased cytotoxicity of the conjugate may be caused by different reasons. For example, it may be caused by the increased uptake of the methotrexate after the derivatization with the cholesterol, whose cellular uptake pathway is involved in LDL receptor, because this is true in the case of cellular uptake of BCH. However, further studies are required to elucidate the mechanism of the increase of anticancer potency.

FIGURE LEGENDS:

Figure 5.1: The chemical structure of methotrexate-cholesterol conjugate

Figure 5.2: Steps of synthesis of methotrexate-cholesterol conjugate

Figure 5.3: Cytotoxicity of methotrexate and methotrexate-cholesterol conjugate on glioma cell line SF-767 at different concentration

Figure 5.4: Microscopic pictures of cell culture incubated with methotrexate (A) and methotrexate-cholesterol conjugate (B) at concentration of 0, 0.125, 1.25, and 0.375 mM.

Figure 5.5: Cytotoxicity of methotrexate-cholesterol conjugate at concentration of 0.125 mM after different time of incubation

Figure 5.6. Microscopic pictures of cell culture of SF-767 after incubation with methotrexate-cholesterol conjugate at 0.125 mM after 0, 1, 2, 3 days.

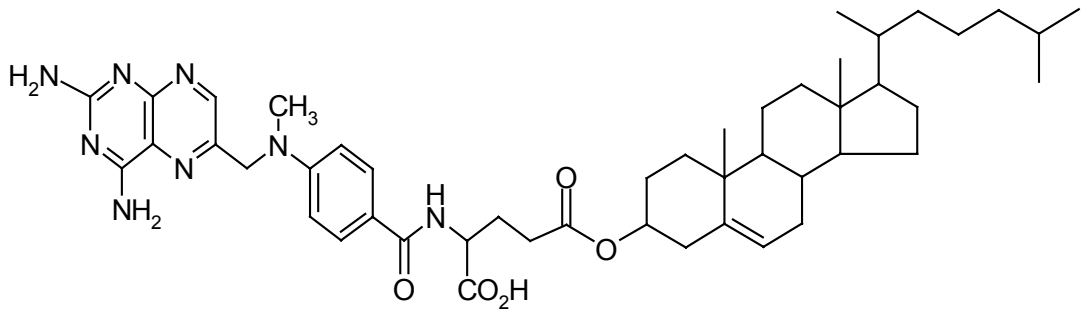


Figure 5.1

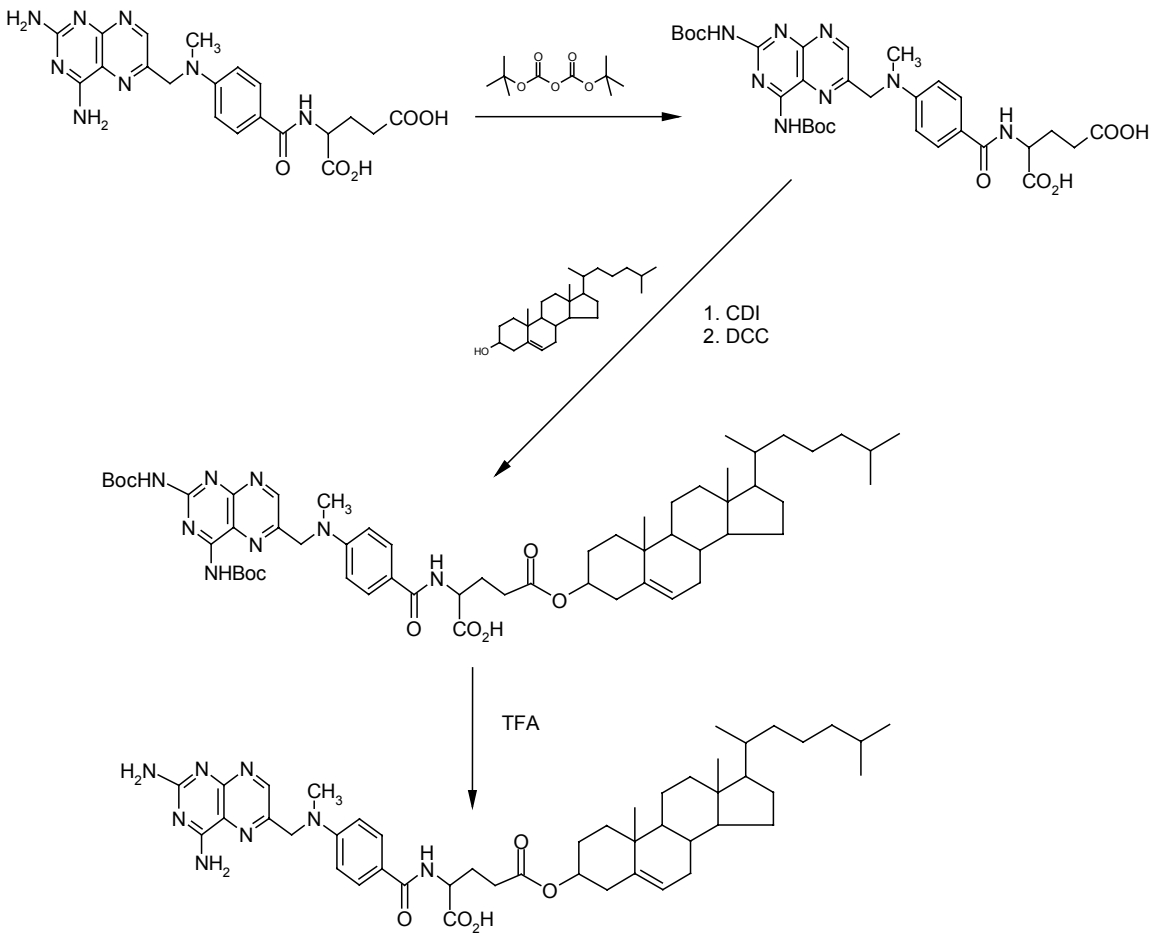


Figure 5.2

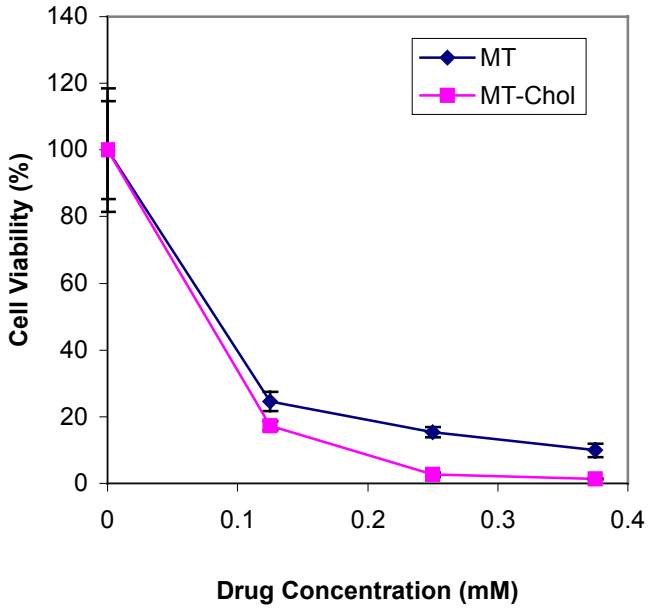
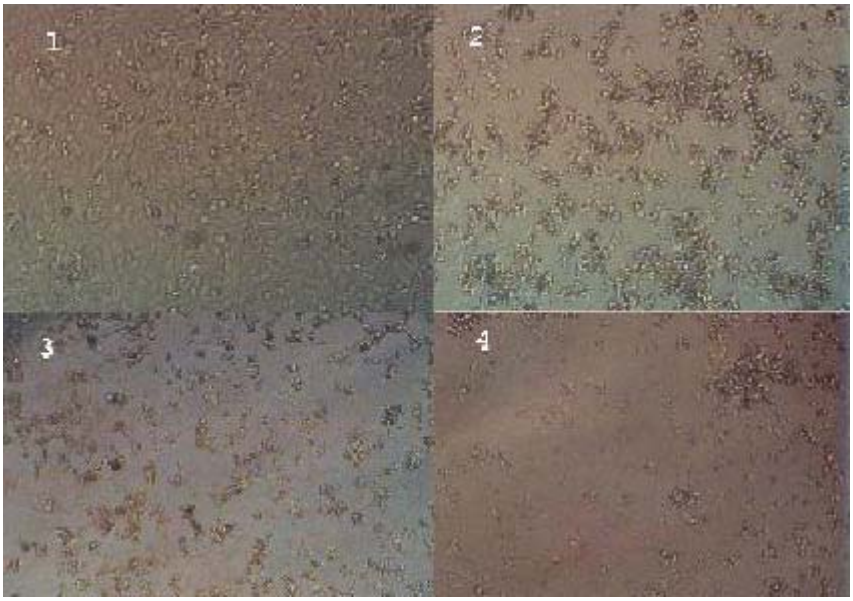
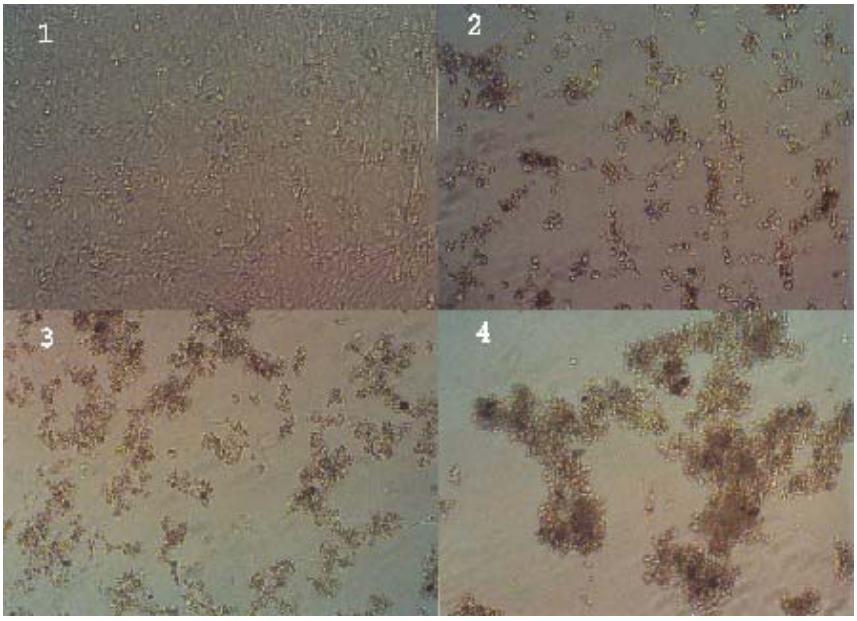

140
120
100
80
60
40
20
0
Cell Viability (%)
MT
MT-Chol
0
0.1
0.2
0.3
0.4
Drug Concentration (mM)

Figure 5.3



(A)



(B)

