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TARGETED DRUG DELIVERY VIA THE FOLATE RECEPTOR

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

the Degree Doctor of Philosophy in the Graduate

School of The Ohio State University

By

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*** * * * ***

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ABSTRACT

The folate receptor (FR) is a highly selective tumor marker overexpressed in greater than 90% of ovarian carcinomas. Two general strategies have been developed for the targeted delivery of drugs to FR-positive tumor cells: 1) coupling to a monoclonal antibody against the receptor and, 2) coupling to a high affinity ligand, folic acid. Folic acid, a high affinity ligand of the FR, retains its receptor binding properties when derivatized via its γ -carboxyl group. Folate conjugation has been successfully applied *in vitro* for the receptor-specific delivery of protein toxins, anti-T-cell receptor antibodies, interleukin-2, chemotherapy agents, γ -emitting radiopharmaceuticals, magnetic resonance imaging contrast agents, liposomal drug carriers, and gene transfer vectors. This dissertation presents novel approaches to using folate receptor-mediated delivery. First, FR-targeted liposomes were evaluated in FR(+) tumor cells as carriers for a lipophilic boron compound for neutron capture therapy (Chapter 2). These carriers were capable of specific receptor binding and FR-mediated endocytosis *in vitro*. Second, novel pH-sensitive liposome formulations based on incorporation of oleyl alcohol were evaluated (Chapter 3). FR-targeting was used in combination with these pH-sensitive liposomes for the purpose of drug delivery and were evaluated *in vitro*. These FR-targeted, pH-

sensitive liposomes, entrapping the membrane-impermeable chemotherapeutic agent, cytosine- β -D-arabinofuranoside (araC), showed ~17-times greater FR-dependent cytotoxicity in a FR(+) cell line compared with araC delivered via FR-targeted non-pH-sensitive liposomes. The third approach involved a novel lipophilic conjugate of folate, folate-polyethylene glycol (PEG)-cholesterol, which was synthesized and evaluated for FR-mediated delivery of liposomes to tumor cells (Chapter 4). As an alternative to phospholipids, cholesterol was found to be an effective anchor for incorporation of a targeting ligand into liposomes. All three approaches demonstrated that the small size, convenient availability, simple conjugation chemistry, and lack of immunogenicity of folic acid make it an ideal ligand for targeted delivery of drug carriers to tumors.

Dedicated to my parents

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3. W. Guo, T. Lee, J. J. Sudimack and R. J. Lee. Receptor-specific delivery of liposomes via folate-PFG-chol, *J. Liposome Res.*, **10**:179-195 (2002).
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FIELDS OF STUDY

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CHAPTER I

INTRODUCTION

1.1. Background

Targeted drug delivery systems promise to expand the therapeutic windows of drugs by increasing delivery to the target tissue as well as the target to non-target tissue ratio. This will in turn lead to a reduction in the minimum effective dose of the drug and the accompanying drug toxicity, and an improvement in therapeutic efficacy at equivalent plasma concentrations. Given the often limited number of targeted receptor sites on any given target tissue, targeted delivery is a particularly attractive approach for agents that have narrow therapeutic windows and/or that are active at very low concentrations.

For targeted delivery to tumor, the cellular target often consists of a membrane-bound tumor-associated antigen (TAA). The receptor for the vitamin folate is a marker for ovarian carcinomas and is frequently overexpressed in a wide range of other types of tumors. It therefore presents an attractive target for tumor-selective drug delivery [1-9].

1.2. Overexpression of Folate Receptors in Human Tumors

Folate receptor (FR), also known as the high affinity membrane folate-binding protein, is a glycosylphosphatidylinositol (GPI)-linked membrane glycoprotein with an apparent molecular weight of 38–40 kDa [10]. Two membrane-bound isoforms of FR have been identified in humans, designated α and β . FRs bind folic acid (an oxidized form of folate) with high affinity. FR α -isoform has a dissociation constant (K_d) for folic acid of ~ 0.1 nM, which is approximately one-tenth that of its K_d for reduced folates (e.g., 5-methyltetrahydrofolate) [11, 12]. The role of FR in cellular folate transport is not well understood, although a "potocytosis" model has been proposed [13]. FRs were found to be clustered in non-coated membrane regions called caveolae. Localization of FRs in caveolae and receptor internalization can be induced by receptor crosslinking and is regulated by cholesterol [13, 14]. Rather than the FR, an anion transporter with a K_t in the micromolar range and sensitivity to the anion transporter inhibitor, probenecid, appeared to be responsible for the transmembrane cellular transport of physiological forms of the folate coenzyme, 5-methyltetrahydrofolate [15].

While elevated expression of FR has frequently been observed in various types of human cancers, the receptor is generally absent in most normal tissues with the exceptions of choroid plexus, placenta, and low levels in lung, thyroid and kidney [8]. Although FR type β has been found on CD34+ cells, the receptor curiously lack affinity for [3 H] folic acid and various folate derivatives [9]. FR type α is frequently overexpressed in tumor cells in culture and epithelial lineaged tumors such as ovarian carcinomas. Several studies show that over 90% of ovarian carcinomas overexpress the FR [4, 7]. Expression of the

receptor is mostly homogeneous in ovarian cancer tissue samples [4]. In a recent study, a monoclonal antibody against the type α receptor, LK26, was used to determine the frequency of FR overexpression in human tumors by indirect immunohistochemical staining [4]. High frequencies of receptor overexpression were found in many types of tumors, including ovarian (52 of 56 cases tested), endometrial (10 of 11), colorectal (6 of 27), breast (11 of 53), lung (6 of 18), renal cell (9 of 18) carcinomas, brain metastases derived from epithelial cancers (4 of 5), and neuroendocrine carcinomas (3 of 21) [4]. In a separate study, quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis was used to determine the mRNA transcript levels of both subtypes of the FR in various human tumor tissues, and [3 H] folic acid binding was also used to determine the FR ligand binding capacity in these tissues [7]. Highly elevated levels of the FR were detected in choriocarcinomas, meningiomas, uterine sarcomas, osteosarcomas, non-Hodgkin's lymphomas, and promyelocytic leukemias [7]. FR type β is frequently overexpressed in non-epithelial lineaged tumors such as sarcomas and acute myeloid leukemias but not in established cell lines of the same origin [16]. The causes of FR overexpression in cancers are unclear. Transfection and expression of FR on NIH/3T3 cells provides cells with the ability to survive in low folate medium and increased cell growth both *in vitro* and *in vivo* [17, 18]. However, another study showed that transfection of cervical carcinoma cells with FR cDNA led to a decrease in the rate of cellular proliferation rather than an increase, suggesting FR expression may not provide a growth advantage to rapidly dividing cells in all cases [19]. Studies also show that high levels of FR expression are associated with increased biological aggressiveness of ovarian

carcinomas as shown by a higher percentage of S-phase cells and increased resistance to chemotherapeutic agents [20]. Thus FR elevation may be a useful prognostic factor.

The prevalence of FR overexpression among human tumors makes it a good marker for targeted drug delivery to these tumors. Two strategies have been developed for FR-specific drug targeting: 1) coupling to monoclonal antibodies (e.g., MOv18) against the FR and 2) coupling to folic acid, in which folic acid functions as the targeting ligand. High affinity FR binding is retained when folate (Figure 1.1) is covalently linked via its γ -carboxyl group to a foreign molecule. The general model for the cellular uptake of drug conjugates targeted to the FR is illustrated in Figure 1.2.

This review summarizes recent studies on the diagnostic and therapeutic applications of targeting the FR *in vitro* and *in vivo* using either folate or monoclonal antibodies against the FR. The drugs or drug carriers evaluated include liposomes, protein toxins, plasmid DNA complexes, imaging, chemotherapeutic, and immunotherapeutic agents [27, 28]. Targeted drug delivery via the FR has been reviewed in several recent articles [21-26].

1.3. Delivery of Radiopharmaceuticals

Radioactive tracer techniques are widely used in the diagnostic imaging of tumors due to their ability to non-invasively probe for tissue pathophysiology. The development of new and improved tumor-selective radiopharmaceuticals is clinically desirable as a means of: i) detecting and/or confirming the presence and location of primary and metastatic lesions; ii) probing biochemical features of neoplastic tissue that have implications for tumor staging and/or subsequent treatment planning, and iii) monitoring tumor response to treatment. Photon emitting radiopharmaceuticals that can be used to generate planar and/or tomographic (SPECT) images comprise the cornerstone of current nuclear medicine practice due to the widespread availability of appropriate radionuclides (e.g., ^{99m}Tc , ^{123}I , ^{67}Ga , and ^{111}In), FDA-approved radiopharmaceuticals, and the required imaging equipment. Actively targeted γ -emitting radiopharmaceuticals promise to greatly improve the specificity and sensitivity of scintigraphy imaging procedures. If sufficient tumor specificity can be achieved, radiopharmaceuticals labeled with an appropriate α - or β -emitting radionuclide can also provide an attractive means for site-selective radiation therapy. Criteria for radionuclide selection in the development of diagnostic or therapeutic radiopharmaceuticals include the physical and biological half-lives of the radionuclide, presence or absence of particle emissions and conversion electrons, as well as the pharmacokinetic and biodistribution properties of the radiopharmaceutical.

Monoclonal antibodies have been evaluated clinically for the tumor-selective delivery of radionuclides for both therapeutic and diagnostic purposes through techniques including radioimmunoscintigraphy (RIS), single-photon-emission computed tomography

(SPECT) and radioimmunotherapy (RIT). Radiolabeled MOv18, a monoclonal antibody against the FR, and its fragments were used in clinical studies in ovarian cancer patients.

1.3.1. Delivery of Radionuclides Utilizing MOv18

Radiolabeled antibodies against the FR have been used in the immunohistochemical detection of tissue FR expression, in planar or tomographic SPECT imaging, as well as in radioimmunotherapy treatment of ovarian carcinomas.

In a recent clinical study, ^{131}I -MOv18 was administered either intravenously (i.v.) or intraperitoneally (i.p.) to 30 patients with ovarian carcinoma.

Radioimmunosciintigraphy (RIS) was carried out in this study to compare the two routes of administration. A more favorable tumor to non-target (T/NT) ratio (2.8 vs. 1.9) and tumor uptake (1.3% vs. 0.8%) resulted when the ^{131}I -MOv18 was delivered intraperitoneally. For diagnostic applications, the i.p. route of administration was not very practical. However, the significant increase in targeting ratio achieved via the i.p. route compared to the i.v. route suggested that it may be more appropriate for certain therapeutic applications. The average sensitivity of this method was shown to be 73% (55 tumors detected out of a total of 75 tumors) and the specificity was 100% [29].

In a separate clinical study, 16 patients with minimal residual disease of ovarian cancer were treated by radioimmunotherapy (RIT) with a single i.p. dose of ^{131}I -MOv18. Complete response was observed in 5 patients, no change in 6 patients, and progressive disease in 5 patients [30]. Furthermore, 1 patient exhibited long-term complete response in a follow-up exam [30].

In single-step radioimmunoimaging protocols, patients are exposed to radiation for a relatively long period of time since it was shown to take approximately 48-72 hours for the optimal tumor/non-target (T/NT) ratios to be reached following radiotracer administration [29]. In order to minimize patient radiation exposure, a two-step method was developed. In a two-step protocol, an initial i.p. injection of biotinylated MOv18 was followed by a waiting period, which allows the MOv18 to localize in tumors, and then an i.p. injection of ^{111}In -streptavidin was administered. SPECT was then performed for tumor imaging. The mean T/NT ratio obtained was higher (9 vs. 2.8) than when the conventional one-step method was utilized [31]. The improvement in tumor targeting was due to the reduced size and accelerated systemic clearance of the radiotracer ^{111}In -streptavidin in comparison with the radiolabeled whole antibody. However, this method has limited appeal for clinical imaging applications due to the requirement for repeated i.p. injections at various time intervals and the potential immunogenicity of the streptavidin.

Finally, a three-step method was developed where patients were first administered biotinylated MOv18, then avidin, and followed by ^{111}In -labeled biotin. The T/NT ratio obtained was 1.7, in contrast to 9 for the two-step method. The relatively poor outcome of this procedure was due to the internalization of the antibody/antigen complexes induced by avidin crosslinking [32]. The receptor-bound avidin, therefore, was no longer accessible to the radiolabeled biotin derivative. While detrimental to tumor imaging, this mechanism of cellular internalization may be exploited for FR-mediated delivery of certain therapeutic agents.

1.3.2. Delivery of Radionuclides Utilizing Low Molecular Weight Folate Conjugates

Radiolabeled low molecular weight folate conjugates compared with radiolabeled antibody have a greatly reduced size and therefore, a much faster systemic clearance. This in turn leads to increased tumor to background ratios. The radionuclides, which have been evaluated as folate conjugates, include ^{67}Ga , ^{111}In , and $^{99\text{m}}\text{Tc}$. The results are summarized as follows:

^{67}Ga and ^{111}In are γ -emitting radionuclides commonly used in medical imaging procedures with half-lives of 78 h and 68 h, respectively. When administered as a citrate complex, ^{67}Ga , forms a high affinity complex with circulating transferrin. Since the transferrin receptor is frequently overexpressed in various types of tumors, the ^{67}Ga -transferrin complex preferentially localizes in the tumor, which allows for tumor imaging [33-35]. Deferoxamine is a commonly used chelator that forms a stable complex with Ga. ^{67}Ga -labeled deferoxamine (DF) conjugates have been frequently used in positron emission tomography (PET) and gamma scintigraphy [36-38]. For FR imaging, folate was conjugated to deferoxamine via its amino group [39]. The resulting conjugate, deferoxamine-folate (DF-folate), was efficiently labeled with ^{67}Ga [39]. ^{67}Ga -DF-folate was shown to have 100 times greater uptake in FR-positive oral carcinoma KB cells than the nontargeted ^{67}Ga -DF [39]. Further, KB cell binding was completely blocked by excess free folate, indicating that binding was specific for the FR [39]. ^{67}Ga -DF-folate was then evaluated for its tumor localizing properties in athymic mice, on a folate free diet, carrying xenograft implants derived from subcutaneously injected KB cells [40, 41]. The tumor to blood ratio obtained at 4 hours post i.v. injection reached 409 to 1, which was far superior

to the various controls, including ^{67}Ga -DF and ^{67}Ga -citrate. Only, the tumor and the kidneys accumulated high levels of the radionuclide conjugate. Radioactivity clearance from the tumor and the kidneys was slower than from other organs, resulting in even higher tumor to blood ratios ($> 1,000$) at 24 h post injection [40]. The undesirable radionuclide accumulation in the kidneys was likely due to the presence of FRs in the kidney proximal tubules. Targeting efficacy was much poorer in mice on the folate-enriched regular rodent diet or when excess free folate was pre-administered as a receptor blocking agent [40]. In addition, the superb imaging property exhibited by ^{67}Ga -DF-folate was primarily attributable to its rapid blood clearance kinetics.

