# Folate Receptor-Mediated Liposomal Delivery of a Lipophilic Boron Agent to Tumor Cells *in Vitro* for Neutron Capture Therapy

**Jennifer J. Sudimack,[1] Dianne Adams,[2] Joan Rotaru,[2] Supriya Shukla,[1] Junhua Yan,[1] Masaru Sekido,[1] Rolf F. Barth,[2] Werner Tjarks,[1] and Robert J. Lee[1,3]**

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***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-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$], in FR-overexpressing tumor cells for neutron capture therapy.

***Methods.*** Large unilamellar vesicles (~200 nm in diameter) were prepared with the composition of egg PC/chol/K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] (2:2:1, mol/mol), with an additional 0.5 mol % of folate-PEG-DSPE or PEG-DSPE added for the FR-targeted or nontargeted 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 with those treated with control liposomes. In contrast, FR-targeted and nontargeted 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.

**KEY WORDS:** folate receptor; drug targeting; neutron capture therapy; boron; liposomes.

## 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 after irradiation of a stable $^{10}B$ isotope with thermal neutrons:

$$^{10}B + {}^{1}n \rightarrow\rightarrow {}^{7}Li + {}^{4}He\ (2.28\ MeV)$$

The resulting kinetic energy is distributed between the ionizing particles, $^4He$ (α particle), and $^7Li^+$ 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 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 use of drug carriers, such as liposomes (10–14, reviewed in 15), and low-density lipoproteins (16,17), as well as covalent conjugates of monoclonal antibodies (18–21) and epidermal growth factor (22–24).

Liposomal delivery of boron is an attractive approach for BNCT because liposomes are capable of carrying relatively large quantities of boron compounds. Low encapsulation efficiencies have resulted in the production of liposomes containing hydrophilic compounds (25). 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 as a result of the increased extravasation and retention in tumor tissues as result of 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 to achieve selective delivery of boron compounds to tumor cells.

In this study, we have evaluated FR-targeted and nontargeted liposomal formulations that incorporate a lipophilic boron agent, K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] (13). Uptake studies were performed 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 to determine the intracellular distribution of boron delivered by the liposomes. These studies are described in detail below.

[1] College of Pharmacy, Rm. 542 LM Parks Hall, The Ohio State University, 500 W. 12th Avenue, Columbus, Ohio 43210.

[2] College of Medicine and Public Health, The Ohio State University, Columbus, Ohio 43210.

[3] To whom correspondence should be addressed. (e-mail: lee.1339@osu.edu)

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## MATERIALS AND METHODS

### Materials

Egg phosphatidylcholine (PC), monomethoxy-polyethylene glycol-2000 (PEG-2000), and distearoylphosphatidylethanolamine (DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Calcein, cholesterol, folic acid dihydrate, Sepharose CL-4B resin, and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] was synthesized, as described previously (13). Tissue culture media, fetal bovine serum (FBS), and antibiotics were purchased from Life Technologies (Rockville, MD, USA). Folate-polyethyleneglycol-DSPE (f-PEG-DSPE) was synthesized as reported previously (26).

### 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% $CO_2$ 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.

### Preparation of Liposomes Incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]

FR-targeted and nontargeted liposomes were prepared using a previously described procedure (27) based on polycarbonate membrane extrusion. Briefly, egg PC, cholesterol, and K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] were dissolved at a 2:2:1 molar ratio in chloroform ($CHCl_3$). The FR-targeted and nontargeted liposome preparations contained an additional 0.5 mole % of f-PEG-DSPE or PEG-DSPE, respectively, which were added to the $CHCl_3$ solution and dried to a thin film in a round-bottom flask. The dried lipid was then hydrated in 1 mL of phosphate-buffered saline (PBS, 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, pH 7.4) and dispersed by mixing. The resulting suspension of multilamellar vesicles were subjected to six 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, Canada). The resulting large unilamellar liposomes were separated from unincorporated K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] 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 in the liposomes was determined by direct current plasma-absorption spectroscopy (DCP-AES), as previously described (28).

FR-targeted and non-targeted liposomes, incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] and encapsulating fluorescent calcein, were prepared as described above, except that the dried lipids were hydrated with an 80 mM calcein solution.

### 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*g*, and resuspended in folate-free culture media at a density of $3 \times 10^7$ cells/mL. Various concentrations of FR-targeted or nontargeted liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] were added to the media and incubated at 37°C with gentle shaking for 2 h. After this, the media were removed by centrifugation at 400*g* for 5 min. To remove unbound, extracellular liposomes, the cells were washed three times with cold PBS by resuspension and centrifugation at 400*g*. The media, wash, and digested cell pellets were collected for boron analysis by DCP-AES.