Figure 5.4

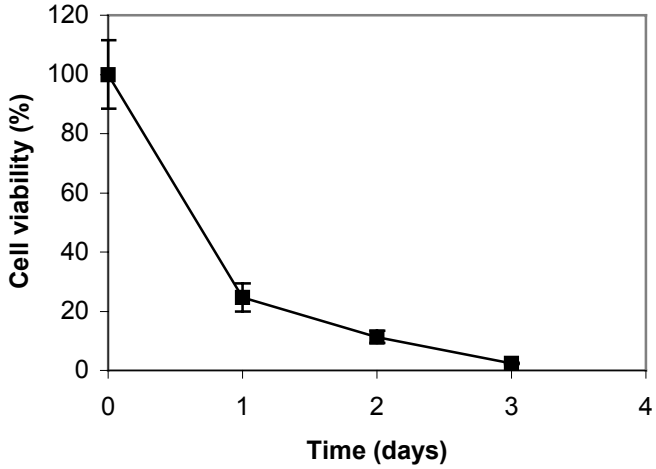


Figure 5.5

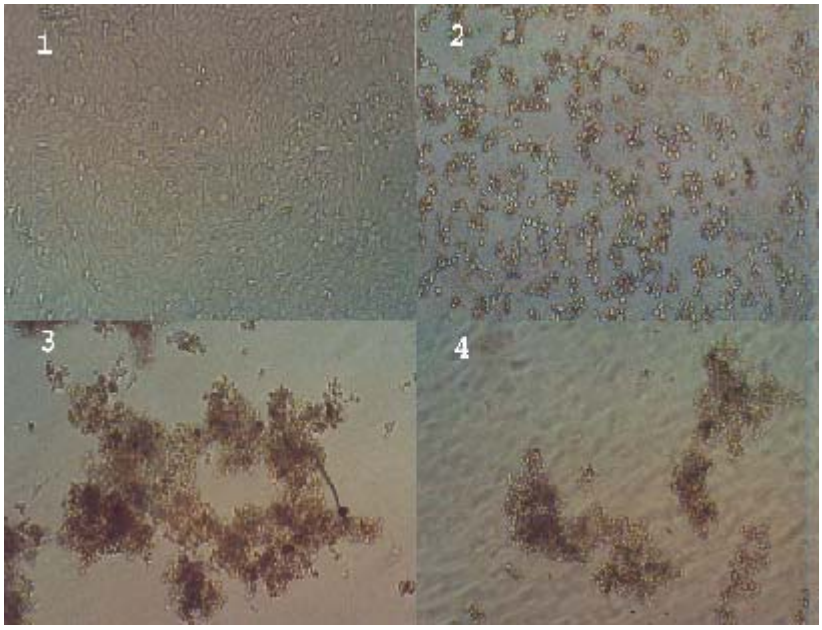


Figure 5.6

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CHAPTER 6

CONCLUSIONS

Malignant glioma represents a very difficult therapeutic challenge. One new therapeutic strategy is gene therapy, which involves the delivery to and expression of the therapeutic genes in the cancer cells. In this research, a novel artificial lipoprotein system has been developed and evaluated for in vitro gene delivery to glioma cell line SF-767. The system mimics the natural lipoprotein in composition and structure but contains lipidized poly-L-lysine to replace the apolipoproteins in the surface of lipoproteins. Such a system can be conveniently formulated from natural lipids, with the ability to control the size and surface charge. With proper ratios among its components, this delivery system can carry genetic materials and deliver them into glioma cells successfully. It demonstrated similar transfection efficiency but a lower cytotoxicity as compared with the commercial Lipofectamine™ gene transfection system. By incorporating other surface proteins or peptides into this system, it may be used for targeted gene or drug delivery. In addition, it could be extended to deliver DNA vaccines.

Since many cancer cells overexpress some cell surface receptors as compared to their normal counterparts, one strategy for cancer therapy is to utilize these receptors to develop targeted drug delivery system. As one of the major circulatory complexes in blood circulation system, LDL is unique in that it is nanoscale particles and it is the carrier of cholesterol and cholesteryl ester in the body. By mimicking the composition and structure of cholesteryl ester, BCH, a carborane-cholesterol conjugate, was synthesized in our laboratory. When formulated into liposomes and incubated with

normal neuron cells and glioma cells, it was found that the cellular uptake of BCH by human glioma cell lines was significantly higher than that by human normal neuron cell line. If the cellular uptake of BCH in liposome formulation is LDL receptor-mediated, the big difference of BCH uptake between the glioma cells and normal neuron cells becomes self-explanatory, since each glioma cell has much higher number of LDL receptors in than that in each normal cell. Thus the elucidation of the mechanism for the uptake of BCH in liposome formulation by the glioma cells turned to be a very important research project for the further development of BCH as a targeted delivery system.

The results from this research have demonstrated that the uptake of BCH in liposome formulation by both glioma cell line was inhibited by monoclonal anti-LDL receptor antibody. In addition, by substituting the normal human serum with lipoprotein deficient serum in the culture medium, the cellular uptake of BCH was also reduced. Divalent metal ions, e.g., calcium ion, are required for the binding of LDL and LDL receptor. By depleting the calcium ion from the culture medium, cellular uptake of BCH was reduced. At low temperature, no significant BCH uptake occurred. These results suggested that LDL receptor play a very important role in the uptake of BCH in liposome formulation by the glioma cell lines SF-763 and SF-767.

In order to demonstrate further whether the cholesterol-drug conjugate can be beneficial for the drug targeting, methotrexate- cholesterol conjugate was synthesized. The cytotoxicity of the methotrexate-cholesterol conjugate to glioma cell line SF-767 was evaluated and compared with that of methotrexate. It was found that the cytotoxicity of methotrexate-cholesterol was higher than that of methotrexate.

APPENDIX

BIOLOGICAL PROTEIN NANOSTRUCTURES AND TARGETED DRUG DELIVERY[1]

[1]Guangliang Pan, Svein Øie, and D. Robert Lu. Submitted to "Cellular Drug Delivery: Principle and Practice" by D. Robert Lu and Svein Øie (eds), Humana Press.

1. Introduction

Targeted drug delivery refers to the site-specific drug delivery that directs drugs mainly to certain cell types within a tissue and to certain molecular complexes or organelles within a cell while avoiding drug loading in non-targeted cells. Targeted delivery of drugs to specific cells involves specific interactions between drugs or drug carriers and cell surface proteins through ligand-receptor interactions or antigen-antibody interactions. Targeted drug delivery to specific molecular complexes or organelles within a cell requires specific interactions of drug with the targeted complexes to lead to the therapeutic effect. In biological systems, these interactions generally occur on various types of biological nanostructures of protein origin and understanding and utilization of the biological nanostructures could lead to significant improvement in drug targeting and drug carriers.

The biological protein nanostructures primarily include protein-lipid, protein-protein, protein-carbohydrate, and protein-nucleic acid complexes. Proteins, one group of the most important biological macromolecules in cells, are smaller nanoscale molecules with typical size range between 1 and 20 nm (1). Through sophisticated interactions with other biomolecules, these protein nanostructures are formed and widely distributed in human body. For example, low-density lipoproteins (LDL), with a diameter of 25-28 nm, are protein-lipid complexes. They are the major circulatory nanostructures in the blood. When used as a drug carrier, these protein-lipid complexes offer certain advantages of being endogenous nanostructures that do not trigger immunological response. They can also escape recognition and elimination by the reticuloendothelial system (RES). On the other hand, glycoproteins, i.e. protein-carbohydrate complexes, are vital structural and

regulatory proteins in viruses and can serve as important therapeutic targets for antiviral drug development. Telomerase, a protein nanostructure formed from protein and nucleic acid, is activated only in cell immortalization and cancer progression. Thus telomerase is an ideal therapeutic target for anticancer therapy. Because protein nanostructures are so critical to various biological and physiopathological activities, they have received wide attention in recent years in the development of drug targeting strategies either as drug carriers or as therapeutic targets. This chapter will focus on two aspects of biological protein nanostructures regarding their involvement in targeted drug delivery: (1) biological protein nanostructures as targeting drug carriers and (2) biological protein nanostructures as therapeutic targets for new drug development.

2. Protein-lipid nanocomplexes (lipoproteins)

Lipoproteins are biological protein-lipid complexes in the nanoscale range. They have spherical shapes consisting of a hydrophobic core and a polar shell which is incorporated with receptor-active proteins. The hydrophobic core contains triglycerides and cholesteryl esters while the polar shell contains phospholipids, unesterified cholesterol, and one or several apolipoproteins. A schematic cross-section diagram of lipoprotein is shown in Figure A.1. Lipoproteins are commonly classified based on their densities which can be determined through gradient ultracentrifugation. The classification thus is related to the respective amounts of lipid and protein in the complex. In an increasing order by density, lipoproteins include chylomicrons, very low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL).