An ^{111}In -labeled diethylenetriaminepentacetic acid (DTPA)-folate was also synthesized and evaluated both *in vitro* and *in vivo* using the KB xenograft tumor model, as described for ^{67}Ga -DF-folate [42]. The tumor to blood ratio obtained at 4 hours post i.v. administration was 82 to 1 [43]. Overall the data obtained with ^{111}In -DTPA-folate was very similar to those for ^{67}Ga -DF-folate. There was, however, a significant reduction in hepatobiliary clearance of this agent compared to ^{67}Ga -DF-folate. The overall clearance kinetics of this agent, therefore, was more rapid and more favorable for tumor imaging. ^{111}In -DTPA-folate is currently being developed as a clinical imaging agent. A phase I/II radioimaging clinical study of ^{111}In -DTPA-folate is being conducted at the University of Texas MD Anderson Cancer Center in ovarian cancer patients.

Compared to ^{111}In and ^{67}Ga , $^{99\text{m}}\text{Tc}$ is considered to be a more favorable radionuclide for radioimaging applications due to its short half-life of 6 hours, a 140 keV γ -radiation energy, which is optimal for available imaging equipment, and low cost. A

novel folate conjugate of a Tc-chelating ligand, 6-hydrazinonicotinamido-hydrazido (HYNIC)-folate, was recently synthesized in our laboratory (Figure 2.3). HYNIC-folate was radiolabeled with ^{99m}Tc using tricine and trisodium triphenylphosphine-3, 3', 3''-trisulfonate (TPPTS) as co-ligands. The receptor binding property of ^{99m}Tc -HYNIC-folate was studied in cultured KB cells. FR-mediated uptake was ~ 300 times the non-specific binding in the presence of 1 mM free folic acid (Figure 2.4). ^{99m}Tc -HYNIC-folate was further evaluated in a C57BL/6 mouse model carrying syngeneic solid tumor grafts derived from 24JK-FBP cells, a methylcholanthrene-induced sarcoma cell line transfected with the human FR type α [44]. Tumor to blood ratios reached 55 and 81 at 4 hours and 24 hours post i.v. injection of the radiotracer, respectively [45]. Tumor uptake of the radioconjugate was blocked by the co-injection of 100 μg of free folic acid, indicating the role of the FR in tumor localization. Tumors were clearly identifiable in gamma-camera images (Figure 2.5). Two other ^{99m}Tc -labeled folate conjugates have been synthesized and evaluated for gamma-imaging, including ^{99m}Tc -DTPA-folate and ^{99m}Tc -ethylenedicysteine-folate, each showing good tumor selectivity [46, 47].

Regardless of the specific radionuclide chelate-folate conjugate used, high levels of the radiotracers were consistently found in the kidneys. This poses a potential challenge for application of low molecular weight folate conjugates in targeted radiotherapy. However, the high selectivity and the rapid clearance kinetics of these conjugates make them ideal candidates for development as imaging agents for the detection of FR positive tumors.

1.4. Delivery of Liposomal Drug Carriers

Liposomes are phospholipid bilayer vesicles. As a drug carrier, the liposome possesses the following properties: 1) the ability to entrap hydrophilic drug molecules in its aqueous interior and/or incorporate hydrophobic molecules in its bilayer(s); 2) long systemic circulation time; 3) efficient uptake by the reticuloendothelial system (RES); and 4) preferential accumulation in solid tumors due to increased endothelial permeability and reduced lymphatic drainage [48-51]. Low liposome encapsulation efficiency is a limiting factor for delivery of hydrophilic drugs and has been addressed in the delivery of an antitumor agent doxorubicin through the recent development of the "remote-loading" method, in which the encapsulation of the drug into the liposome is dependent on a pH-gradient [52]. Modification of the liposome surface with polyethyleneglycol (PEG) has resulted in reduced RES uptake and further prolonged circulation time [53-60].

The therapeutic efficacy of drug carrying liposomes can potentially be greatly improved if they could be targeted selectively to the tumor. Monoclonal antibodies and their fragments have been attached to the liposome surface to construct immunoliposomes [54-57]. The clinical potential of immunoliposomes is limited because: 1) the targeting ligand is immunogenic. The elicitation of a human-antimouse IgG antibody (HAMA) response precludes repeated administration of immunoliposomes; 2) covalent attachment of a protein to a liposome is technically challenging. The conjugation chemistry for attaching antibodies to lipids is cumbersome and may result in partial denaturation of the targeting ligand; 3) proteinaceous ligands are prone to denaturation upon exposure to organic solvent, limiting the methods available for liposome preparation; 4)

immunoliposomes are often unstable and tend to lose immunoreactivity upon storage and/or become rapidly cleared upon injection via F_c receptor-mediated RES uptake; and 5) few tumor-specific antibodies are available in quantities needed for drug targeting.

For effective targeting of liposomes, folate has to be attached via a long PEG spacer (e.g., PEG M_r 3350) to overcome the steric hindrance encountered at the cell surface [61]. In cultured KB cells, liposome uptake appeared to saturate at $\sim 2.5 \times 10^5$ sites/cell [61]. The cellular uptake of folate-PEG-liposomes was saturable and could be blocked with a high concentration (1mM) of free folic acid [60]. The requirement for high concentrations of free folate for effective receptor blocking indicated that folate-targeted liposomes had very high affinity for receptor-bearing tumor cells, possibly due to its capacity to interact with cellular FRs via multivalent interaction [61, 62]. Folate-targeted liposomes encapsulating a fluorescent dye, calcein, were taken up by cultured KB cells via receptor-mediated endocytosis and displayed punctate fluorescence patterns when examined under a fluorescence microscope [61]. Folate-PEG-liposomes entrapping doxorubicin were evaluated in cultured KB cells for uptake and cytotoxicity [63]. Cellular doxorubicin uptake following incubation with these liposomes was 45-fold higher than that of non-targeted liposomes and 1.6 times higher than free doxorubicin, and the cytotoxicity of these liposomes was 86 times and 2.7 times higher, respectively [63]. The cellular uptake of the folate-PEG-liposomes was unaffected by the presence of physiological concentrations of folate in the culture medium [63].

Folate-targeted liposomes have also been used to deliver an antisense oligodeoxyribonucleotide, which inhibits the expression of epidermal growth factor

receptor (EGFR) [64]. The main obstacles to the therapeutic application of antisense oligodeoxyribonucleotides were poor cellular uptake, short plasma half-life, and lack of tissue specificity. Folate-PEG-liposome-encapsulated oligonucleotides were efficiently delivered as indicated by increased cellular uptake. These liposomal oligos were also shown to effectively suppress the expression of EGFR in KB cells and to inhibit cell growth [64].

Folate conjugates and folate-targeted liposomes are internalized into a low-pH endosomal compartment by FR-bearing cells via receptor-mediated endocytosis. The rates of liposome content release following endocytosis and cytosolic drug delivery are affected by the environmental pH and intraliposomal buffer strength [65]. Further, the rate of endosomal release of liposome-entrapped drug molecules could be greatly increased by the utilization of a pH-sensitive lipid composition or attachment of pH-sensitive fusogenic peptides to the surface of liposomes [66, 67].

Folate offers many unique advantages as a targeting ligand for liposomes over antibodies. It is non-immunogenic, is not prone to denaturation, has good stability, has simple and defined conjugation chemistry, is highly specific for tumors, is available in large quantities, and is compatible with the use of organic solvent during preparation. Due to the relatively large size (50–200 nm in diameter) of liposomes and their tendency to passively accumulate in tumor tissues, it is difficult to predict whether the incorporation of a targeting ligand could further improve liposome uptake by solid tumors in terms of overall tumor accumulation. Studies with immunoliposomes have been few and results have been mixed. While there was generally very limited increase in tumor accumulation

due to targeting based on percent injected dose per gram of tumor tissue, significantly improved therapeutic efficacy has been observed in some cases. For example, intravenously administered anti-HER2 immunoliposomes carrying doxorubicin have shown significantly improved tumor penetration, intracellular accumulation, and antitumor activity compared with non-targeted control liposomes, which primarily accumulated in the interstitial space [57]. Therefore, beneficial effects of liposome targeting are likely found at the cellular and subcellular levels.

1.5. Delivery of Gene Transfer Vectors

Gene therapy presents a promising approach for the treatment of human diseases. A series of potential therapeutic DNA constructs for cancer gene therapy have been developed including those coding for suicide genes, cytokines, tumor-suppressor genes, antisense genes/oligonucleotides, and ribozymes. The clinical application of gene therapy, however, has been limited by the availability of suitable gene transfer vectors. An ideal gene transfer vector should be safe, stable, cost-effective to produce in clinically relevant quantities, and capable of efficient and tissue-specific gene delivery.

1.5.1. Viral Vectors

Most of the gene transfer vectors currently under clinical evaluation are based on genetically engineered replication-defective viruses, e.g., retroviral vectors, adenoviral vectors, and adeno-associated viral vectors. While often quite efficient in gene transfer, viral vectors suffer from oncogenic risks associated with random insertion into the host genome (for retro- and adeno-associated viruses) and elicitation of a host immune response, resulting in the elimination of the transduced cells and preventing repeated use of the vector due to induction of neutralizing antibodies (for adenoviruses). There is also the risk of genetic recombination inside the cell that leads to the generation of infectious viral particles. Viral vectors are also limited in gene-carrying capacity and are challenging to manufacture and purify in clinically relevant quantities. Due to these limitations, there has been considerable interest in the development of non-viral vectors based on synthetic components. Viral vectors can be targeted through attachment of a ligand. Douglas et. al.

showed that folate coupled to F_{ab} fragments against the adenoviral fiber was able to redirect the viral vector to specifically transfect cells that are positive for the FR [80].

1.5.2. Non-Viral Vectors

Existing synthetic non-viral vectors often consist of DNA complexes of cationic liposomes and/or cationic polymers [68,69]. They have the common advantages of being non-immunogenic, easy to produce, and non-restrictive on the size of the DNA molecule it carries. The structure and gene transfer activity of these complexes depend on both vector composition and the formulation method. Monovalent cationic lipids form a different type of DNA complex (lipoplexes) than cationic lipids with a multivalent cationic head group or when cationic lipid is used in conjunction with a polycation (lipopolyplexes). Under freeze-fracture electron microscopy, monovalent cationic lipid/DNA complexes (lipoplexes) often appear as "spaghetti-like" structures consisting of lipid-coated DNA double helix strands. The multivalent cationic lipid/DNA complexes and lipopolyplexes are often more compact in size and more active in gene transfer (in the absence of serum) [70]. However, the transfection activity of these complexes is usually highly sensitive to the presence of serum in the culture media. The gene transfer efficiencies of non-viral vectors are often much lower than viral vectors, especially when administered *in vivo*. Also, the performance of these vectors is quite variable depending on the route of injection. Major problems with existing non-viral vectors are vector aggregation, non-specific tissue uptake, rapid systemic clearance, and undesirable organ distribution patterns determined by the first-pass effect.

A common flaw in the design of positively charged DNA complexes for gene transfer is that the formulations are usually optimized for gene transfer *in vitro*. In tissue culture, cellular uptake of the DNA complexes is mediated by charge interaction between the positively charged DNA complexes and the negatively charged cellular membrane. This interaction further induces cellular internalization via endocytosis of the DNA complexes. However, when administered into the blood stream, the DNA complexes are placed in an environment rich in negatively charged plasma proteins and circulating blood cells. The positive charge of the complex is instantly neutralized and the complexes aggregated and accumulate in the lung by pulmonary embolism and/or in the liver due to first pass effect. Since the positive charge of the DNA complex is primarily contributing to the extent of vector aggregation and is not likely to be available for target cell interaction, negatively charged DNA complexes and naked DNA are often more active than the *in vitro* optimized gene transfer formulations, which invariably are positively charged. The fate of the DNA complex is also highly dependent on the route of administration. Intranasal, intraperitoneal, and intratumoral administration expose the vector to different biological environments; therefore, different vector formulations may be needed for optimal gene delivery efficiency for differing modes of administration.

Attachment of a targeting ligand and/or components that promote endosome escape greatly improve the gene transfer efficiency of nonviral vectors [71-76]. In addition, folate-conjugated polylysine coupled to inactivated viral particles mediated efficient receptor-specific gene delivery *in vitro* [75,76].

1.5.3. Anionic Liposome-entrapped Polyplexes (LPDII)

Neutral or anionic liposomes have also been evaluated for plasmid DNA delivery. However, they suffer from low DNA entrapment efficiency (usually less than 20%) due to the large molecular size and large negative charge of plasmid DNA. Protocols for DNA entrapment into these liposomes are often cumbersome and require the use of high lipid concentrations for high DNA entrapment, which also leads to the generation of a large number of empty vesicles. Although anionic pH-sensitive immunoliposomes deliver DNA into lymphoma ascites tumors, their practical usefulness in gene therapy has yet to be demonstrated.

In the LPDII system, DNA is first complexed to polylysine and this cationic complex is then mixed with anionic pH-sensitive liposomes carrying a receptor targeting ligand to form liposome-entrapped polyplex particles by a charge-mediated self-assembly process [77]. Folic acid has been used as the targeting ligand for the receptor-mediated gene delivery to tumor cells.

Folate-targeted pH-sensitive liposomes entrapping polyplexes (folate-LPDII) were capable of highly efficient and receptor-specific gene transfer. Folate-LPDII particles were prepared by mixing anionic liposomes composed of dioleoylphosphatidylethanolamine (DOPE)/cholesteryl hemisuccinate (CHEMS)/folate-PEG-DOPE (60:40:0.1, m/m) and the cationic DNA/polylysine (1:0.75, w/w) complexes. Vector self-assembly was mediated by charge interactions. The resulting vector particles were spherical and had a mean diameter of 70-80 nm as determined by negative-stain electron microscopy. The vector particles could be formulated at a variety of anionic

lipid/DNA polyplex charge ratios. At low lipid/polyplex ratios, the overall charge of the vector was positive, cellular uptake and transfection were mediated by non-specific charge interaction. Meanwhile, at high lipid/polyplex ratios, the vector was negatively charged and required the presence of a targeting ligand and transfected only cells that expressed the corresponding receptor. The negatively charged particles were particularly interesting due to their potential for improved compatibility with blood components and tissue-specific targeted gene delivery.

Folate-targeted LPDII vectors carrying a firefly luciferase reporter gene driven by the Rous sarcoma viral promoter (pRSVL) have been evaluated for their *in vitro* gene transfer activity in cultured KB cells. DNA/polylysine polyplexes alone showed very little transfection activity. High transfection activities were observed with folate-LPDII vectors formed at lipid/DNA ratios of 4, 6, 10, and 12 [77]. But the transfection activity of only vectors formed at high lipid/DNA ratios (10 & 12) could be partially blocked by 1 mM free folic acid [77]. Complete free folate blocking was not expected since these vectors, similar in size to large unilamellar liposomes, are likely to interact with FR-bearing cells via multivalent binding, resulting in much greater cellular affinity. These data suggest that tumor cell transfection by folate-LPDII is mediated by the FR at lipid/DNA ratios that give anionic particles but not at ratios that produce cationic particles, due to the presence of overriding charge mediated cellular uptake. The transfection activity correlates well with the level of ¹²⁵I-labeled DNA uptake by these same cells [77]. The role of the FR was further confirmed by duplicating the above studies in the receptor-negative CHO cells,

where transfection was only observed with cationic folate-LPDII vectors where uptake is mediated by charge interaction [77].