### 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 $1 \times 10^7$ cells/mL. For the competitive binding assays, 1 mM of free folate was added to the incubation media. Various concentrations of FR-targeted or nontargeted liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] were added to the KB cell suspension and incubated at 37°C with gentle shaking for 2 h. After incubation, the cell samples were prepared as described above for the F98 cell binding study.

To determine the intracellular distribution of cell-associated boron-containing liposomes, cells treated with FR-targeted and nontargeted 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-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]-incorporated liposomes encapsulating 50 μM calcein. The cell pellets were then resuspended in 1 mL of PBS after incubation and cell washing. KB cell images were obtained in both the fluorescence (dark field) and phase-contrast (bright field) modes using a 40× objective. Digital images were collected and analyzed using the NIH Image 1.6 (Springfield, VA, USA) software.

KB cells that had been treated with K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]-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 after resuspension in PBS. All fluorescence measurements were performed using a Perkin-Elmer LS-5B spectrofluorometer operated with an FTWinlab (Morena Valley, CA, USA) software program. The excitation and emission wavelengths were set at 490 nm and 520 nm, respectively.

### Intracellular Distribution of Boron in KB Cells

Subcellular fractionation experiments were performed using a previously described method (29). Briefly, KB cells were suspended by treatment with 5 mM EDTA and pelleted by centrifugation at 400*g*. KB cells ($2.0 \times 10^8$) were then resuspended in serum- and folate-free RPMI 1640 media and separated into two aliquots of $2.0 \times 10^7$ cells/mL. FR-targeted

or nontargeted liposomes, incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$], 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*g* for 5 min and the cells were washed three times with cold PBS. During the last wash, the cells were counted in a hemocytometer to determine cell viability and final cell concentration before fractionation. The cells were centrifuged at 400*g* for 3 min and then resuspended in 7 mL of cold distilled water and lysed by sonication on ice. The lysates were centrifuged at 1000*g* for 10 min and the pellet was resuspended in a 0.25 M sucrose/1.8 mM $CaCl_2$/1% Triton X-100 solution. A sucrose gradient was made by adding an equal volume of 0.34 M sucrose/0.18 mM $CaCl_2$ solution to the bottom of the tube, pushing up the lighter solution. The nuclear pellet was obtained after centrifugation at 600*g* for 10 min. The first supernatant was centrifuged at 3500*g* for 10 min to obtain the mitochondrial pellet. The supernatant fraction was again centrifuged at 16,000*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.

## RESULTS

### Characterization of Liposomes Incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]

FR-targeted and nontargeted large unilamellar vesicles (~200 nm in diameter) incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] (Fig. 1) into the liposomal bilayer were prepared and analyzed. As shown in Fig. 2, the FR-targeted and nontargeted liposomes showed ~100% incorporation efficiency, as evidenced by the absence of a free boron fraction. Furthermore, 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.

### Cellular Uptake of Boronated Liposomes *in Vitro*

F98 and KB cells were incubated with FR-targeted and nontargeted liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] for 2 h at 37°C. To determine whether FR-mediated delivery was responsible for cellular uptake of boronated liposomes, FR (–) F98 cells were exposed to FR-targeted and nontargeted K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]-containing liposomes. As shown in Fig. 3, identical amounts (<0.2 μg of $^{10}B/10^7$ cells) of boron were delivered by FR-targeted and nontargeted liposomes to F98 cells.

To determine whether there was specific targeting to an

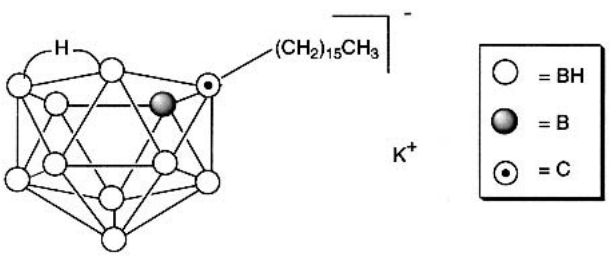


**Fig. 1.** The structure of K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$], which was synthesized as described in the Materials and Methods section.