Since lipoproteins are taken up in varying amount through ligand-receptor interactions by different type of cells, they may be utilized as biocompatible nanoscale

carriers for targeted drug delivery. For example, in hepatocytes, remnant receptor and asialoglycoprotein receptor can recognize chylomicrons and lactosylated HDL, respectively, in a molecular-specific manner. LDL receptors on cell surfaces can specifically recognize LDL and its expression can be up-regulated or down-regulated depending on the type and state of these cells. By incorporating bioactive molecules into lipoproteins or modified lipoproteins, targeted drug delivery may be achieved resulting in more bioactive molecules taken up by a select type of cells, e.g. cancer cells. Each class of lipoproteins has its unique biological property and thus can be utilized individually for targeted drug delivery.

2.1 Chylomicron for drug targeting

Chylomicrons are the largest lipoprotein complexes (80-500 nm) in human body. Their main function is to transport dietary lipids from the intestine to the liver and adipose tissue. Assembled in the intestine from the absorbed dietary lipids and the apolipoproteins synthesized by the intestinal epithelium, they are transported out of the epithelial cells to the tissue fluid and further carried by lymphatic system for general circulation. When they enter the blood stream, their compositions of phospholipids and proteins are changed greatly through the hydrolysis of triglycerides and the component exchange with other lipoproteins in the plasma to form chylomicron remnants. Chylomicron remnants are mainly taken up by parenchymal cells in liver (2). When they are oxidized, chylomicrons can be taken up by liver endothelial cells and Kupffer cells (3). The uptake of chylomicron remnants by various cells is LDL receptor-mediated, which requires apoE protein as the ligand on the chylomicron remnant particles (3,4).

When associated with chylomicrons, many lipophilic drugs and xenobiotics can be absorbed via the intestinal lymphatic system (5). This route can circumvent the first pass effect in the intestine and, more importantly, it can be used for drug targeting to liver cells because the liver is the destination of chylomicrons. Targeted drug delivery to the liver can help treat many critical diseases such as alcohol-induced liver disorders, chronic liver diseases and cirrhosis, virus-induced liver diseases (heptatitis), liver tumor, familial hypercholesterolemia, and type III hyperlipoproteinemia. To effectively treat viral infection, antiviral drugs or prodrugs can be incorporated into chylomicrons and targeted to the liver. For example, by incorporating a nucleoside analogue iododeoxyuridine into recombinant chylomicrons, the antiviral drug was selectively delivered to liver parenchymal cells (6). The liver is also an excellent target for gene therapy for diseases caused by metabolic defects. Gene therapy involving viral vector is in general limited by the high immunogenicity and poor safety profile. Utilizing the hydrophobic core of the chylomicrons, Hara et al. incorporated a hydrophobic DNA complex into reconstituted chylomicron remnants and the DNA was successfully delivered to liver cells (7-8).

2.2 Very low density lipoprotein (VLDL) for drug targeting

VLDL particles have a size range of 30-80 nm. They are assembled in the endoplasmic reticulum and matured in Golgi apparatus of hepatocytes before secretion (9). After entering into the plasma, VLDL particles are catabolized by a series of biochemical actions including apolipoprotein exchange with apoC-I, apoC-II, apoC-III, and apoE, lipolysis by triglyceride lipase and cell-surface receptor-mediated uptake by cells. As lipolysis proceeds, VLDL particles become smaller and smaller and eventually are converted to IDL. Some of the IDL particles are rapidly taken up by hepatocytes via

a receptor-mediated mechanism and others undergo further hydrolysis before being converted to LDL.

The catabolism cascade of VLDL suggests the possibility of using VLDL as a drug carrier for targeted delivery. Because some cancer cells overexpress the receptors for apo-E, a protein ligand present on the surface of VLDL, VLDL can potentially serve as an antineoplastic drug carrier. *In vitro* experiments demonstrated that VLDL could effectively incorporate cytotoxic drugs, 5-fluorouracil (5-FU), 5-iododeoxyuridine (IudR), doxorubicin (Dox), and vindesine, and the resultant complex showed effective cytotoxicity to human carcinoma cells (10). By mimicking the composition and structure of VLDL, Shawer et al. developed a VLDL-resembling phospholipid nanoemulsion system which could carry a new antitumor boron compound for targeted delivery to cancer cells (11).

2.3 Low-density lipoprotein (LDL) for drug targeting

LDL (25-30 nm) is not directly synthesized in human body. Instead, most of them are formed through the VLDL pathway. LDL is the major circulatory lipoprotein for the transport of cholesterol and cholesteryl esters. Cholesterol required for cell membrane construction is mainly obtained from LDL. LDL can be internalized by cells via LDL receptor-mediated endocytosis, a process that is determined by the availability of LDL receptors (12). Apolipoprotein apoB-100 is the major ligand in LDL for recognition and binding by LDL receptor (13). Once it is internalized, LDL is distributed to lysosomes in which cholesteryl esters are hydrolyzed (Figure A.2). Since cholesterol is required for cell growth and LDL is the main carrier for cholesterol in blood circulation, most cells can take up LDL through a receptor-mediated mechanism. It is estimated that 60-80% of

LDL can be cleared from plasma by LDL receptor-mediated pathways (14-15). As compared to chylomicron, VLDL, and IDL, LDL has a longer serum half-life of 2 to 4 days (16). Thus, among various lipoproteins, LDL has a distinctive advantage to be used as drug carrier for targeted delivery and has been widely studied.

2.3.1. LDL for anticancer drug targeting

It has been demonstrated that many tumor cells overexpress LDL receptors for the uptake of LDL particles to meet their increased requirement for cholesterol in cell membrane construction (17- 22). Subsequently, a significant amount of work has been carried out to examine LDL as a candidate for antitumor drug carriers. Many lipophilic anticancer drugs have been incorporated into LDL particles for the purpose of drug targeting to various tumors (10, 23). When the antineoplastic drugs, methotrexate and floxuridine (FdUrd), were oleyl derivatized and incorporated into LDL particles, they were effectively delivered into the hepatocellular carcinoma cell line Hep G2 (24). The serum half-life of these drugs carried by LDL particles was considerably prolonged as compared to the free drug. Photodynamic therapy (PDT) of tumors is a recently developed therapeutic approach. It is based on the generation of highly cytotoxic oxygen species through the irradiation of photosensitizer such as porphyrins, chlorins, and phthalocyanines at selected wavelength. The efficacy of this therapy is dependent on the specific uptake of these photosensitizers by tumor cells. Using LDL as a carrier, photosensitizers were successfully targeted to tumor cells (25-27).