The study further showed that the pH-sensitive liposomal composition was essential for the observed transfection pattern. pH-sensitive liposomes are generally composed of a fusogenic lipid, usually DOPE, and a pH-sensitive component that is anionic at extracellular pH but becomes charge neutral at the acidic endosomal pH, such as CHEMS. DOPE is a cone-shaped lipid molecule that favors the transition from bilayer to hexagonal II phase thereby facilitating membrane fusion and/or disruption. This lipid phase transition is blocked by the presence of the negatively charged CHEMS. Upon protonation at endosomal pH, CHEMS becomes uncharged, and therefore can no longer stabilize the bilayer structure that allows for DOPE-mediated membrane fusion. Replacing DOPE with the cylindrical lipid DOPC completely abolishes transfection activity of the folate-LPDII vectors [77]. Also, replacing CHEMS with DOPS, a lipid that does not become extensively protonated at endosomal pH, eliminates transfection activity at high lipid/DNA ratios where negatively charged vectors formed [77].

The structure of folate-targeted LPDII has been studied by negative-stain electron microscopy. The vector particles appeared spherical and consisted of a high electron density core representing the DNA polyplex, and a low electron density coat representing lipid components. When dual isotopically labeled folate-LPDII particles were fractionated on a sucrose gradient, most of the lipid (labeled with ^3H) co-migrated with the plasmid DNA (labeled with ^{125}I) as an intermediate density band (DNA polyplexes have high density and liposomes have low density).

Under optimal *in vitro* conditions, negatively charged folate-LPDII vectors are more efficient in transfection than lipoplexes and are specific for receptor-positive tumor cells. The plasmid DNA in LPDII particles is also more optimally condensed compared to cationic liposome lipoplexes, resulting in the formation of smaller and colloiddally more stable particles. The negatively charged LPDII particles should also be more compatible with the *in vivo* environment, which is abundant with negatively charged components. Compared to anionic liposomes containing passively entrapped DNA, LPDII formulation has the advantage of quantitative DNA incorporation and high transfection activity without contamination by empty lipid vesicles. The main limitation of existing LPDII formulation is the high level of serum sensitivity. A possible explanation is that the fusogenic activity of DOPE is inhibited by the presence of serum. Further improvements are needed for LPDII formulations to be used for *in vivo* gene transfer. For *in vivo* applications, a potential limitation is clearance by the reticuloendothelial system (RES); however, this may be partially overcome by coating vector particles with polyethyleneglycol (PEG). This strategy has previously been applied to produce long-circulating sterically stabilized liposomes.

Folate has also been conjugated to polyethylenimine (PEI, branched polymer, M_r ~60 kDa) via a PEG linker. PEI, linear or branched, is a cationic polymer ideally suited for the formation of compact charge complexes with plasmid DNA. In addition to its DNA condensing properties, this polymer also possess endosomalytic activities due to its strong buffering capacity at endosomal acidic pH. In this study, folate conjugates of polyethylenimine were synthesized and evaluated for their ability to mediate receptor-

specific gene transfer *in vitro*. The transfection efficiency of the folate-PEG-PEI/DNA complexes was highly dependent on the PEI nitrogen to DNA phosphate (N/P) ratio, and was partially receptor-dependent [78, 79]. This conjugate is simpler than the LPDII system and produces DNA complexes of smaller sizes; it may be useful for *in vivo* tumor cell-specific gene delivery.

Antisense oligodeoxynucleotides have been specifically delivered to tumor cells *in vitro* via direct conjugation to folate [81]. Folate was attached to the 3' terminus of an anti-c-fos oligodeoxynucleotide. Its cellular uptake in cells that overexpress the FR was approximately 8-fold higher with the folate-conjugated oligo compared to the oligo alone. The folate-conjugated oligo also exhibited a much greater tumor inhibitory effect.

1.6. Delivery of Protein Toxins

Protein toxins, including plant ribosome inactivating proteins and bacterial toxins, conjugated to folate effectively kill cells overexpressing the FR without damaging the surrounding normal cells [82]. This approach is attractive due to the extreme potency of these toxins to cause cell death. Usually, delivery of a few hundred molecules per cell is sufficient to ensure cellular death. This is very important for targeted delivery systems where the capacity for targeting is limited by the number of receptors on the target cell.

Momordin, a ribosome-inactivating toxin, was conjugated to folate and was utilized in a co-culture with malignant and normal cell lines *in vitro* [83, 84]. The momordin-folate treatment resulted in the selective killing of the malignant cells without damaging the normal cells. The IC_{50} for the folate conjugates was reduced to 10^{-9} M. In addition, the momordin-folate did not significantly affect the rate of proliferation of the normal cells at the concentrations tested [84].

Other folate-conjugates, including folate-truncated *pseudomonas* exotoxin and folate-truncated diphtheria toxin, also selectively kill malignant cell lines in co-cultures [85]. LysPE38-folate conjugate (a truncated *Pseudomonas* exotoxin-folate) killed malignant cells six times faster and was 10-times more potent than the momordin-folate conjugate [85]. The augmented activity was attributed to the translocation domain, which allowed efficient endosomal escape of the folate conjugate following receptor binding and endocytosis.

Folate conjugated protein toxins are significantly smaller than the corresponding immunotoxins. This should lead to more favorable pharmacokinetics and reduced immunogenicity.

1.7. Delivery of Immunotherapy Agents

Immunotherapy via receptor targeting presents yet another promising approach for the treatment of tumors. A number of strategies are currently being explored, including the use of humanized anti-FR antibody, chimeric anti-FR transfected tumor-infiltrating lymphocytes, bispecific antibodies against the T-cell receptor, and interleukin-2 fusion protein.

Monoclonal antibodies directed against tumor-associated antigens mediate lysis of tumor cells by effector immunocytes, such as macrophages, natural killer cells or monocytes. Humanized chimeric antibodies are often used instead of murine antibodies due to the variable Fc receptor affinity and undesirable human anti-mouse antibody (HAMA) response associated with the use of murine antibodies.

Humanized chimeric MOv18 and MOv19 monoclonal antibodies were evaluated for lytic activity against FR-positive tumor cells [86]. An equal amount of lysis of the target ovarian cancer cell line (IGROV1) resulted when murine MOv19 was compared to the chimeric MOv19. Chimeric MOv18 resulted in higher levels and more consistent tumor lysis than murine MOv18. The chimeric analogs were found to bind FR with similar affinity and only one chimeric antibody was required per FR to achieve effective cell kill [86].

A chimeric MOv18 antibody/T-cell receptor construct, which consists of T-cell receptor-transmembrane and signaling chains fused to the variable domains from the MOv18 monoclonal antibody, was also evaluated for tumor immunotherapy [44].

C57BL6 mice carrying FR transfected 24JK-FBP cells were treated with tumor-infiltration

lymphocytes transfected with the above chimeric construct [44]. A significant increase in survival resulted in mice treated with MOv-TIL (tumor-infiltrating lymphocytes) over those treated with saline, nontransduced TIL, or TIL transduced with a control anti-trinitrophenol chimeric receptor gene. MOv-TIL treatment also resulted in fewer lung metastases in mice injected intravenously with tumor cells [44].

MOv18-derived bispecific antibodies have also been investigated for potential use in tumor immunotherapy. Bispecific antibody OC/TR were constructed using MOv18 (recognizing the FR) and a monoclonal antibody against the T cell receptor (recognizing CD3). T lymphocytes retargeted using $F_{(ab)_2}$ fragments of the bispecific monoclonal antibody OC/TR lyse ovarian carcinoma cells [87, 88]. In a clinical trial, the treatment of *in vitro*-activated autologous peripheral blood T lymphocytes retargeted with OC/TR combined with IL-2 were evaluated in 19 patients with ovarian cancer for potential therapeutic efficacy. Complete response, complete intraperitoneal response, stable disease, and progressive disease were shown in 3, 1, 7, and 5 patients, respectively. Complete responses lasted from 18 up to 26 months. In addition, the HAMA response was evident at the end of treatment in 21 of 25 patients; however, tumor regression was evident in this study when the bi-specific antibody-retargeted T lymphocytes were used [88].

As an alternative to bispecific antibodies, folate conjugated anti-T-cell receptor antibody fragments are smaller in size, easier to prepare, and is highly potent in mediating the lysis of tumor cells, which express either FR isoform [89, 90]. The affinity of these folate/anti-T-cell receptor conjugates is only one-tenth of the affinity of free folate [90].

Finally, interleukin 2 (IL-2) has been fused to a single-chain Fv of MOv19. This construct specifically accumulates in tumors in a mouse tumor model and exhibit significant antitumor activity [91]. This approach provides local delivery of IL-2 without causing systemic toxicity.

1.8. Summary

In addition to the above mentioned areas, folate has been used in the targeting of both chemotherapeutic agents [92] and paramagnetic magnetic resonance imaging contrast agents [91] with very promising results.

Efficacy of targeted drug delivery is greatly affected by the size of the drug conjugate or drug carrier. Larger constructs are often immunogenic, prone to RES uptake, have prolonged systemic circulation time, are slow to extravasate, and exhibit significant passive targeting effects in solid tumors. Therefore, in order to achieve high tumor to normal tissue ratios, low molecular weight conjugates are greatly preferred. For the targeted delivery of large particles such as liposomes and gene transfer vectors, it is often difficult to demonstrate a significant increase in overall tumor accumulation. Targeting nonetheless may be beneficial at the cellular and/or subcellular level.

Folate as a targeting ligand offers many potential advantages over macromolecules such as monoclonal antibodies. These include: 1) small size of the targeting ligand, which often leads to favorable pharmacokinetic properties of the folate conjugates and reduced probability of immunogenicity thus allowing for repeated administration; 2) convenient availability and low cost; 3) relatively simple and defined conjugation chemistry; 4) high receptor affinity and lack of normal tissue receptor expression, thus high tumor tissue specificity; 5) the receptor/ligand complex can be induced to internalize via endocytosis, which may facilitate the cytosolic delivery of therapeutic agents, and 6) high frequency of folate receptor overexpression among human tumors, thus providing a wide range of tumor targets.

Therefore, folate-based targeting systems show great potential for future clinical, diagnostic and therapeutic applications.

Fig. 1.1. The structure of folic acid. The α - and γ -carboxyl groups are labeled.

Fig. 1.2. A schematic diagram of the folate receptor-mediated endocytosis pathway.

Fig. 1.3. Structure of 6-hydrazinonicotinamido-hydrazido (HYNIC)-folate.

Fig. 1.4. ^{99m}Tc -HYNIC-folate uptake by cultured tumor cells.

Fig. 1.5. Gamma camera image of a mouse treated with ^{99m}Tc -HYNIC-folate.

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CHAPTER 2

FOLATE RECEPTOR-MEDIATED LIPOSOMAL DELIVERY OF A LIPOPHILIC BORON AGENT TO TUMOR CELLS *IN VITRO* FOR NEUTRON CAPTURE THERAPY

2.1. ABSTRACT

Purpose. This study was aimed at the *in vitro* evaluations of folate receptor (FR)-targeted liposomes as carriers for a lipophilic boron agent, K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁], in FR-overexpressing tumor cells for neutron capture therapy.

Methods. Large unilamellar vesicles (LUVs; 200 nm in diameter) were prepared with the composition of egg PC/chol/K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁] (2:2:1, mol/mol), with an additional 0.5 mol % of folate-PEG-DSPE or PEG-DSPE added for the FR-targeted or non-targeted liposomal formulations, respectively.

Results. Boron-containing, FR-targeted liposomes readily bound to KB cells, an FR-overexpressing cell line, and were internalized via FR-mediated endocytosis. The boron uptake in cells treated with these liposomes was approximately 10-times greater compared to those treated with control liposomes. In contrast, FR-targeted and non-targeted liposomes showed no difference in boron delivery efficiency in F98 cells, which

do not express the FR. The subcellular distribution of the boron compound in KB cells treated with the FR-targeted liposomes was investigated by cellular fractionation experiments, which showed that most of the boron compound was found in either the cytosol/endosomal or cell membrane fractions, indicating efficient internalization of the liposomal boron.

Conclusion. FR-targeted liposomes incorporating the lipophilic boron agent, $K[nido-7-CH_3(CH_2)_{15-7,8-C_2B_9H_{11}}]$, into its bilayer were capable of specific receptor binding and receptor-mediated endocytosis in cultured KB cells. Such liposomes warrant further investigations for use in neutron capture therapy.

2.2. INTRODUCTION

Recent studies have reported amplified expression of the folate receptor (FR) in various types of human cancers, including breast, uterine, ovarian, and lung carcinomas (1). The receptor is generally absent in most normal tissues with the exception of choroid plexus, placenta, and low levels are expressed in lung, thyroid and kidney (2). The prevalence of FR overexpression among human tumors, however, makes it a good marker for targeted drug delivery. Furthermore, FRs bind folic acid (an oxidized form of folate) with high affinity. For example, the FR α -isoform has a dissociation constant (K_d) for folic acid of ~ 0.1 nM, which is approximately 10-fold lower than its K_d for reduced folates (e.g., 5-methyltetrahydrofolate) (3). Furthermore, the FR has been shown to mediate the internalization of folate-derivatized liposomes into acidic endosomal compartments (4). Targeted delivery via the FR has been reviewed extensively (5, 6). Incorporation into FR-targeted liposomes, therefore, could present a useful approach for the targeted delivery of a variety of therapeutic agents to tumor cells, including ^{10}B for neutron capture therapy.

Boron neutron capture therapy (BNCT), a binary cancer treatment, is based upon the capture reaction that occurs following irradiation of a stable ^{10}B isotope with thermal neutrons:

The resulting kinetic energy is distributed between the ionizing particles, ${}^4\text{He}$ (α particle) and ${}^7\text{Li}$ ions, which have effective ranges of < 10 μm in tissue. Therefore, cellular damage is limited to those cells that have taken up the ^{10}B -containing agent and is equally

lethal to oxic and hypoxic cells. However, relatively large intracellular accumulations of boron (approximately 20-30 μg of ^{10}B per gram of tumor) are necessary to produce cell death (7). BNCT requires the selective localization of boron within tumor cells in order to maximize damage to the tumor and minimize damage to surrounding normal tissue (8). The targeting of ^{10}B -containing agents to tumors has been reviewed, recently (9). Such targeting strategies have included the employment of drug carriers, such as liposomes (10-15, rev. in 16), and low density lipoproteins (17, 18), as well as covalent conjugates of monoclonal antibodies (19-22) and epidermal growth factor (23-26).

Liposomal delivery of boron is an attractive approach for BNCT, since liposomes are capable of carrying relatively large quantities of boron compounds. Low encapsulation efficiencies have resulted in the production of liposomes containing hydrophilic compounds (27). However, lipophilic boron compounds incorporated within the liposome bilayer result in an increase in the overall efficiency of incorporation of a ^{10}B -containing agent. Therefore, the gross boron content of the liposomes is increased in the formulation (13). In addition, liposomes show selective localization to tumors due to the increased extravasation and retention in tumor tissues due to a porous endothelial lining and reduced lymphatic drainage. However for an ideal ^{10}B -delivery system, liposomes should not only be able to selectively localize to the tumor tissue, but also exhibit favorable cellular and subcellular distributions. Therefore, a cellular marker that would differentiate malignant from normal cells could be targeted in order to achieve selective delivery of boron compounds to tumor cells.