2.3.2. LDL for brain drug targeting

The blood-brain barrier (BBB) is a semi-permeable barrier that allows certain types of molecules to pass through but not others, depending on the lipophilicity,

molecular size and electric charge. It is a significant barrier for many drugs such as antibiotics, neuropeptides, and antineoplastic agents. In order to overcome this barrier, a number of methods have been employed including the use of prodrugs, antibody and drug carrier systems such as liposomes (28-30). Because the brain involves a variety of receptor-mediated transport systems to control the entry and exit of hydrophilic molecules and macromolecules, such systems can be utilized for brain drug targeting and transport. It is known that LDL receptors exist on endothelial cells of brain capillaries for LDL endocytosis (31-33). Thus LDL can potentially be used as carriers for those drugs that are unable to pass through BBB freely.

2.3.3. Acetylated LDL for drug targeting

When chemically altered lipoproteins appear in the circulation of the human body, the RES system is activated to remove these altered lipoproteins if they are recognized as foreign substances. The process involves the scavenger receptors on the cell surface of human macrophage (34). Unlike T4 lymphocytes, which lead to collapse of the immune system when they are infected by human immunodeficiency virus (HIV), HIV infected macrophages allow HIV to replicate for a long period of time. Macrophages play a very important role in HIV dissemination to various organs and to other parts of the immune system (35-36). Experiments have shown that when antiviral drugs, e.g. AZT, are incorporated into chemically altered LDL, such as acetylated LDL, the HIV infected macrophages can be targeted (37).

2.3.4. Lactosylated LDL for drug targeting

In the liver, Kupffer cells play a critical role during inflammation through enhanced expression of adhesion molecules, often resulting in the harmful infiltration of

neutrophils into the liver. In addition, the production of inflammation mediators, such as interleukins and tumor necrosis factors by Kupffer cells, causes a cascade of events that are related to serious physiological problems (38). Because only Kupffer cells express galactose particle receptors in the liver, lactosylated LDL became a good candidate for drug targeting to Kupffer cells (39). A cholesterol-conjugated oligonucleotide, which is a potent inhibitor to the gene expression of intercellular adhesion molecule-1, was associated with lactosylated LDL and the antisense oligonucleotide was efficiently delivered into Kupffer cells (40), indicating the specific uptake of the encapsulated content by Kupffer cells.

2.3.5. Oxidized LDL for drug targeting

Atherosclerosis is responsible for more deaths than any other disease in Western countries. One important hallmark of this disease is the appearance of lipid-loaded macrophages in the vessel wall. Current available therapies such as percutaneous angioplasty and bypass surgery are limited by recurrence or worsening of the atherosclerotic process. Photodynamic therapy involving various photosensitizers was considered as a promising new therapy in recent years (41). One obstacle to this therapy is how to efficiently deliver photosensitizers into macrophage cells. It is known that a high level of scavenger receptors is expressed on the cell surface of macrophages with the atheroscelosis plaque (34). These scavenger receptors can be good candidates for targeted delivery. It has been shown that photosensitizer aluminum phthalocyanine chloride associated with oxidatively modified LDL (OxLDL) was selectively delivered to macrophages (42).

2.3.6. Surface modified LDL for gene delivery

The success of gene therapy is dependent on a safe and efficient gene delivery system. Most of the current gene therapy protocols are based on viral gene delivery vectors, which may cause long term safety problems (43). Although many non-viral gene delivery vectors have been widely investigated, most of them were limited by low transfection efficiency. Lipoprotein has been used to construct a new gene delivery system to increase the safety and efficiency. Kim et al. developed a Terplex system, which had a diameter about 100 nm. The Terplex system was formed through the balanced hydrophobic and electrostatic interactions among LDL, lipidized poly(L-lysine), and plasmid DNA (44-45). This system has demonstrated its efficiency by delivering both plasmid DNA and antisense oligonucleotide to smooth muscle cells and lung fibroblasts. As an endogenous nanoparticle, LDL played a key role in the internalization of the Terplex system into the target cells via LDL receptor-mediated endocytosis.

2.4. High-density lipoprotein (HDL) for drug targeting

HDL is the smallest lipoprotein with a diameter of 7-11 nm. It shares common structural characteristics with other lipoproteins. However, its polar shell contributes more than 80% of the total mass. Newly synthesized HDL hardly contains any cholesteryl ester molecules. Cholesteryl esters are gradually added to the particles via enzymatic reaction by lecithin:cholesterol acyltransferase (LCAT), a 59-kD glycoprotein associated with HDLs. The cholesteryl esters in HDL can also be transferred to VLDL and LDL via another associated protein, cholesteryl ester transfer protein. The uptake of HDL into cells appears to occur in a similar way as for LDL. However, cholesterol uptake from HDL also involves more selective means than wholesale uptake as its

cholesteryl esters can be transferred into the cells (46). Although the function of HDL in human body is not well defined, in general it transports excess cholesterol and cholesteryl esters from various tissue cells to the liver. The major advantage of utilizing HDL for drug delivery and targeting is their small size and fast internalization by tumor cells. Among various lipoproteins, HDL has the smallest size. This makes it easier to pass through the vascular pores to reach the target tissue and quicker to be internalized by the cells. HDL has mainly been used for the incorporation of water insoluble anticancer drugs for targeting (47-48). When the anticancer drug Taxol was incorporated into HDL, stable complexes were formed for cancer cell targeting (48).

2.5. Artificial lipoprotein for drug delivery and targeting

Endogenous lipoprotein for drug delivery has usually been purified from plasma by gradient ultracentrifugation. These lipoproteins are limited in availability and loading additional transport or gene transfection enhancers has been problematic. In order to overcome such limitations, the concept of artificial lipoprotein can be utilized. Previously, several research groups have attempted to develop artificial lipoproteins (49-52). However, these studies have primarily been focused on the incorporation of natural apo-B protein into lipid microemulsion for biochemical and metabolism research, and few of them on drug delivery and targeting.

By mimicking the structure of natural lipoproteins, artificial lipoproteins have been extensively investigated in our laboratory to incorporate different lipidized proteins or peptides for a diversified drug delivery and targeting strategy. The artificial lipoproteins consist of two structural portions, a hydrophobic core and a polar shell containing surface proteins. The hydrophobic core is mainly composed of triolein and