In this study, we have evaluated FR-targeted and non-targeted liposomal formulations that incorporate a lipophilic boron agent, $\text{K}[\text{nido-7-CH}_3(\text{CH}_2)_{15}\text{-7,8-}$

C₂B₉H₁₁] (13). Uptake studies were carried out in order to determine the boron levels achievable with each of the liposomal formulations in FR (+) KB cells, using FR (-) F98 cells as a control. Furthermore, *in vitro* subcellular fractionation experiments were performed with liposome-treated KB cells, in order to determine the intracellular distribution of boron delivered by the liposomes. These studies are described in detail below.

2.3. MATERIALS AND METHODS

2.3.1. Materials

Egg phosphatidylcholine (PC), monomethoxy-polyethylene glycol-2000 (PEG-2000), and distearoylphosphatidylethanolamine (DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Calcein, cholesterol (Chol), folic acid dihydrate, Sepharose CL-4B resin, and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO). K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁] was synthesized, as described previously (13). Tissue culture media, fetal bovine serum (FBS), and antibiotics were purchased from Life Technologies (Rockville, MD). Folate-polyethyleneglycol-DSPE (f-PEG-DSPE) was synthesized, as reported previously (28).

2.3.2. Cell Culture

KB cells, a human oral cancer cell line (ATCC# CCL-17) that overexpresses the FR, were given to us by Dr. Philip Low, Purdue University, West Lafayette, IN. KB cells were cultured as a monolayer in folate-free RPMI 1640 media supplemented with penicillin, streptomycin, and 10 % FBS in a humidified atmosphere containing 5 % CO₂ at 37 °C.

F98 glioma cells were cultured as a monolayer (ATCC# CRL-2397) in DMEM media supplemented with penicillin, streptomycin, and 10 % FBS under the same conditions as those used for KB cells.

2.3.3. Preparation of Liposomes Incorporating K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁]

FR-targeted and non-targeted liposomes were prepared using a previously described procedure (29) based on polycarbonate membrane extrusion. Briefly, egg PC, chol, and K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁] were dissolved at a 2:2:1 molar ratio in chloroform (CH₃Cl). The FR-targeted and non-targeted liposome preparations contained an additional 0.5 mole % of f-PEG-DSPE or PEG-DSPE, respectively, which were added to the CH₃Cl solution and dried to a thin film in a round-bottom flask. The dried lipid was then hydrated in 1 ml phosphate-buffered saline (PBS, 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4) and dispersed by mixing. The resulting suspension of multilamellar vesicles were subjected to 6 cycles of freezing and thawing, then briefly sonicated, and extruded through a 0.2 µm pore size polycarbonate membrane using a handheld LiposoFast™ Extruder (Avestin Inc., Ottawa, ON). The resulting large unilamellar liposomes were separated from unincorporated K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁] by gel-filtration on a Sepharose CL-4B column equilibrated with PBS. The mean diameter of the extruded liposomes (containing 35 mg of total lipid) was determined by photon correlation spectroscopy on a NICOMP Particle Sizer Model 370. The final boron concentration of in the liposomes was determined by direct current plasma-absorption spectroscopy (DCP-AES), as previously described (30).

FR-targeted and non-targeted liposomes, incorporating K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁] and encapsulating fluorescent calcein, were prepared as described above, except that the dried lipids were hydrated with an 80 mM calcein solution.

2.3.4. Boronated Liposomal Delivery in Cultured F98 Cells

F98 cells, an FR (-) cell line (data not shown), were washed with PBS and resuspended by treatment with 5 mM EDTA, and then were further diluted in incubation media, pelleted by centrifugation at 400 x g, and resuspended in folate-free culture media at a density of 3×10^7 cells/ml. Various concentrations of FR-targeted or non-targeted liposomes, incorporating K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁], were added to the media and incubated at 37 °C with gentle shaking for 2 h. Following this, the media were removed by centrifugation at 400 x g for 5 min. In order to remove unbound, extracellular liposomes, the cells were washed 3-times with cold PBS by resuspension and centrifugation at 400 x g. The media, wash, and digested cell pellets were collected for boron analysis by DCP-AES.

2.3.5. Boronated Liposomal Delivery in FR-bearing KB Cells

KB cells were washed with PBS and treated the same as above for the F98 cell binding studies, except that the cells were resuspended in folate-free culture media at a density of 1×10^7 cells/ml. For the competitive binding assays, 1 mM of free folate was added to the incubation media. Various concentrations of FR-targeted or non-targeted liposomes, incorporating K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁], were added to the KB cell suspension and incubated at 37 °C with gentle shaking for 2 h. Following incubation, the cell samples were prepared as described above for the F98 cell binding study.

In order to determine the intracellular distribution of cell-associated boron-containing liposomes, cells treated with FR-targeted and non-targeted liposomes encapsulating calcein were examined by fluorescence microscopy using a Zeiss Axioskop

Epifluorescence Microscope with an Optonics three chip, low light level color CCD camera attachment. The cells were processed as described above, except that they were treated with K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁]-incorporated liposomes encapsulating 50 μ M calcein. The cell pellets were then resuspended in 1 ml PBS following incubation and cell washing. KB cell images were obtained in both the fluorescence (dark field) and phase-contrast (bright field) modes using a 40 X objective. Digital images were collected and analyzed using the NIH Image 1.6 (Springfield, VA) software.

KB cells that had been treated with K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁]-incorporated liposomes encapsulating calcein were evaluated by fluorescence spectrophotometry. The cells were treated as described above, except they were lysed with 0.1 % (v/v) Triton X-100 following resuspension in PBS. All fluorescence measurements were performed using a Perkin-Elmer LS-5B spectrofluorometer operated with an FTWinlab (Morena Valley, CA) software program. The excitation and emission wavelengths were set at 490 nm and 520 nm, respectively.

2.3.6. Intracellular Distribution of Boron in KB Cells

Subcellular fractionation experiments were performed using a previously described method (31). Briefly, KB cells were suspended by treatment with 5 mM EDTA and pelleted by centrifugation at 400 x g. KB cells (2.0×10^8) were then resuspended in serum- and folate-free RPMI 1640 media and separated into two aliquots of 2.0×10^7 cells/ml. FR-targeted or non-targeted liposomes, incorporating K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁], were added to the media and were incubated at 37 °C with gentle shaking for 2 h. After the incubation period, the media were removed by

centrifugation at 400 x g for 5 min and the cells were washed 3-times with cold PBS. During the last wash, the cells were counted in a hemocytometer in order to determine cell viability and final cell concentration before fractionation. The cells were centrifuged at 400 x g for 3 min, then resuspended in 7 ml of cold distilled water, and lysed by sonication on ice. The lysates were centrifuged at 1000 x g for 10 min and the pellet was resuspended in a 0.25 M sucrose/1.8 mM CaCl₂/1 % Triton X-100 solution. A sucrose gradient was made by adding an equal volume of 0.34 M sucrose/0.18 mM CaCl₂ solution to the bottom of the tube, pushing up the lighter solution. The nuclear pellet was obtained after centrifugation at 600 x g for 10 min. The first supernatant was centrifuged at 3500 x g for 10 min to obtain the mitochondrial pellet. The supernatant fraction was again centrifuged at 16,000 x g for 20 min to obtain the lysosomal pellet. Finally, the final supernatant contained the cytosol in addition to the endosomes. The samples were collected for boron analysis, by DCP-AES.

2.4. RESULTS

2.4.1. Characterization of Liposomes Incorporating K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁]

FR-targeted and non-targeted large unilamellar vesicles (LUVs) (~ 200 nm in diameter), incorporating K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁] (Fig. 2.1) into the liposomal bilayer, were prepared and analyzed. As shown in Fig. 2.2, the FR-targeted and non-targeted liposomes showed ~ 100% incorporation efficiency. Further, the liposomal formulations were stable when stored at 4 °C in PBS for 8 weeks, as evidenced by an absence of boron leakage or change in particle size.

2.4.2. Cellular Uptake of Boronated Liposomes *In Vitro*

F98 and KB cells were incubated with FR-targeted and non-targeted liposomes, incorporating K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁], for 2 h at 37 °C. In order to determine whether FR-mediated delivery was responsible for cellular uptake of boronated liposomes, FR (-) F98 cells were exposed to FR-targeted and non-targeted K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁]-containing liposomes. As shown in Fig. 2.3, identical amounts (< 0.2 µg of ¹⁰B/10⁷ cells) of boron were delivered by FR-targeted and non-targeted liposomes to F98 cells.

In order to determine if there was specific targeting to an FR (+) KB cell line, the cells were exposed to FR-targeted and non-targeted liposomes. Further, a competitive binding assay was performed using 1 mM free folic acid. As shown in Fig. 2.4, uptake of the FR-targeted liposomes by KB cells was approximately 10-times higher than the non-targeted liposomes over the entire range of boron concentrations. In addition, the

level of boron uptake achieved by FR-targeted liposomal delivery was reduced by 75 % by co-incubation with 1 mM free folate.

Comparison of the uptake efficiencies of the FR-targeted and non-targeted liposomes, incorporating K[nido-7-CH₃(CH₂)₁₅-7,8-C₂B₉H₁₁], taken up by either KB or F98 cells are shown in Fig. 2.5. The percent cellular uptake of boron was highest with the lowest boron concentration, and as concentration increased, the percent uptake decreased. Furthermore, the boron uptake in KB cells resulting from the delivery of FR-targeted liposomes was 35- to 64-times greater than that taken up by F98 cells, depending on the initial boron concentration, thereby establishing that uptake was receptor-specific.

The intracellular distribution of cell-associated FR-targeted boronated liposomes (¹⁰B concentration = 24 µg/ml) encapsulating calcein was studied by fluorescence microscopy. As shown in Fig. 2.6, calcein fluorescence in KB cells exposed to FR-targeted liposomes was distributed throughout the cell including the plasma membrane, intracellular vesicles, and cytosol. Cells treated with the FR-targeted liposomes in combination with 1 mM free folic acid, exhibited much less fluorescence than FR-targeted liposomes, alone. However, the calcein fluorescence was distributed around the plasma membrane, as well as the cytosol/endosomal compartments as demonstrated by punctate fluorescence, which also was seen with the FR-targeted liposomes, alone. The cells exposed to non-targeted liposomes were shown to be associated with the cell surface, however very little fluorescence was apparent.

The amount of calcein fluorescence was further quantified by fluorescence spectrophotometry. After the KB cells had been treated with 0.1 % (v/v) Triton X-100, the fluorescence of the cell lysates were measured. The difference in calcein

fluorescence between the FR-targeted and non-targeted liposomes (^{10}B concentration = 24 $\mu\text{g}/\text{ml}$) associated with KB cells was approximately 10.5-times different, which was comparable to the differences in boron uptake at the same initial boron concentration.

2.4.3. Subcellular Distribution of $\text{K}[\text{nido-7-CH}_3(\text{CH}_2)_{15}\text{-7,8-C}_2\text{B}_9\text{H}_{11}]$ in Cultured KB Cells

The intracellular distribution of the boron compound was investigated by subcellular fractionation experiments. KB cells were incubated with FR-targeted and non-targeted liposomes, incorporating $\text{K}[\text{nido-7-CH}_3(\text{CH}_2)_{15}\text{-7,8-C}_2\text{B}_9\text{H}_{11}]$, for 2 h at 37 $^\circ\text{C}$ at a boron concentration of 24 $\mu\text{g}/\text{ml}$, since the greatest difference in boron uptake resulted at this concentration. Cell viability was greater than 90 % after a 2 h incubation. The intracellular distribution of the boron delivered by either FR-targeted or non-targeted liposomes is shown in Fig. 2.7. Delivery of FR-targeted liposomes resulted in a total boron uptake of 587 $\mu\text{g } ^{10}\text{B}/10^9$ KB cells. In addition, the cytosol/endosomes (~ 42% uptake) and the plasma membrane (~ 34% uptake) had the highest uptake of the various subcellular fractions. However, the lysosomal (~ 12% uptake), mitochondrial (~ 11% uptake), and nuclear (~ 1% uptake) fractions showed much reduced boron uptake in comparison to the other subcellular fractions.

The overall boron uptake resulting from the delivery by non-targeted liposomes was shown to be much lower (42.9 $\mu\text{g } ^{10}\text{B}/10^9$ KB cells). The highest levels of boron were detected in the lysosomes (~ 33% uptake), mitochondria (~ 23% uptake) and on the plasma membrane (~ 22% uptake). In addition, boron delivered by non-targeted liposomes, was undetectable in the cytosol/endosomes and the nuclear fractions. Overall,

the boron uptake resulting from FR-targeted ($587 \mu\text{g } ^{10}\text{B}/10^9 \text{ KB cells}$) liposomal delivery exhibited much greater boron content in the subcellular fractions compared to non-targeted ($42.9 \mu\text{g } ^{10}\text{B}/10^9 \text{ KB cells}$) liposomes. These results demonstrate that FR-targeting specifically increased boron uptake by KB cells.

2.5. DISCUSSION

In this study, we have evaluated FR-targeted and non-targeted liposome formulations that incorporated the lipophilic boron agent, $K[nido-7-CH_3(CH_2)_{15}-7,8-C_2B_9H_{11}]$. This includes a characterization of stability and incorporation efficiency of these liposomes. Furthermore, we have examined the FR-specific uptake of these boron-containing liposomes *in vitro* and elucidated the subcellular distribution of boron in KB cells, an FR-overexpressing cell line. $K[nido-7-CH_3(CH_2)_{15}-7,8-C_2B_9H_{11}]$, a lipophilic boron agent that has been studied previously (13), was incorporated into the liposomal bilayer of FR-targeted and non-targeted liposomes. Further, the intracellular distribution of ^{10}B in KB cells *in vitro*, was determined. Cells were exposed to either of the liposomal formulations (^{10}B concentration = $24 \mu g \text{ }^{10}B/ml$) for 2 h, following which boron concentrations were determined. The lipophilic boron compound showed remarkable intracellular accumulation in KB cells and was not removed by extensive washing. Due to the lipophilic properties of the compound, it is possible that upon uptake, $K[nido-7-CH_3(CH_2)_{15}-7,8-C_2B_9H_{11}]$ was embedded in the membranes of the subcellular organelles. The resulting intracellular boron levels were $587 \mu g \text{ }^{10}B/10^9$ cells and $42.9 \mu g \text{ }^{10}B/10^9$ cells for FR-targeted and non-targeted boron-containing liposomes, respectively. Assuming that 10^6 cells weigh ~ 1 mg and a boron level of $20-30 \mu g \text{ }^{10}B/g$ of tissue would be sufficient for therapy, both FR-targeted and non-targeted liposomes would be able to provide enough boron for BNCT, provided that the compound was allowed to accumulate in the tumor for at least 2 h.

The potential *in vivo* use of FR-targeted liposomes for BNCT is dependent on the ability of these liposomes to selectively target FR (+) tumors. As shown in Fig. 2.3, the

FR-targeted liposomes were not preferentially taken up by FR (-) F98 cells, *in vitro*. However, FR-targeted liposomes exhibited over 10-times greater boron uptake than the non-targeted type in KB cells *in vitro*, as evidenced by quantitation of boron and fluorescence microscopy. In addition, the free folate competitive binding assay, resulted in a much lower boron uptake from the FR-targeted liposomes, indicating that the observed uptake was FR-dependent. Furthermore, these results also were consistent with the fluorescence micrographs, shown in Fig. 2.6. Whereas, the non-targeted liposomes only showed minimal fluorescence around the plasma membrane, which was indicative of non-specific cellular binding, the FR-targeted liposomes showed much greater membrane binding in addition to punctate intracellular fluorescence, which was indicative of FR-specific binding and endocytosis into intracellular vesicles.

An important factor in determining the effect of BNCT is the intracellular distribution of ^{10}B within tumor cells. The nucleus, and even more specifically the DNA, are the targets of choice (32). A boron concentration, which is 2.5-times higher than that localized in the cell nucleus and uniformly distributed within the target cell, however, has been shown to be as effective as targeting the nucleus (32). In our study, subcellular fractionation revealed that most of the intracellular boron delivered by FR-targeted liposomes was associated with either the plasma membrane or cytosol/endosomal fractions. The plasma membrane contained a large fraction of the overall boron delivered, indicating that the FRs bound and retained the FR-targeted liposomes. In addition, boron also accumulated in the cytosol/endosomes, indicating that the boron-containing moieties were capable of entering the cell via FR-mediated endocytosis and most likely were associated with the endosomal membranes. Furthermore, it has been

shown that the subcellular distributions of boron and calcein, as detected by quantitative analysis and fluorescence microscopy, overlapped (Fig. 2.6 and Fig. 2.7). For example, the association of boron to the cell membrane resulting from the delivery of FR-targeted liposomes, was demonstrated both by quantitative determination of boron and in the fluorescence micrographs, which showed increased fluorescence associated with the KB cell membranes. Furthermore, the resulting difference in boron uptake and fluorescence in KB cells between FR-targeted and non-targeted liposomes was the same (approximately 10-times difference between FR-targeted and non-targeted) at the same boron concentration.

In order for BNCT to be successful, it is necessary to obtain approximately 20-30 μg of ^{10}B per gram of tissue in the tumor cells. In this study, an f-PEG linker was utilized as a targeting ligand to direct boron-containing liposomes to FR-overexpressing cells. Folate, as a low molecular weight agent with high FR affinity, possesses several advantages as a targeting ligand. It is readily available and exhibits superior physiochemical stability with exposure to adverse storage conditions, organic solvents, and repeated freezing and thawing. Production with folate is consistent and relatively inexpensive since the necessary conjugation chemistry is well-defined.

In conclusion, since folate is endocytosed, targeted moieties can accumulate intracellularly due to receptor recycling. In addition, due to a variety of FR (+) tumors, multiple tumor types could be targeted for neutron capture therapy. We have demonstrated that the introduction of FR-targeting greatly increased the uptake of boron into KB cells. This suggests that strategies to further improve the intracellular boron uptake in malignant cells, should focus on the utilization of liposomes, which are capable

of carrying large quantities of ^{10}B -containing agents, in combination with FR-targeting. Further studies, including *in vivo* biodistribution, on the boron-containing, FR-targeted liposomes are warranted to further assess the potential of these delivery vehicles.

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Fig. 2.1. The structure of $K[nido-7-CH_3(CH_2)_{15}-7,8-C_2B_9H_{11}]$, which was synthesized as described in the Materials and Methods.

Fig. 2.2. Incorporation efficiency of the lipophilic boron agent into PEG-ylated folate receptor (FR)-targeted and non-targeted liposomes. Entrapment of the boron compound was determined by loading a 0.5 ml sample onto a 10 ml Sepharose CL-4B column. Fifteen consecutive 1 ml fractions were eluted with PBS, and the amount of boron was determined by DCP-AES.

Fig. 2.3. Cellular uptake of boron delivered by FR-targeted and non-targeted liposomes in cultured F98 cells. Cells were incubated with the boron-containing liposomes for 2 h at 37 °C, as described in the Materials and Methods. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

Fig. 2.4. Cellular uptake of boron delivered by FR-targeted and non-targeted liposomes in cultured KB cells. A competitive binding assay was performed using 1 mM free folate. Cells were incubated with the boron-containing liposomes for 2 h at 37 °C, as described in the Materials and Methods. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

Fig. 2.5. The boron uptake efficiency by cultured A. F98 cells and B. KB cells after treatment with three different concentrations of boron-containing, FR-targeted or non-targeted liposomes. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

Fig. 2.6. Fluorescence micrographs of the selective uptake of FR-targeted boron and calcein-containing liposomes by KB cells. A suspension of KB cells were incubated with FR-targeted and non-targeted, boron (24 $\mu\text{g/ml}$)- and calcein (50 μM)-containing liposomes and photographed in both the fluorescence (dark fields) and the phase contrast mode (bright fields) on a microscope as described in the Materials and Methods. Top, cells treated with FR-targeted liposomes; middle, cells treated with FR-targeted liposomes + 1mM free folate; bottom, cells treated with non-targeted liposomes. Left panels: micrographs taken in the fluorescence mode; right panels: the same field viewed in the phase contrast mode.

Fig. 2.7. Boron content of various subcellular fractions of KB cells, measured by DCP-AES after exposure to either FR-targeted or non-targeted, boron-containing liposomes for 2 h at 37 °C, as described in the Materials and Methods. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

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CHAPTER 3

A NOVEL pH-SENSITIVE LIPOSOME FORMULATION CONTAINING OLEYL ALCOHOL

3.1. ABSTRACT

pH-sensitive liposomes are designed to undergo acid-triggered destabilization. First generation pH-sensitive liposomes, based on the cone-shaped lipid dioleoyl phosphatidylethanolamine (DOPE), have been shown to lose fusogenicity in the presence of serum. Here, we report the design and evaluation of novel serum-resistant pH-sensitive liposome formulations that are based on the composition of egg phosphatidylcholine (PC), cholesteryl hemisuccinate (CHEMS), oleyl alcohol (OAlc), and Tween-80 (T-80). When loaded with the fluorescent probe calcein, these liposomes exhibited excellent stability at pH 7.4 and underwent rapid destabilization upon acidification as shown by calcein dequenching and particle size increase. Adjusting the mole percentages of T-80 and OAlc in the formulation could regulate the stability and pH-sensitive properties of these liposomes. Liposomes with a higher T-80 content exhibited greater stability but were less sensitive to acid-induced destabilization. Meanwhile, formulations with a higher OAlc content exhibited greater content release in

response to low pH. The pH-triggered liposomal destabilization did not produce membrane fusion according to an octadecylrhodamine B chloride (R₁₈) lipid-mixing assay. Compared to DOPE-based pH-sensitive liposomes, the above formulations showed much better retention of their pH-sensitive properties in the presence of 10% serum. These liposomes were then evaluated for intracellular delivery of entrapped cytosine- β -D-arabinofuranoside (araC) in KB human oral cancer cells, which have elevated folate receptor (FR) expression. FR, which is amplified in many types of human tumors, has been shown to mediate the internalization of folate-derivatized liposomes into an acidic intracellular compartment. FR-targeted OAlc-based pH-sensitive liposomes, entrapping 200 mM araC, showed ~17 times greater FR-dependent cytotoxicity in KB cells compared to araC delivered via FR-targeted non-pH-sensitive liposomes. These data indicated that pH-sensitive liposomes based on OAlc, combined with FR-mediated targeting, are promising delivery vehicles for membrane impermeable therapeutic agents.

3.2. INTRODUCTION

Liposomes are phospholipid bilayer vesicles that have been studied extensively as potential drug carriers. Major obstacles to liposomal drug delivery have been the slow drug release and lack of fusogenic activity of regular liposomes following internalization into the endosomal compartment. These factors contribute to the diminished efficiency of cytosolic delivery [1], especially when the encapsulated molecules are large or highly hydrophilic [2-4]. In order to overcome this obstacle, pH-sensitive liposomes have been developed that are stable at physiological pH but are destabilized upon acidification following cellular internalization, thereby, promoting the release of their contents into the cytosol [5-7].

Most reported formulations of pH-sensitive liposomes are composed of dioleoylphosphatidylethanolamine (DOPE), which has a strong propensity to form a non-bilayer structure due to its cone-shape geometry, and a weakly acidic amphiphile, such as cholesteryl hemisuccinate (CHEMS), which confers stability to the bilayer phase at neutral pH [8-12]. Under acidic conditions, CHEMS becomes partially protonated thus losing its negative charge and, therefore, its ability to stabilize the bilayer structure, which is based on electrostatic repulsion. This further results in the destabilization and/or fusion of the liposomes. The DOPE-based pH-sensitive liposomes have been shown to improve the cytoplasmic delivery of membrane-impermeable therapeutic agents [7,13,14]. However, the potential application of these liposomes as drug carriers *in vivo* is hampered by their relatively poor stability in the presence of serum [15-18]. Attempts to increase the serum stability of the DOPE-containing liposome formulations by incorporating a

polyethyleneglycol (PEG)-derivatized lipid resulted in significantly reduced pH-sensitivity [19].

The folate receptor (FR) is known to be overexpressed in a wide variety of solid tumors as well as in myeloid leukemias [20-22]. Folate conjugates of various therapeutic and diagnostic entities, including liposomes, have been shown to be internalized by cells via FR-mediated endocytosis into an acidic endosomal compartment [23-25]. In addition, folate-derivatized liposomes have been shown to selectively increase the delivery of cytotoxic drugs into tumor cells with elevated FR expression [26]. However, highly hydrophilic agents, such as cytosine- β -D-arabinofuranoside (araC), delivered by liposomes might be sequestered in the endosomal compartment resulting in a slow rate of cytosolic release. Combining FR-targeting, which promotes cellular endocytosis, with pH-sensitive liposomes, which promotes endosomal drug release, therefore, presents an attractive approach for improving the cytosolic delivery of araC.

In this study, we report the design and evaluation of novel pH-sensitive liposome formulations based on the use of oleyl alcohol (OAlc) in combination with egg phosphatidylcholine (PC) as the membrane destabilizing components. The stability and pH-sensitivity of the liposomes in buffer and in 10% serum were determined by fluorescence dequenching assays. Furthermore, the OAlc-containing liposomes, targeted to the FR, were evaluated for the intracellular delivery of araC. The possible mechanism for the pH-sensitivity of these liposomes is also discussed.

3.3. MATERIALS AND METHODS

3.3.1. Materials

Egg PC and DOPE were purchased from Avanti Polar Lipids (Alabaster, AL). Octadecylrhodamine B chloride (R₁₈) was purchased from Molecular Probes (Eugene, OR). Calcein, CHEMS, araC, folic acid dihydrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sepharose CL-4B resin, Tween-80 (T-80), and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). OAlc was purchased from Aldrich Chemical Co. (St. Louis, MO). Polycarbonate membranes and the handheld LiposoFast™ Extruder were obtained from Avestin Inc. (Ottawa, ON). Tissue culture media were purchased from Life Technologies (Rockville, MD). Folate-PEG-cholesterol (f-PEG-Chol) was synthesized as reported previously [27].

3.3.2. Cell Culture

KB cells, a human oral cancer cell line that expresses amplified FR, were obtained as a gift from Dr. Philip Low at Purdue University (West Lafayette, IN). The cells were cultured at 37 °C as a monolayer in folate-free RPMI 1640 media supplemented with antibiotics and 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂.

3.3.3. Liposome Preparations

Liposomes encapsulating calcein were prepared using a procedure based on polycarbonate membrane extrusion, as described previously [27]. Briefly, a chloroform solution of the lipid mixture with the desired composition was dried into a thin film in a

round-bottom flask on a rotary evaporator, and then further dried under vacuum. The lipid mixture was then hydrated in 80 mM calcein. The suspension was subjected to 6 cycles of freezing and thawing, briefly sonicated, and then extruded through a 0.1 μm pore-size polycarbonate membrane using a handheld LiposoFastTM Extruder.

Untrapped calcein was separated from the liposomes by gel filtration on a Sepharose CL-4B column equilibrated in phosphate-buffered saline (PBS, 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4). The mean diameter of the extruded liposomes was determined by photon correlation spectroscopy on a NICOMP Particle Sizer Model 370. The final calcein concentrations in the liposome preparations were calculated based on absorbance at 495 nm using a molar extinction coefficient of 80,000 M⁻¹ cm⁻¹.

3.3.4. Membrane Fusion Assay

The membrane fusion assay was carried out as described previously [28, 29]. Briefly, labeled liposomes were prepared by incorporating a lipid soluble probe, R₁₈, at 5.7 mole% of the total lipid, a concentration that results in fluorescence self-quenching. Both labeled and unlabeled liposomes were prepared at a total lipid concentration of 2 mM. These were then mixed at a volume ratio of 1:4 (labeled:unlabeled). The liposome mixture was diluted to 5 μM lipids in the appropriate buffer, followed by a 10 min incubation at 37 °C. R₁₈ fluorescence intensity was then measured at the excitation and emission wavelengths of 560 nm and 590 nm, respectively.

3.3.5. Fluorescence Dequenching Assay

Liposome content release was characterized using a calcein dequenching assay, as described previously [19]. All fluorescence measurements were performed using a Perkin-Elmer LS-5B spectrofluorometer operated with an FTWinlab (Morena Valley, CA) computer program. The excitation and emission wavelengths were set at 490 nm and 520 nm, respectively. Calcein-loaded liposomes containing 40 nmoles of lipid were added to 2 mL of PBS (adjusted to pH 7.0 and 7.4) or sodium acetate buffer (100 mM NaCl, 10 mM acetate, pH 5.0, 5.5, 6.0, and 6.5) in a disposable cuvette. After a 10 min incubation at 37 °C, buffer pH was adjusted to 7.4 and calcein fluorescence was measured. The fluorescence was then re-measured following the addition of 0.1% Triton X-100, which caused 100% calcein leakage from the liposomes. The percent of calcein release was calculated based on the equation:

$$\% \text{ Calcein Release} = ((I_{\text{pH}} - I_0)/(I_{100} - I_0)) * 100 \%$$

where I_0 was the fluorescence at pH 7.4, I_{pH} was the fluorescence intensity following incubation at acidic buffer pHs, and I_{100} was the fluorescence after the addition of Triton X-100. In addition, the changes in the mean particle size of the OAlc liposomes in an acidic environment were measured at preset time intervals.

3.3.6. Determination of Liposome Stability in the Presence of Serum

Liposomes entrapping 80 mM calcein and containing 40 nmoles of lipid were added to 2 mL buffer at various pH values in the presence or absence of 10% newborn calf serum. After a 10 min incubation at 37 °C, calcein fluorescence was measured and percentage of calcein release calculated, as described above. In addition, liposome particle size measurements were also performed at various time points.