cholesterol oleate. The polar shell is composed of egg phosphatidylcholine, lysophosphatidylcholine, cholesterol, and lipidized protein or peptide. The fatty chains on the lipidized proteins and peptides serve as anchors to interact with the phospholipid chains and to form stable protein-lipid nanocomplexes. An early trial of such a system for drug delivery and targeting was through the constitution of a lipidized poly-L-lysine onto phospholipid nanoemulsion particles for gene delivery to tumor cells (53). The incorporation of sufficient amount of palmitoyl poly-L-lysine (p-PLL) molecules onto the nanoemulsion particles led to positively charged complexes that were able to electrostatically interact with negatively charged DNA molecules. As demonstrated by Figure A.3, plasmid DNA migrated towards the positive anode because they were negatively charged (Lane 1). When plasmid DNA was incubated with p-PLL (Lane 2), no DNA migration band was observed. The binding of DNA molecules by p-PLL could block the intercalation of ethidium bromide molecules into the DNA molecules and thus no fluorescence emission occurred. When different ratios of p-PLL to nanoemulsion (i.e. the p-PLL to triolein ratio) were incubated, they demonstrated different DNA carrying capability (Lane 3 to Lane 7). A high ratio of p-PLL to nanoemulsion could tightly bind all the DNA molecules and no free DNA migration band appeared (Lane 3 to Lane 6). When the ratio of p-PLL to nanoemulsion became sufficiently low (0.0625:1 as the p-PLL to triolein ratio), plasmid DNA started to escape from the complex and free DNA bands (Lane 7) appeared on the agarose gel. Since the cell surface is normally negatively charged, the uptake of exogenous particles is largely affected by the surface charge of the particles. Positively charged particles appeared to be required for the cellular uptake of the particles. However, excess positive charge on the particles could induce cellular

toxicity and limit its use as DNA carrier. Thus particles with a properly balanced charge are required for the cellular uptake. The surface charge of the nanoemulsion/p-PLL/DNA complexes were measured and their zeta potentials are shown in Figure A.4. The zeta potentials of the particles increased with the increase of the amount of p-PLL when a fixed amount of DNA was used. Among the nanoemulsion/p-PLL/DNA complexes examined for the gene transfection, the complex with the zeta potential of 8.47 ± 1.85 mV resulted in the highest transfection efficiency. Such complexes demonstrated similar transfection efficiency as the Lipofectamine™, a commercial gene transfection product. The artificial lipoprotein complex, however, had much lower toxicity (Figure A.5).

In recent years, cancer drug delivery and targeting have become a very active research area. Many cancer cells overexpress specific receptor proteins or peptides, which can recognize and bind specific ligands. For example, during the development of a tumor, angiogenic endothelial cells overexpress α_v integrin, which can specifically recognize cyclic peptides containing Arg-Gly-Asp motif (54). Many other specific ligand-receptor binding has also been found in tumor cells (55-57). In order to direct anticancer drug to the specific cellular site, an effective delivery system becomes critically important. The nanoscale size of the artificial lipoproteins and their capability of incorporating different recognition protein ligands may present a practical solution for anticancer drug targeting and effective gene delivery.

3. Protein-protein complexes and drug targeting

Protein-protein interactions are critical events for a wide range of physiological and pathological processes. The biological formation of protein nanostructures through protein-protein interactions must be controlled in a precise manner in order to function

properly. In viruses, virtually all the cellular processes including the formation of protein nanostructures during replication and assembly involve protein-protein interactions (58). In the human immune system, the proper interaction between CD4 and CD8, the cell surface proteins expressed on T-cells, with T-cell receptor (TCR) and major histocompatibility complex (MHC) Class I or II is required to activate T-cells (59). The importance of such protein-protein interactions, as in the examples of viral replication and assembly and immune activation in human body makes these nanocomplexes to be ideal therapeutic targets for new drug developments.

There are many types of nanoscale protein-protein complexes including homodimers, heterodimers, antigen-antibody complexes, enzyme-inhibitor complexes, and multicomponents such as viral coat protein and ribosomes (60). The formation of these protein nanostructures via protein-protein interactions generally involves large and relatively flat surface areas with numerous contact sites, making it difficult to use small drug molecules to block such processes. However, these interactions require precise control in order to form pathophysiologically functional complexes. This presents an opportunity for therapeutic drug design and development. In herpes virus (HSV), HSV ribonucleotide reductase (RR) is a tetramer ($\alpha_2\beta_2$) that consists of two large R1 subunits and two small R2 subunits (61-62). The formation of an intact tetramer through proper interactions is important for the survival of HSV. When a synthetic peptide YAGAVVNDL was introduced into cells, the activity of RR was inhibited without causing significant side effect in the host cells (63-64). The proper formation of other protein nanocomplexes in HSV, such as DNA polymerase (heterodimer) and helicase-

primase complex, are also essential for the virus. Therefore, these nanocomplexes are also being considered as potential therapeutic targets.

In HIV, protease, intergrase, and reverse transcriptase are all homodimer nanostructures formed by protein-protein interactions. Protease has been one of the primary therapeutic targets for AIDS chemotherapy being critical for viral maturation. All successful inhibitors of HIV-1 protease to date are peptide mimetics that bind to the active site of the protease. Because of specific mutation within the HIV-1 genome (65), drug-resistant proteases appeared in many HIV-1 strains. A good alternative to this therapeutic strategy would be agents that can block dimeriazation of protease (66-67). The assembly of other important protein nanostructures, such as intergrase and reverse transcriptase, can also be inhibited by many peptides at the dimeric interface (68-69). These types of protein-protein interactions are also widely observed in other types of viruses and have been considered as therapeutic target for drug development (70-71)

Although the formation of protein nanostructures through protein-protein interactions is critical to many physiopathological processes, it has been difficult to develop effective drug compounds to inhibit these processes. Recent design and screening strategies include rational structure-based drug design, peptide display technology, and *in vivo* genetic selection systems (72-74). However, many issues relating to drug delivery, such as cell permeability, intracellular localization and physicochemical stability, must be resolved. Subsequently, various systems including scrape loading, electroporation, and delivery systems have been investigated. Among the delivery systems, liposomes, polycationic peptides, viruses and proteins of eukaryotic, bacterial or viral origin have been studied (75-78). A good example of utilizing protein as delivery

vehicle is the B subunit of *E. coli* heat-labile enterotoxin, which is able to deliver bioactive peptide to the cells to disrupt viral protein-protein complex (79).

4. Protein-carbohydrate complexes and drug targeting

As one of the major groups of biological molecules, carbohydrates are unique in that they can have many branches and their monomeric units can connect to each other in different linkages in contrast to proteins and nucleic acids, which are exclusively linear and have only one type of linkage (amide linkage in proteins and 3'-5' phosphodiester linkage in nucleic acids). Most carbohydrates exist as nanoscale complexes with proteins (glycoproteins) or lipids (glycolipids). The complex sugar chains of glycoproteins and glycolipids play very important roles in the control of cellular functions and cell-cell recognitions, and therefore extensive investigations into the assembly of carbohydrate complexes may yield important information for drug targeting development.