3.3.7. Cytotoxicity Assay

The delivery of araC by OAlc liposomes was evaluated in KB cells. Preparation of araC-containing liposomes was carried out by the same method used for the preparation of calcein-containing liposomes described above, except 200 mM araC was used to hydrate the dried lipid mixture instead of calcein. In addition, 0.5 mole% of f-PEG-Chol was incorporated into the lipid mixture for the preparation of FR-targeted liposomes. The araC concentrations in the liposome preparations were calculated based on absorbance at 280 nm using a molar extinction coefficient of $475 \text{ M}^{-1} \text{ cm}^{-1}$, after solubilizing the lipid membranes in a methanol:water (2:1) mixture. The encapsulation efficiency of these liposomes was found to be approximately 15%.

The cytotoxicity of various liposomal araC formulations was determined by the MTT assay, as described previously [30]. Briefly, KB cells were seeded in 96-well plates to reach ~ 25% confluence at the time of the study. The cells were treated in triplicate with 1:4 serial dilutions of the various araC formulations. Following a 2 h incubation at 37 °C, the cells were then washed with PBS. Fresh media, containing 10% fetal bovine

serum, were then added and the cells were cultured for another 48 h. MTT was then added to the culture medium at a concentration of 0.6 mg/mL and the cells were incubated at 37 °C for an additional 2 h. The medium was then removed and the cells were dissolved in acidified isopropanol and the absorption at 495 nm was measured using an automatic plate reader, as an indicator of cell viability. The concentration of araC leading to 50% cell killing (IC_{50}) was calculated from a concentration dependent cell viability curve.

3.4. RESULTS

3.4.1. Formulation of pH-sensitive liposomes containing OAlc

All liposome compositions are described in molar ratios in this article. The pH-sensitive liposomes were designed using a primary lipid composition of egg PC/CHEMS (50:50) combined with varying amounts of T-80 and OAlc. T-80 was found be necessary since, in its absence, stable liposomes could not form at an OAlc to egg PC molar ratio above 1:2.

3.4.2. Effects of T-80 and OAlc Content on Acid-Induced Liposome Destabilization

We predicted that the incorporation of T-80 would confer greater stability and reduced pH-sensitivity to egg PC-based liposomes, whereas increasing OAlc content would have the opposite effect. To evaluate this hypothesis, calcein-containing large unilamellar vesicles (LUV) (~ 100 nm in diameter) composed of egg PC/CHEMS/T-80/OAlc with two different T-80 contents and escalating OAlc-to-PC ratios were prepared and evaluated for pH-dependent calcein release.

First, liposomes with varying amounts of OAlc and a high T-80 content were compared for pH-dependent content release. As shown in Figure 3.1A, liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:5:80) released 33% of their contents after a 10 min incubation at 37 °C at pH 5.0. In contrast, liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:5:20) showed only 6% calcein release under identical conditions. These findings suggested that elevated OAlc content resulted in increased pH-sensitivity of the liposomes.

Secondly, the above study was repeated using liposomes with a low T-80 content. Once again, the liposomes with higher OAlc content showed greater calcein release at acidic pH, as shown in Figure 3.1B. Furthermore, the overall amount of calcein release was much higher compared to the above-described liposomes with a high T-80 content. For example, liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:2:80) showed 83% calcein release at pH 5. These findings suggested lowering T-80 content increased the pH-responsiveness of the liposomes.

Thirdly, we also examined non-pH-sensitive liposomes composed of egg PC/CHEMS/T-80 (50:50:2) using the same calcein dequenching assay. As anticipated, these liposomes showed only minimal calcein release (< 5%) at low buffer pH (data not shown). All liposome formulations evaluated in this study were also found to be stable (showing < 5% calcein leakage) over a period of 8 weeks in PBS (pH 7.4) when stored at 4 °C.

3.4.3. Effect of Buffer pH on Liposome Particle Size

As shown in Figure 3.2, liposomes composed of egg PC/CHEMS/T-80/OAlc at molar ratios of 50:50:2:80 or 50:50:5:80, showed a 10- and a 15-fold increase in mean diameter, respectively, after a 30 min incubation at pH 5.0. The increase in particle size was time dependent. No changes in particle sizes were observed when these liposomes were kept in pH 7.4 buffer.

3.4.4. Membrane Fusion Assay

To determine the likely mechanism of acid-induced liposomal destabilization, a membrane fusion assay based on lipid mixing was performed using liposomes containing a self-quenching concentration of the lipophilic probe, R₁₈. The composition of the R₁₈-labeled liposomes was egg PC/CHEMS/T-80/OA1c/R₁₈ (50:50:2:80:5.7), whereas, the unlabeled liposomes were composed of egg PC/CHEMS/T-80/OA1c (50:50:2:80). Meanwhile, liposomes composed of DOPE/CHEMS/R₁₈ (60:40:5.7) and DOPE/CHEMS (60:40) were used as a positive control for the above assay. As shown in Figure 3.3, the OA1c-containing liposomes did not show a significant increase in R₁₈ fluorescence at any pH, suggesting a lack of membrane fusion. In contrast, R₁₈ fluorescence from the DOPE/CHEMS liposomes gradually increased with decreasing pH, suggesting liposomal fusion. The degree of observed R₁₈ fluorescence dequenching was much greater at pHs below 6, which correspond to the pK_a of CHEMS.

3.4.5. Stability and pH-sensitivity of OA1c Liposomes in the Presence of Serum

For pH-sensitive liposomes to be utilized for *in vivo* drug delivery, they must retain both stability and pH-sensitive properties in the presence of serum. It has been previously reported that serum protein binding to liposomes negatively affects the acid-triggered destabilization of DOPE-based pH-sensitive liposomes [15-18]. We, therefore, examined the effect of serum on liposomes composed of egg PC/CHEMS/T-80/OA1c (50:50:2:80), using liposomes composed of DOPE/CHEMS (60:40) as a reference control formulation [31]. As shown in Figure 3.4, the liposomes containing OA1c retained most

of their pH-sensitive properties upon incubation with serum. Calcein release from these liposomes after a 10 min incubation at pH 5 was 56% in the presence of 10% serum. In contrast, liposomes composed of DOPE/CHEMS showed only 20% release under the same conditions. It was noted that for both OAlc and DOPE-based liposomes, serum exposure adversely affected the degree of calcein release in response to low buffer pH. The OAlc-based liposomes, however, were affected to a lesser degree, with a decrease of calcein leakage from 83% to 56%, compared to a decrease from 84% to 19% for the DOPE-based liposomes. Serum exposure at pH 7.4 did not significantly affect liposomal particle size over 24 h (data not shown).

3.4.6. Delivery of araC to KB cells by FR-targeted OAlc-based pH-sensitive

Liposomes

To assess the potential utility of OAlc liposomes for increasing cytosolic drug delivery, cytotoxicity studies were carried out using FR-targeted liposomes entrapping araC in cultured KB cells. F-PEG-Chol, an FR-specific targeting ligand, was incorporated in the liposome formulation to facilitate FR-mediated cellular uptake. No significant change in the pH-sensitivity of the targeted liposomes, as evaluated by the calcein dequenching assay, was observed with these liposomes (data not shown).

All araC-containing liposome formulations were found to be stable (< 5% leakage) over 6 weeks in PBS (pH 7.4) when stored at 4 °C. As shown in Table 3.1, FR-targeted pH-sensitive liposomal araC showed significantly elevated cytotoxicity, with an IC_{50} of 78 μ M. When 1 mM free folic acid was present during drug incubation, the

cytotoxicity was reduced by ~ 4-fold, with an IC_{50} of 312 μ M, indicating that the observed araC delivery was FR-dependent. In contrast, FR-targeted non-pH-sensitive liposomal araC showed a much lower cytotoxicity towards KB cells (IC_{50} of 1,312 μ M). In the absence of receptor targeting, liposomal araC showed very low cytotoxicity ($IC_{50} > 10,000$ μ M), even when compared to the free drug. This was presumably due to the lack of a cellular uptake mechanism for the liposome entrapped drug. Empty pH-sensitive liposomes did not contribute significantly to cellular cytotoxicity (data not shown), as demonstrated by the resulting cytotoxicity of the free drug/empty liposome combination. These results indicated that both FR-targeting and a pH-sensitive liposomal composition were required for the efficient intracellular delivery of membrane-impermeable drugs, such as araC.

3.5. DISCUSSION

In this study, we evaluated novel pH-sensitive liposome formulations that were based on the incorporation of a fatty alcohol, OAlc. This includes a detailed characterization of the pH-sensitivity and serum stability of these liposomes. Furthermore, we have examined the FR-specific intracellular delivery of the anticancer drug, araC, utilizing this novel liposomal formulation.

Previously reported formulations for pH-sensitive liposomes mostly incorporate the lipid DOPE as the principal component to promote bilayer destabilization. As shown in Figure 4, these liposomes tend to lose most of their pH-sensitivity in the presence of 10% serum. Alternative compositions with greater resilience to serum are, therefore, needed for *in vivo* drug delivery. It has been suggested that OAlc, an unsaturated fatty alcohol, is capable of forming a hydrogen bond through its hydroxyl to an oxygen atom on the phosphate group on the PC molecule, resulting in the formation of a complex with geometry similar to that of DOPE, as illustrated in Figure 3.5 [32]. This in turn could result in a lowering of the energy barrier for the lipid transition from a lamellar phase to a hexagonal II phase, which is required for membrane destabilization. However, a lipid mixing assay failed to show membrane fusion activity for the OAlc-containing liposomes in response to low buffer pH. Therefore, the observed pH-dependent leakage among these liposomes entrapping calcein might primarily be due to membrane destabilization, without inducing bilayer fusion.

Since the OAlc content in the lipid composition determines the extent of OAlc/PC complex formation, it should, therefore, be possible to increase the pH-sensitivity of the

liposomes by increasing the OAlc content in the formulation. This concept has been demonstrated by the marked increase in calcein leakage when the OAlc content was increased in the liposomal formulation, as shown in Figure 3.1.

The potential *in vivo* use of pH-sensitive liposome formulations is dependent on retention of their stability and pH-sensitivity in the presence of serum. As shown in Figure 3.4, OAlc-containing liposomes demonstrated excellent stability and pH-sensitivity in the presence of serum. In addition, the degree of pH-sensitivity could be conveniently tuned by altering OAlc content in the formulation. Incorporation of T-80, which introduced a steric barrier on the surface of the liposomes, resulted in increased liposome stability while reducing its pH-dependent aggregation. Consequently, maintaining a delicate balance between these two factors is very important in creating an effective pH-sensitive liposomal formulation that is optimal for drug delivery.

AraC is a cytosine analogue widely used clinically as an anti-leukemic drug. AraC, which is highly hydrophilic, is taken up by cells via a nucleoside transporter [33, 34]. As a cell cycle-dependent agent, araC, showed relatively poor cytotoxicity when added to cultured KB cells as a free drug (IC_{50} of 4375 μ M) using an exposure time of 2 h. We, therefore, examined the effect of encapsulation of araC into FR-targeted liposomes, both pH-sensitive and non-pH-sensitive, on the *in vitro* cytotoxicity of this agent. As shown in Table 3.1, both FR-targeting and the use of a pH-sensitive formulation were found to be critical for maximizing liposomal araC cytotoxicity.

Since folic acid has high affinity for the FR and is stable, small, non-immunogenic, and readily available, it is ideal for exploration as a targeting ligand.

Moreover, the FR is frequently overexpressed among human tumors, including ~ 70% of acute myeloid leukemias, while being absent in most normal tissues. FR-targeted liposomes can be readily produced in sufficient quantities for future clinical development. Therefore, FR-targeted OAIC-liposomes, such as those entrapping araC, might have potential utility in the treatment of myeloid leukemias. FR-targeted delivery to solid tumors could also be possible given the high serum stability of the OAIC-containing liposomes, if prolonged systemic circulation and efficient endothelial extravasation could be achieved with the liposomes.

In addition to intracellular drug delivery, the pH-sensitive liposomal formulation described here may also constitute a drug delivery vehicle to pathological tissues, such as cancer, inflammation and infection sites, and ischemic areas, in which the pH is known to be lower than normal tissue [35]. Besides low molecular weight drugs such as araC, OAIC liposomes might find utility in the delivery of bioactive polypeptides, antisense oligodeoxyribonucleotides, as well as plasmid DNA in the delivery of gene therapy. Further studies on these liposomes, therefore, are warranted to explore these potential areas of application.

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Liposome Formulation*	IC₅₀ (μM)
FR-Targeted, OAlc pH-sensitive liposomal AraC	78
FR-Targeted, OAlc pH-sensitive liposomal AraC + 1 mM free folic acid	312
FR-Targeted, Non-pH-sensitive liposomal AraC	1,312
Non-Targeted, Non-pH-sensitive liposomal AraC	> 10,000
Free AraC + Empty FR-Targeted, OAlc liposomes	4,375

* The liposome compositions were: FR-targeted, OAlc pH-sensitive liposomes: egg PC/CHEMS/T-80/OAlc/f-PEG-Chol (50:50:2:80:0.5); FR-targeted, non-pH-sensitive liposomes: egg PC/CHEMS/T-80/f-PEG-Chol (50:50:2:0.5); Non-targeted, non-pH-sensitive liposomes: egg PC/CHEMS/T-80 (50:50:2).

Table 3.1. Cytotoxicity of various araC formulations in KB cells.

Fig. 3.1. Acid-induced calcein release from OAlc-containing liposomes with either high (Panel A) or low (Panel B) T-80 contents. Liposomes encapsulating 80 mM calcein were incubated for 10 min at 37 °C in buffers with a series of different pH values. Percent leakage of calcein was calculated based on fluorescence measured before and after addition of Triton X-100, as described in Materials and Methods. Each data point represents the mean of three parallel experiments; error bar = 1 standard deviation. The compositions of the liposomes used were egg PC/CHEMS/ T-80/OAlc at molar ratios of: in Panel A, (●), 50:50:5:20; (■), 50:50:5:40; (▲), 50:50:5:80; and in Panel B, (●), 50:50:2:20; (■), 50:50:2:40; (▲), 50:50:2:80.

Fig. 3.2. pH-Dependent particle size increase of OAlc liposomes incubated at pH 5.0 buffer. The liposomes were composed of egg PC/CHEMS/T-80/OAlc at molar ratios of (O) 50:50:5:80 or (●) 50:50:2:80.

Fig. 3.3. Acid-induced R_{18} fluorescence dequenching in pH-sensitive liposomes. Mixtures of labeled and unlabeled liposomes (at 1:4 ratio) were incubated for 10 min at 37 °C in buffers with a series of different pH values. The compositions of the liposomes used were (●) DOPE/CHEMS (60:40) and (○) egg PC/CHEMS/ T-80/OA/c (50:50:2:80). Each data point represents the mean of three parallel experiments; error bar = 1 standard deviation.

Fig. 3.4. Effect of serum on the pH-sensitivity of OAlc liposomes. Liposomes composed of either egg PC/CHEMS/T-80/OAlc (50:50:2:80) or DOPE/CHEMS (60:40) were compared for pH-dependent calcein release in the presence or absence of 10% serum. Each data point represents the mean of three parallel experiments; error bar = 1 standard deviation. Conditions used: (▲), DOPE/CHEMS liposomes in the presence of 10% serum; (Δ), DOPE/CHEMS liposomes in buffer; (●), OAlc liposomes in the presence of 10% serum; (○), OAlc liposomes in buffer.