Glycoproteins are one of the major components in the outer surface of mammalian cells. They play critical roles in many important biological processes such as cell growth, fertilization, cell adhesion, immune responses, bacterial and viral infections, degradation of blood clot, and inflammation. The majority of glycoproteins are formed by covalent attachment of carbohydrates to the nitrogen atom (provided by asparagines residue) or oxygen atom (provided by serine or threonine residue) in proteins. The proteins are glycosylated as they move through the lumen of endoplasmic reticulum (ER) and Golgi apparatus in the cells, mostly by glycosidase and glycotransferase. The type and extent of glycosylation is dependent on the type and nature of proteins, cells and tissues (80).

The fusion of HIV envelope with host cell membranes is a critical step for HIV to enter the cells. The envelope glycoproteins of HIV are highly glycosylated. HIV-1 gp120

contains 20-25 glycosylation sites and the carbohydrates contribute about 50% of the apparent molecular weight. Blocking of the protein glycosylation can significantly interfere with the normal life cycle of HIV (81). Many sugar analogues have been screened for the anti-HIV activity *in vitro*. One of these analogues, N-butyldeoxygalactonojirimycin (NB-DNJ) has been shown to be a potent inhibitor of infection with minimal cytotoxicity. In hepatitis B virus, although there are only two glycosylation sites on the glycoprotein, the viral replication and assembly was inhibited by the treatment of NB-DNJ. Gp41, another HIV envelope glycoprotein, also plays an important role in the fusion of HIV envelope with host cell membranes. Corresponding peptide and non-peptide inhibitors to gp41 have been developed (82). Knowledge about the HIV-1 envelope glycoprotein has provided insight into the possibilities for design of novel HIV vaccines (83). Protein-carbohydrate nanostructures in HIV-1 have currently become the most important therapeutic targets for the development for anti-HIV drugs.

The molecular targets for new anticancer agents include inducers of cell differentiation, cell cycle arrest, apoptosis, and signaling pathways for growth factors and cytokines. Because the protein glycosylation pathways are ubiquitous in cancer cells, they provide excellent opportunities for anticancer drug targeting. For example, alkaloid swainsonine, a Golgi alpha-mannosidase II inhibitor, is the first inhibitor to be selected for clinical test (84). Because p-glycoproteins are multidrug transporters that result in multidrug resistance in cancer chemotherapy, inhibitors targeting this protein have been developed (85). Protein glycosylation pathways are not only the ideal targets for drug development to treat cancers, but are also excellent targets in other diseases. For example, platelets play an important role in the pathophysiology of certain diseases such as acute

myocardial infarction and diabetes mellitus. Platelet activation and aggregation is caused by the activation of the glycoprotein IIb/IIIa receptor. Thus glycoprotein IIb/IIIa has been considered as a therapeutic target in these diseases (86-87).

5. Protein-nucleic acid complexes and drug targeting

Nucleic acids (DNA and RNA) are linear polymers of nucleotides with linkages of 3' to 5' by phosphodiester bridges. The genetic information for making all functional macromolecules are stored by the cellular DNA and accessed through the transcription of information into RNA. A typical DNA double helix has a diameter of 2 nm with varying length depending on organism. RNA occurs in various forms with different important biological functions such as messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), and small nuclear RNA (snRNA).

The protein-nucleic acid nanostructures resulting from the interactions between protein and nucleic acids are critical to almost all aspects of genetic activity within an organism including DNA replication, transcription, packaging, rearrangement, and repair (88). Based on the structural motif for DNA binding, there are four types of major protein-DNA complexes, i.e. Helix-Turn-Helix (HTH), Zinc Finger (ZF), Basic Leucine Zipper (B-Zip), and Basic Helix-Loop-Helix (B-HLH). Although the proteins in protein-DNA complexes are very diversified, the basic goal is to achieve a precise complementarity of the molecular shapes. This requires specific chemical recognition between proteins and their particular DNA targets. Thus it has been proven possible to design proteins with novel recognition specificities for the purpose of breaking the normal protein-DNA binding (89). On the other hand, a specific DNA or DNA complex can also be designed to bind the protein. For example, peptide nucleic acid (PNA), a DNA mimic in which the nucleobases are attached to a pseudopeptide backbone, was

used to arrest transcription within a gene sequence and to provide an artificial open complex to promote transcription (90).

Certain potent drug molecules interfere with DNA transcription by binding to the transcription factors and thus obstructing the specific DNA binding (91). For example, doxorubicin can bind to NIL2A, a basic leucine-zipper transcription factor, to inhibit the formation of NIL2A/DNA complex. Echinomycin can bind to EGR1, a Cys2His2 zinc finger transcription factor, and inhibit the formation of EGR1/DNA complex. Intercalators such as nogalamycin and hedamycin, G/C-rich minor groove binding drugs such as chromomycin A3, and A/T-specific drugs such as pluramycin can effectively inhibit the transcription factor-DNA complex formation (91-92).

Interaction between protein and RNA plays very important roles in post-transcriptional RNA processing and protein biosynthesis. For example, spliceosomes, complexes of small nuclear RNAs and different proteins, are responsible for the precise formation of messenger RNAs. Ribosomes, the complexes of proteins with RNAs, are the agents for protein synthesis. For these complexes of proteins and RNAs, either component can be the target for chemotherapy. For example, peptide nucleic acid (PNA) can be targeted to mRNA to block protein synthesis in an antisense strategy. PNA can also be targeted to the RNA components of ribonucleoproteins (RNPs) to inhibit their enzymatic activities (90).

One of the most interesting protein-RNA complexes for drug targeting is telomerase, a protein-RNA complex that elongates telomeric DNA and appears to play an important role in cellular immortalization (93). Telomeres are nucleoprotein structures at the end of human chromosomes. They play a fundamental role in the regulation of

cellular lifespan (94). The tandemly repeated DNA sequence of telomeres is specified and controlled by telomerase, which is tightly repressed in the vast majority of normal cells but becomes activated during cell immortalization and in cancers (94-95). Telomerase has received much attention as a novel and potentially highly specific target for the development of new anticancer therapeutics (96-97).

6. Concluding remarks

Nanoscale protein complexes in the biological system, including protein-lipid, protein-protein, protein-carbohydrate, and protein-nucleic acid complexes, are ubiquitous in living systems. They play essential roles in various biochemical and genetic activities in cells and viruses. As a result, they become very important objects in pharmaceutical and biomedical research and development, especially in the development of targeted drug delivery systems. The understanding of the formation, structure, and function of these protein nanostructures are essential for the development of targeted therapeutic delivery systems either using these nanostructures as drug carriers or treating these nanostructures as the therapeutic targets. With the rapid advancement in life sciences, pharmaceutical and biomedical sciences, and nanoscience and nanotechnology, more and more efficient targeted drug delivery strategies based on protein nanostructues can be developed.