Fig. 3.5. Structures of: A. The proposed PC/OA1c complex. B. DOPE. Note that, in both structures, the hydrophilic moieties occupy a relatively small volume compared to the lipophilic region. This should promote the formation of non-bilayer structures and liposome destabilization.

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CHAPTER 4

RECEPTOR-TARGETED DELIVERY OF LIPOSOMES VIA FOLATE-PEG-CHOL

4.1. ABSTRACT

A novel lipophilic conjugate of folate, folate-PEG-Chol, was synthesized and evaluated for receptor-mediated targeting of liposomes to tumor cells. Liposomes composed of DSPC/Chol/PEG-DSPE/folate-PEG-Chol (60/34/5/1, m/m) were taken up by cultured folate receptor-bearing KB cells via a saturable mechanism. Cellular binding of these liposomes could be competitively inhibited by free folic acid with an IC_{50} of 0.39 mM, indicating an extraordinarily high binding affinity. Fluorescence micrographs of KB cells treated with targeted liposomes encapsulating calcein showed that they were distributed both on the cell surface and in intracellular vesicular compartments. Targeted liposomes carrying doxorubicin were shown to be 38-times more toxic to KB cells than non-targeted control liposomes. A biodistribution study in receptor-positive tumor-bearing C57BL/6 mice showed no significant differences between the tumor uptake of folate-PEG-liposomes and non-targeted control liposomes. This study has demonstrated that cholesterol could be used as an alternative to phospholipids as an effective anchor for incorporation of a targeting ligand into liposomes.

4.2. INTRODUCTION

Liposomes are phospholipid bilayer vesicles with a potential application as drug carriers. Liposomal doxorubicin is currently in clinical use and has shown significant advantages over free doxorubicin, including reduced cardiotoxicity, increased blood circulation time, and increased tumor accumulation (1). The therapeutic potential of liposomes could theoretically be further extended if they can be specifically targeted to the tumor. Previous studies with tumor-targeted immunoliposomes have produced mixed results (2-6). Liposomes have a natural tendency to passively accumulate in solid tumors due to their increased endothelial permeability and reduced lymphatic drainage. Consequently, targeting of liposomes often does not result in increased tumor accumulation. However, studies also show that at the cellular and subcellular level, targeted liposomes produced a much more favorable pattern of distribution. For example, anti-HER2 immunoliposomes showed greater tumor penetration and cellular internalization than non-targeted liposomes (5). In addition, these immunoliposomes also showed much greater therapeutic efficacy (5).

In the current study, liposomes targeted to the folate receptor (FR) are evaluated. The FR is a glycosylphosphatidylinositol (GPI)-anchored high affinity membrane folate binding protein overexpressed in a wide variety of human tumors, including greater than 90% of ovarian carcinomas (7-9). Meanwhile, normal tissue distribution of FRs are highly restricted, making it a useful marker for targeted drug delivery to tumors (7-9). Monoclonal antibodies against the FR (MOv18) have been evaluated for applications in scintigraphy and tomography imaging, radiotherapy, and immunotherapy of ovarian cancer in clinical studies (10-14). Folic acid, a high affinity ligand for the FR ($K_d \sim 10^{-10}$

M), retains its receptor binding property when covalently derivatized via its γ -carboxyl. Folate conjugates are taken into receptor-bearing tumor cells via FR-mediated endocytosis (15). Folate-conjugation, therefore, presents another useful strategy for targeted drug delivery into receptor-positive tumor cells. Folic acid is potentially superior to antibodies as a targeting ligand due to its small size, lack of immunogenicity, availability, and simple, defined conjugation chemistry. Receptor-specific delivery has been shown with folate conjugates of protein toxins (16), anti-T-cell receptor antibodies (17), liposomes (18-20), radioimaging agents (21), antisense oligodeoxynucleotides (22), and gene transfer vectors (23).

Folate-mediated targeting of liposomes has previously been accomplished by the incorporation of a small amount of folate-polyethyleneglycol-distearoyl-phosphatidylethanolamine (folate-PEG-DSPE) (19). In this study, we explored the potential use of a novel conjugate, folate-PEG-Chol, for liposome targeting. The efficacy of targeting was evaluated in cell culture by both fluorescence microscopy and fluorimetry using liposomes carrying calcein. Further, liposomes encapsulating doxorubicin were evaluated for their *in vitro* cytotoxicity. Finally, a biodistribution study in FR(+) tumor-bearing mice was carried out to evaluate the tumor localizing properties of these FR-targeted liposomes.

4.3. MATERIALS AND METHODS

4.3.1. Materials

Folic acid dihydrate, calcein, cholesterol (Chol), dicyclohexylcarbodiimide (DCC), diethylenetriaminepentaacetic (DTPA) anhydride, N-hydroxysuccinimide (NHS), polyoxyethylene-bis-amine (PEG-bis-amine, MW ~ 3350), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Sephadex G-25 resin were purchased from Sigma Chemical Co (St. Louis, MO). Cholesteryl chloroformate was purchased from Aldrich Chemical Co (Milwaukee, WI). Distearoylphosphatidylcholine (DSPC), methoxy-PEG2000-DSPE, and distearoylphosphatidylethanolamine (DSPE), were purchased from Avanti Polar Lipids (Alabaster, AL). Doxorubicin hydrochloride and $^{111}\text{In Cl}_3$ were obtained from the Ohio State University Medical Center Nuclear Pharmacy. BCA protein assay kit was purchased from Pierce Chemical Co (Rockford, IL). Polycarbonate membrane and the handheld LiposoFast™ Extruder were obtained from Avestin Inc. (Ottawa, ON). Tissue culture media were purchased from Life Technologies (Rockville, MD). All reagents were used without further purification.

4.3.2. Cell Culture

Human oral cancer KB cells, which overexpress the folate receptor, were obtained as a gift from Dr. Philip Low at Purdue University (West Lafayette, IN). 24JK-FBP cells, a mouse sarcoma cell line transfected with the human folate receptor α gene, were obtained from Dr. Patrick Hwu at the National Cancer Institute (Bethesda, MD) (24). Both cell lines were cultured as monolayers in folate-free RPMI 1640 media supplemented with antibiotics and 10% fetal bovine serum in a humidified atmosphere

containing 5% CO₂ at 37°C. These media contained a final folate concentration similar to that of human plasma. Regular tissue culture media were not used since they contain μ M levels of folate, i.e., orders of magnitudes higher than that of the physiological level, potentially blocking the receptor binding of folate conjugates and causing receptor down regulation.

4.3.3. Synthesis of Folate-PEG-Chol

First, for synthesis of NHS-folate, 500 mg of folic acid was dissolved in 10 mL dimethylsulfoxide (DMSO) along with a 1.1 molar excess of N-hydroxysuccinimide (NHS); 1.1 molar excess of dicyclohexylcarbodiimide (DCC) was then added and the reaction mixture stirred overnight in darkness at room temperature. The insoluble byproduct, dicyclohexylurea was removed by filtration through glass wool. The filtrate containing the DMSO solution of NHS-folate was stored at -20°C until use in further synthesis.

Folate-PEG-amine was synthesized by reacting PEG-bis-amine (MW ~3350) with 1.1 molar excess of NHS-folate in DMSO. Briefly, 500 mg PEG-bis-amine was dissolved in 2 mL DMSO; 1.4 mL of the above-synthesized folate-NHS was then added. The reaction mixture was incubated overnight at room temperature in darkness. The progression of the reaction was monitored by the depletion of primary amines measured by a ninhydrin assay. Approximately 50% of the initial amines were converted at the end of the reaction. DMSO and low molecular weight by-products were removed from the reaction mixture by gel-filtration on a Sephadex G-25 column equilibrated in deionized water. The folate-PEG-amine product, which contained trace amounts of folate-PEG-

folate and PEG-bis-amine, was loaded onto a Diethylaminoethyl (DEAE)-Trisacryl anion-exchange column and eluted with an NH_4HCO_3 gradient. Folate-PEG-amine was eluted at $\sim 20 \text{ mM } \text{NH}_4\text{HCO}_3$, lyophilized and stored at -20°C . Folate and free amine concentrations were calculated based on absorbance of folate at 363 nm using a molar extinction coefficient of $6500 \text{ M}^{-1} \text{ cm}^{-1}$ and a ninhydrin assay, respectively. The ratio of folate to amine groups was $\sim 1:1$.

To synthesize folate-PEG-Chol, a 1.1 molar excess of cholesteryl chloroformate was added to folate-PEG-amine in chloroform. The reaction was carried out overnight at room temperature. Formation of folate-PEG-cholesterol was monitored by the disappearance of the free amino group, which was determined by a ninhydrin assay. The product, folate-PEG-Chol, was dried under vacuum, washed twice with ether to remove any residual cholesteryl chloroformate, re-dissolved in chloroform and stored at -20°C . The purity of the product was analyzed by silica gel thin-layer chromatography (TLC) using a solvent system composed of chloroform/methanol (7/3, v/v). The final folate-PEG-Chol concentration was calculated based on absorbance of folate at 363 nm using a molar extinction coefficient of $6500 \text{ M}^{-1} \text{ cm}^{-1}$.

4.3.4. Liposome Preparation

Liposomes were prepared with compositions of DSPC/Chol/PEG-DSPE/folate-PEG-Chol (60/34/5/1, m/m) (targeted), DSPC/Chol/PEG-DSPE/folate-PEG-DSPE (60/34/5/1, m/m) (targeted positive control), or DSPC/Chol/PEG-DSPE (60/34/6, m/m) (non-targeted control). Synthesis of folate-PEG-DSPE was performed as described previously (19).

For preparation of liposomes encapsulating calcein, a chloroform solution of the lipid mixture with the above compositions containing 60 mg of DSPC were dried into a thin film in a round-bottom flask on a rotary evaporator, and then further dried for 2 h under vacuum. The lipid mixture was then hydrated and suspended in 2 mL of 50 mM calcein solution. The lipid suspension was then subjected to 6 cycles of freezing and thawing, and then extruded 10-times through a 0.1 μm pore-size polycarbonate membrane using a handheld LiposoFastTM Extruder. The calcein containing liposomes were then purified on a 10 mL Sepharose CL-4B size-exclusion column equilibrated in phosphate-buffered saline (PBS, 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4). The mean diameter of the extruded liposomes was 104 ± 16 nm, determined on a NICOMP Particle Sizer Model 370. The final calcein concentrations in the liposome preparations were calculated from absorbance at 495 nm using a molar extinction coefficient of $80,000 \text{ M}^{-1} \text{ cm}^{-1}$. Phospholipids content was measured using a colorimetric assay as described previously.

For preparations of liposomes encapsulating DOX, a previously described pH-gradient-driven “remote loading” method was used (25). DOX concentration was calculated from the absorbance at 495 nm using a molar extinction coefficient of $10,650 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 7.5. Loading efficiency obtained was approximately 95%, with a DOX/lipid weight ratio of 1/25. The DOX-loaded liposomes were sterilized by passing through a 0.2 μm cellulose acetate filter, stored at 4°C, and used within 3 days of preparation.

4.3.5. Uptake of Liposomes by Cultured KB Cells

For liposome binding studies, KB cells were seeded in 24-well tissue culture plates and grown for 24 h to reach ~ 100% confluence. Targeted or control liposomes encapsulating calcein were then added to the cells in 0.5 mL culture medium at various concentrations. Incubation was performed at either 4°C or 37°C for 4 h. In competitive binding assays, varying concentrations of free folic acid was also included in the incubation media. Following incubation, the cells were carefully washed 3-times with cold PBS, and lysed in 0.5 mL PBS containing 1% Triton X-100. The lysis procedure results in the complete fluorescence dequenching of cell-associated calcein. The fluorescence intensity of the cellular lysates was determined at 490 and 510 nm excitation and emission wavelengths, respectively, using a Perkin Elmer Luminescence Spectrometer LS50B. Relative fluorescence values were converted to phospholipid concentrations based on a standard curve of phospholipid concentration versus calcein fluorescence measured in the cell lysis buffer.

4.3.6. Fluorescence Microscopy

To visualize liposome uptake by fluorescence microscopy, KB cells cultured as a monolayer in 75 cm² flasks were harvested by treatment with trypsin/EDTA solution. The trypsinized KB cells were pelleted by centrifugation, resuspended in fresh culture media at a density of 10⁶ cell/mL, and then aliquoted into 1.5-mL microcentrifuge tubes. Cells were then incubated for 2 h at 37°C under gentle shaking with calcein-containing folate-targeted or control liposomes diluted in culture media with a final calcein concentration of 50 µM. The cells were then washed 3-times with PBS and examined

under a Zeiss Axioskop Epifluorescence Microscope with an Optronics three chip, low light level color CCD camera attachment. Digital images were collected and analyzed using the NIH Image 1.6 software, and printed on a Tektronix Phaser 450 photographic printer.

4.3.7. Cytotoxicity Study

The cytotoxicity of liposomal and free DOX was determined by MTT assay. KB cells were seeded in 96-well plates to reach ~ 25% confluence at the time of the study. The cells were treated in triplicate with 1:4 serial dilutions of the various formulations for 2 h at 37°C, and then washed 4-times with PBS. Fresh, serum-containing media were then added and the cells were cultured for an additional 48 h. Cell viability was then determined by the MTT assay, as described previously (19).

4.3.8. Preparation of ^{111}In -labeled Liposomes

To prepare ^{111}In -labeled liposomes for biodistribution studies, targeted and non-targeted control liposomes with the above described compositions were prepared with the addition of 1 mole% of DTPA-DSPE. The synthesis of DTPA-DSPE was performed by reacting equimolar amounts of DTPA anhydride with DSPE overnight. The liposome preparation was performed according to the same procedure as described above except PBS alone was used for lipid hydration. The resulting liposomes had a mean diameter of 103 ± 14 nm. For radiolabeling, 200 μL of 100 mg/mL sodium citrate was added to 100 μL of the liposome sample containing 1.5 mg phospholipids, followed by the addition of 1 mCi of $^{111}\text{InCl}_3$ in 500 μL of 50 mM HCl. The labeling mixture incubated at 37°C for

30 min. The ^{111}In -labeled liposomes were then purified by gel filtration on a Sepharose CL-4B column. The labeled liposome fractions were pooled and diluted to a final lipid concentration of $500\mu\text{g/mL}$. The radiochemical yield was $\sim 90\%$.

4.3.9. Animal Model and Biodistribution Studies

Female C57BL/6 mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were placed on a folate-free rodent diet for 10 days prior to inoculation of tumor cells. Normal diet was not used due to its high folate content of 4 mg/kg , which would result in a plasma folate concentration much greater than the physiological concentration in humans. Approximately 5×10^5 FR (+) 24JK-FBP tumor cells were then inoculated subcutaneously in the shoulder region of each mouse in a volume of $100\text{ }\mu\text{L}$ using a 27-gauge needle. Biodistribution studies were performed 2 weeks after tumor cell implantation, when all mice developed a significant tumor mass. In groups of three, the mice were injected intravenously with $100\text{ }\mu\text{L}$ of ^{111}In -labeled liposomes containing $50\mu\text{g}$ of phospholipids and $\sim 25\text{ }\mu\text{Ci}$ of radioactivity, with or without co-administration of 1 mg of free folic acid. Animals were sacrificed at 24 h post injection and major organs were collected including blood, heart, lung, kidneys, spleen, liver, muscle, and tumor. Harvested tissue samples were weighed and counted for radioactivity in an automatic gamma counter. The biodistribution of ^{111}In was calculated as a percentage of the injected dose per gram of tissue weight (%ID/g).