Figure legends:

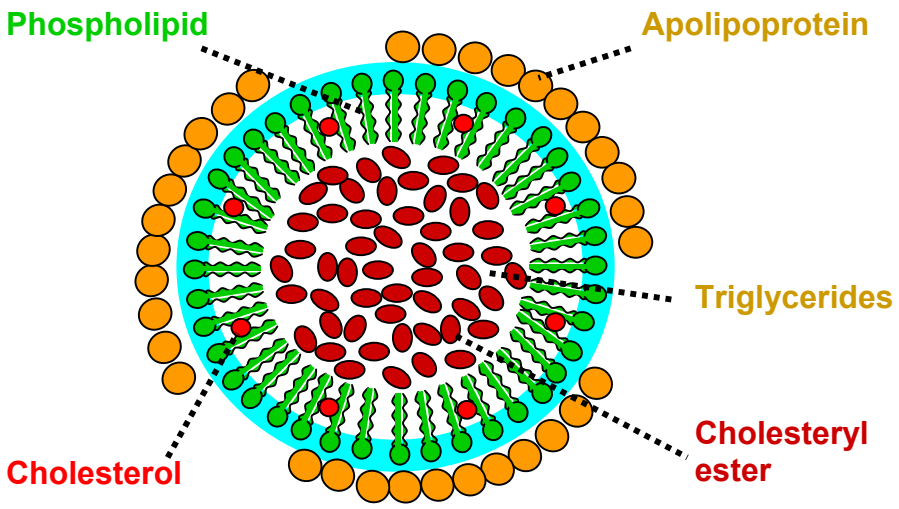
Phospholipid
Apolipoprotein
Triglycerides
Cholesteryl ester
Cholesterol

Figure A.1

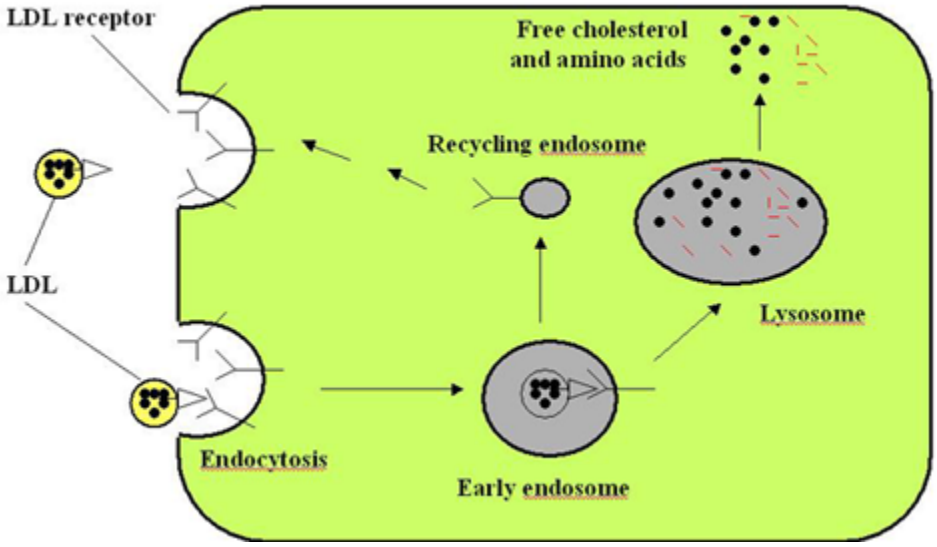
LDL receptor
Free cholesterol
and amino acids
Recycling endosome
LDL
Lysosome
Endocytosis
Early endosome

Figure A.2

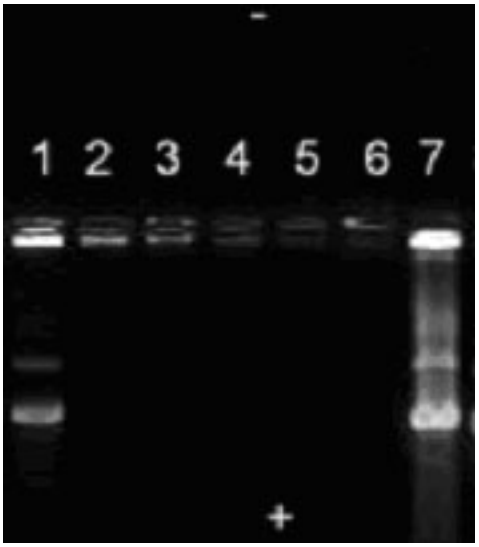

Figure A.3

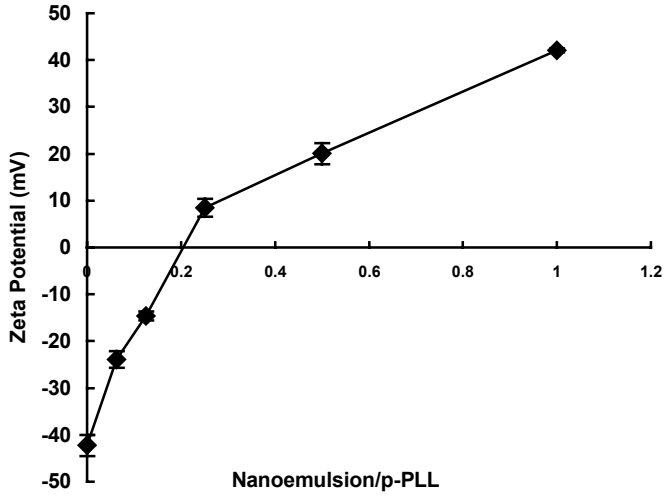
Zeta Potential (mV)
50
40
30
20
10
0
-10
-20
-30
-40
-50
0
0.2
0.4
0.6
0.8
1
1.2
Nanoemulsion/p-PLL

Figure A.4

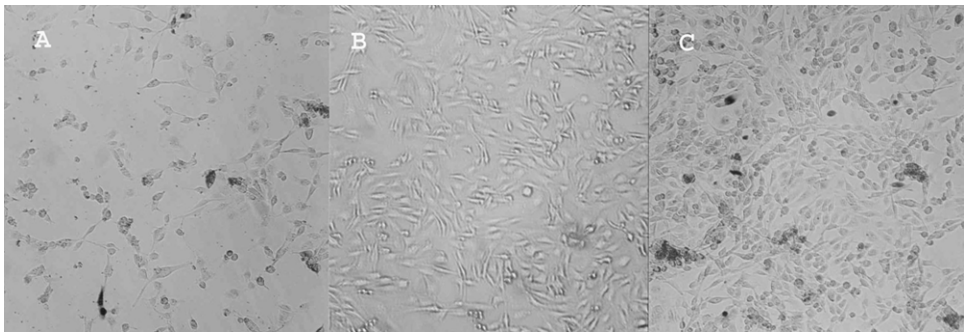

Figure A.5

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