4.4. RESULTS

4.4.1. Synthesis of Folate-PEG-Chol

Folate-PEG-Chol was synthesized by coupling folate-PEG3350-amine to cholesteryl chloroformate as shown in Fig. 4.1. When analyzed by silica-gel thin-layer chromatography, the folate-PEG-Chol product appeared as a single spot with an R_f value of 0.86. Compared to the synthesis of folate-PEG-DSPE (19), the above synthetic procedure was much simpler.

4.4.2. Liposome Uptake by Cultured KB Cells

To assess the efficacy for targeting the folate receptor, liposomes containing 1 mole% folate-PEG-Chol encapsulating calcein, a membrane impermeable fluorescent dye, were prepared. Liposomes containing 1 mole% folate-PEG-DSPE with calcein were used as a positive control. Human oral cancer KB cells, which overexpress the FR, were incubated with various liposome samples at 37°C for 4 h. The cell-associated calcein was then quantified by fluorescence spectroscopy following the lysis with 1% Triton X-100. As shown in Fig. 4.2, uptake of folate-PEG-Chol-targeted liposomes was 20-times greater than that of non-targeted liposomes. Presence of 1.5 mM free folic acid reduced the level of cellular uptake by more than 80%, suggesting a folate receptor-mediated cellular uptake mechanism. There appeared to be no significant differences in targeting efficiency between DSPE and cholesterol-anchored folate molecules, although liposomes targeted with folate-PEG-Chol exhibited a higher level of cellular uptake (~1.2-2 folds) than those with the same mole percentage of folate-PEG-DSPE (Fig. 4.2). Cellular

uptake was virtually unaffected by the presence of 20% fetal bovine serum (data not shown).

KB cell uptake of folate-PEG-Chol targeted liposomes was saturable at 4°C with an apparent $B_{\max}$ of 2.5×10^4 pmoles phospholipids/mg protein (Fig. 4.3). When incubation was performed at 37 °C, a temperature permissible to both receptor binding and endocytosis, the level of cellular uptake for the targeted liposomes was approximately 2-fold greater. As demonstrated by Dr. Guo, uptake of folate-PEG-Chol-containing liposomes could be competitively inhibited by excess free folate, (Fig. 4.4). The folate concentration required to reduce cellular liposome uptake by 50% was 0.39 mM folate, i.e., orders of magnitudes higher than its K_d (~ 0.1 nM) for the receptor. The much stronger binding of targeted liposomes was probably due to the multivalent interaction between the liposome-associated folates and the clusters of cellular FRs. Based on the surface area of a 100-nm liposome and an estimated area per DSPC headgroup of $90 \text{ \AA}^2$, 1 mole% folate-PEG-Chol provides ~ 1150 molecules per liposome. Assuming that ~50% of these are on the outer surface of the liposomes, ~ 575 molecules of folate were available for receptor binding. Since lipids in a bilayer could diffuse freely and the sizes of these liposomes were sufficiently large, it is conceivable that multiple attachments may be formed between the liposome and a cluster of FRs on the surface of the KB cell. Competitive binding of ligands to a receptor can be described by the equation: $K_i = IC_{50}/(1+[ligand]/K_{d-app})$. Based on a K_i of 0.1 nM for folic acid, the K_{d-app} for these folate-targeted liposomes was calculated to be 1.5×10^{-15} M.

4.4.3. Intracellular Distribution of Fluorescent Liposomes

Intracellular distribution of calcein-containing liposomes targeted with folate-PEG-Chol was also examined in KB cells by fluorescence microscopy. Fluorescent micrographs of suspended KB cells obtained following a 4 h incubation at 37°C are shown in Fig. 4.5. For cells treated with folate-PEG-Chol-targeted liposomes, the overall cellular fluorescence was very high and the liposomes were distributed both on the cell surface and in intracellular vesicles (panel 1). In contrast, incubation with liposomes in the presence of 1.5 mM free folate resulted in a much greatly reduced level of cellular fluorescence (panel 5), and cells treated with non-targeted control liposomes showed very little fluorescence above the background (panel 3).

4.4.4. Cytotoxicity of Targeted Liposomes carrying DOX

DOX is one of the most commonly used anticancer drugs and the first drug to be formulated in liposomes for the clinical treatment of human tumors. DOX was loaded into the liposomes via a transmembrane pH-gradient using a “remote-loading” procedure. KB cells were incubated with liposomal or free DOX at 37°C for 2 h. Cell viability was measured by an MTT assay. DOX concentrations resulting in 50% cell killing (IC_{50}) were determined from concentration-dependent cell viability curves. As determined by Dr. Guo, the IC_{50} of folate-PEG-Chol-targeted liposomal DOX was 0.41 μ M, which was 1.9- and 38-times lower than free DOX and DOX in control liposomes, respectively (Table 4.1). When 1 mM free folate was present during the incubation, the IC_{50} of DOX loaded in the targeted liposome sample increased to 4.23 μ M. The superior cytotoxicity of folate-targeted DOX-containing liposomes was presumably due to increased cellular

uptake mediated by binding to the FR. These data were consistent with those obtained previously with DOX liposomes targeted with folate-PEG-DSPE (19).

4.4.5. Tumor Localizing Properties of Folate-Targeted Liposomes

To assess the potential for liposome targeting *in vivo*, C57BL/6 mice carrying the syngeneic 24JK-FBP cell-derived subcutaneous implants were used as a model. The liposomes contained 1 mole% DTPA-DSPE and were labeled with ^{111}In . As with calcein-containing liposomes, ^{111}In -labeled liposomes containing folate-PEG-Chol were efficiently taken up by cultured KB and 24JK-FBP cells, which could be blocked with free folate (data not shown). For biodistribution studies, the ^{111}In -labeled liposome samples were administered intravenously. Two groups of mice also received 1 mg of free folic acid co-injected to block receptor-mediated tumor uptake. Animals were sacrificed 24 h post injection and tissues were harvested for gamma counting. As shown in Fig. 4.6, both non-targeted and folate-PEG-Chol-targeted liposomes showed similar patterns of biodistribution *in vivo*, with liver and spleen being the primary organs of liposome accumulation. Tumor uptake of the liposomes was moderate, with tumor-to-blood ratios of 6.5 ± 3.1 and 7.1 ± 2.4 for targeted and non-targeted control liposomes, respectively. Free folate had little effect on biodistribution of either of the liposome formulations. These data suggest that the presence of a targeting ligand did not significantly improve the overall level of uptake in solid tumors.

4.5. DISCUSSION

Targeted liposomes containing a newly synthesized lipophilic folate conjugate, folate-PEG-Chol, were characterized for their receptor targeting properties in cultured KB cells. Although cholesterol has previously been used as an anchor for PEG in long-circulating liposomes (26), the use of cholesterol as a lipophilic anchor for liposome targeting has not been reported previously. It is possible that cholesterol, which normally packs tightly in a phospholipid bilayer, could serve to stabilize the structural integrity of the membrane when used as a lipophilic anchor for a targeting ligand. A long spacer based on PEG3350 was incorporated between folate and cholesterol based on the previous observation that folate, when directly attached to the lipidic anchor, could not be recognized by the cellular FR (18). Moreover, a more recent study showed that better receptor targeting efficiency was observed with a longer PEG spacer (20). Compared to the synthesis of folate-PEG-DSPE, the synthesis of folate-PEG-Chol was considerably simpler, involving only two steps (19). Although concerns have been raised over the possible exchange of cholesterol between liposomes and circulating lipoproteins, such mechanism of extraction may not affect chemical derivatives of cholesterol, as suggested by the observation that presence of serum did not abolish receptor-specific cellular uptake of the targeted liposomes (27).

Human oral cancer KB cells, which overexpress the FR, were used as an *in vitro* model to study the receptor-targeting efficacy of folate-PEG-Chol containing liposomes. No significant differences in overall cellular uptake levels were found between these liposomes and targeted liposomes containing folate-PEG-DSPE. Cellular uptake of folate-PEG-Chol-targeted liposomes could be competitively blocked by free folic acid.

However, the liposomes exhibited extraordinarily high apparent binding affinity, presumably due to their capacity for multivalent binding to the FRs. The K_{d-app} value obtained was $\sim 10^5$ -times less than that of the K_d for folic acid. The measured apparent affinity of the liposomes for KB cells was likely affected by kinetic factors since free folate had a much faster rate of diffusion and was relatively unobstructed by the steric hindrance which may adversely affect the cellular binding of liposomes. The dissociation constant, K_d , can also be defined as k_{off}/k_{on} , and is affected by the free energy of the ligand/receptor complex and the rate of diffusion. Since the liposomes are much larger than low molecular weight folate conjugates, the total system free energy is much greater and the rate of diffusion much slower. These could in turn lead to a higher apparent K_d . The apparent affinity of the liposomes to the tumor cell is therefore influenced by both the multivalency of the binding, and the factors discussed above.

The design of targeted delivery systems with multivalent ligand could minimize receptor binding competition from endogenous free ligands, which may compromise tumor targeting of monovalent folate conjugates. Meanwhile, the *in vivo* target of these delivery systems may extend to weaker binders of folate, such as the prostate-specific membrane antigen (PSMA), a folate hydrolase overexpressed in prostate carcinomas as well as the neovasculature of a wide variety of human solid tumors (28).

Data from this study also showed that liposome binding to KB cells was saturable. Based on a mean cell diameter of $15\ \mu\text{m}$, the available membrane area for a surface-attached KB cell is $\sim 400\ \mu\text{m}^2$. No more than 5×10^4 100 nm liposomes should be able to fit into this area. The number of liposomes bound per cell at saturation determined in this study was 4.4×10^4 , i.e., a close match with the available cellular surface area. This

suggests that targeted liposomes binding to cells that greatly overexpress the FR is limited by available surface area rather than the number of FRs.

The potential use of these targeted liposomes in drug delivery was further assessed using DOX-containing liposomes. The DOX liposomes targeted with folate-PEG-Chol showed 38-times greater cytotoxicity compared to non-targeted liposomes. The increase in cytotoxicity due to targeting could be partially reversed by the addition of 1 mM free folic acid. Free folate could not completely abolish the effect of targeting due to the extraordinarily high affinity of the liposomes for the cells. These data were consistent with previous results obtained with liposomes targeted with folate-PEG-DSPE. The differences in receptor-mediated drug delivery efficacy among these two targeted liposome formulations could be attributed to differences in the lipid compositions of the liposomes, which may affect the rate of DOX release from the liposomes. Free DOX is known to accumulate rapidly in cells by passive diffusion, therefore, it was not surprising that even the targeted liposomes showed only slightly higher toxicity compared to free DOX in our cell culture study. *In vivo*, however, liposomal DOX is expected to exhibit much longer blood residence time and lower acute toxicity compared to free DOX.

Preliminary studies were also carried out to characterize the *in vivo* tumor localizing properties of FR-targeted liposomes in syngeneic solid tumor models. Our data showed no statistically significant differences between the biodistribution of the targeted and the non-targeted liposomes, including selectivity for the tumor. This is consistent with previous data obtained with immunoliposomes. Due to the increased endothelial permeability and reduced lymphatic drainage in solid tumors, the benefit of receptor targeting is not likely derived from increased overall tumor uptake. The high

liver and spleen uptake levels could possibly be partially attributed to the presence of 1 mole% DTPA-DSPE, a negatively-charged lipid needed for radiolabeling with ^{111}In . However, previous studies with HER2-targeted immunoliposomes containing DOX did show that the targeted liposomes exhibited better tumor penetration, tumor cell uptake, and therapeutic efficacy (5). Therefore, further studies are warranted to study the intratumoral and subcellular distribution of folate-PEG-liposomes.

Folate as a targeting ligand is stable, small, non-immunogenic, and readily available. Therefore, folate-PEG-liposomes have the potential to be produced in clinically relevant quantities and constitute a good candidate for future clinical development. Although the use of these targeted liposomes in the treatment of solid tumors is questionable, these drug carriers may be highly effective in targeting systemic malignancies, such as myeloid leukemias (28), which is known to have high incidence of FR expression. Another exciting new possibility is the targeting of tumor epithelium via PSMA (29). Targeting epithelium does not require extravasation and may greatly improve the delivery efficiency of antiangiogenic or thrombotic factors. Further *in vivo* studies are warranted to study the tumor targeting potential of folate-PEG-liposomes.

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DOX FORMULATION	IC₅₀ (μM) (n = 3)
Folate-PEG-liposome	0.41
Folate-PEG-liposome + 1 mM folic acid	4.23
Non-targeted liposome	15.4
Free DOX	0.78

Table 4.1. Comparison of cytotoxicity of various DOX formulations in KB cells

Fig. 4.1. Scheme for the synthesis of folate-PEG-Chol.

Fig. 4.2. Uptake of folate-targeted liposomes by cultured KB cells. The cells were treated in triplicates with various liposomes containing 20 μ M calcein at 37°C for 4 h. The formulations were: A) non-targeted liposomes; B) liposomes containing 1 mole% folate-PEG-DSPE; C) liposomes containing 1 mole% folate-PEG-DSPE in the presence of 1.5 mM free folic acid; D) liposomes containing 1 mole% folate-PEG-Chol; and E) liposomes containing 1 mole% folate-PEG-Chol in the presence of 1.5 mM free folic acid.

Fig. 4.3. Uptake of folate-targeted liposomes by cultured KB cells: dependence on concentration. The cells were treated in triplicates with various concentrations of liposomes at 4°C for 4 h.

Fig. 4.4. Uptake of folate-targeted liposomes by cultured KB cells: competition by free folate. The cells were treated in triplicates with folate-PEG-Chol-targeted liposomes containing 20 μ M calcein at 4°C for 4 h in the presence of various concentrations of free folate. One hundred percent uptake was defined as liposome uptake in the absence of free folate.

Fig. 4.5. Fluorescence micrographs of KB cells treated with calcein-containing liposomes targeted with folate-PEG-Chol. KB cells, as a trypsinized suspension, were incubated with various types of liposomes at 50 μ M in calcein for 2 h at 37°C. Left panels indicate cells visualized in the fluorescence mode; right panels show the same fields in the phase-contrast mode. Cells were treated with 1,2) folate-PEG-Chol-targeted liposomes; 3,4) non-targeted liposomes; or 5,6) folate-PEG-Chol-targeted liposomes in the presence of 1mM free folate.

Fig. 4.6. Biodistribution of folate-PEG-Chol-targeted liposomes in tumor-bearing mice. C57BL/6 mice ($n = 3$) bearing subcutaneous 24JK-FBP cell-derived tumors were treated with ^{111}In -labeled liposomes in groups of 3. The mice were sacrificed at 24 h after intraperitoneal injection.

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