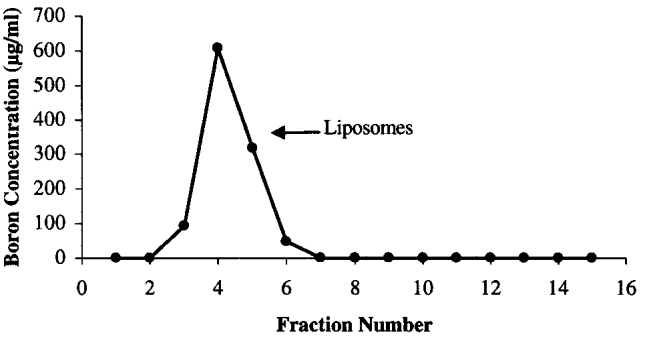


**Fig. 2.** Incorporation efficiency of the lipophilic boron agent into PEG-ylated folate receptor–targeted and nontargeted liposomes. Entrapment of the boron compound was determined by loading a 0.5-mL sample onto a 10-mL Sepharose CL-4B column. Fifteen consecutive 1-mL fractions were eluted with phosphate-buffered saline, and the amount of boron was determined by direct current plasma-absorption spectroscopy.

FR (+) KB cell line, the cells were exposed to FR-targeted and nontargeted liposomes. Furthermore, a competitive binding assay was performed using 1 mM free folic acid. As shown in Fig. 4, uptake of the FR-targeted liposomes by KB cells was approximately 10 times higher than the nontargeted 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 coincubation with 1 mM free folate.

Comparison of the uptake efficiencies of the FR-targeted and nontargeted liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] taken up by either KB or F98 cells are shown in Fig. 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.

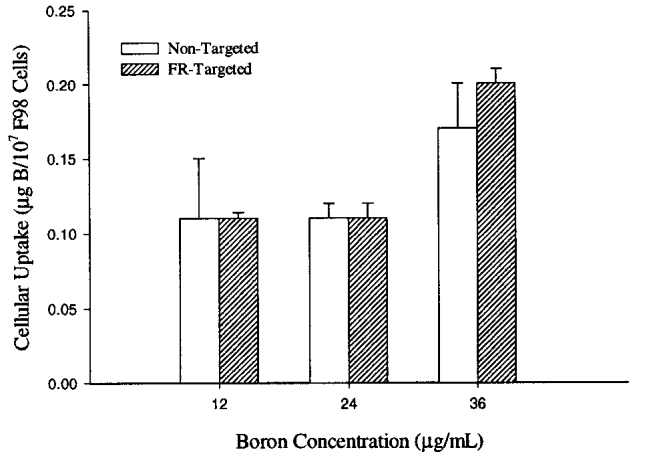


**Fig. 3.** Cellular uptake of boron delivered by folate receptor–targeted and nontargeted liposomes in cultured F98 cells. Cells were incubated with the boron-containing liposomes for 2 h at 37°C, as described in the Materials and Methods section. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

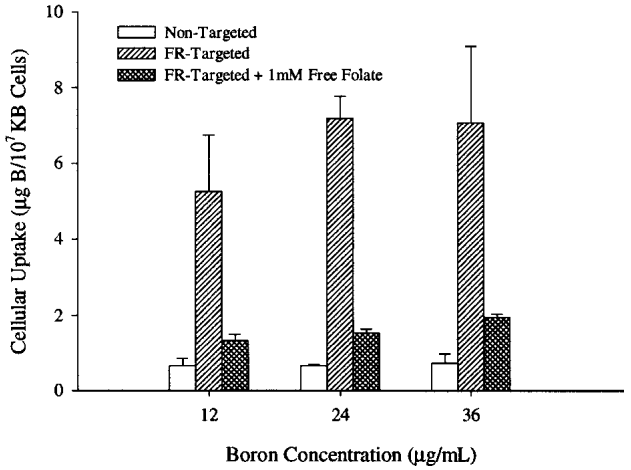


**Fig. 4.** Cellular uptake of boron delivered by folate receptor–targeted and nontargeted liposomes in cultured KB cells. A competitive binding assay was performed using 1 mM free folate. Cells were incubated with the boron-containing liposomes for 2 h at 37°C, as described in the Materials and Methods section. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

The intracellular distribution of cell-associated FR-targeted boronated liposomes ($^{10}$B concentration = 24 μg/mL) encapsulating calcein was studied by fluorescence microscopy. As shown in Fig. 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 nontargeted 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 nontargeted liposomes ($^{10}$B concentration = 24 μg/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.

### Subcellular Distribution of K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] 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 nontargeted liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] for 2 h at 37°C at a boron concentration of 24 μg/mL because 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 nontargeted liposomes is shown in Fig. 7. Delivery of FR-targeted liposomes resulted in a total boron uptake of 587 μg $^{10}$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.

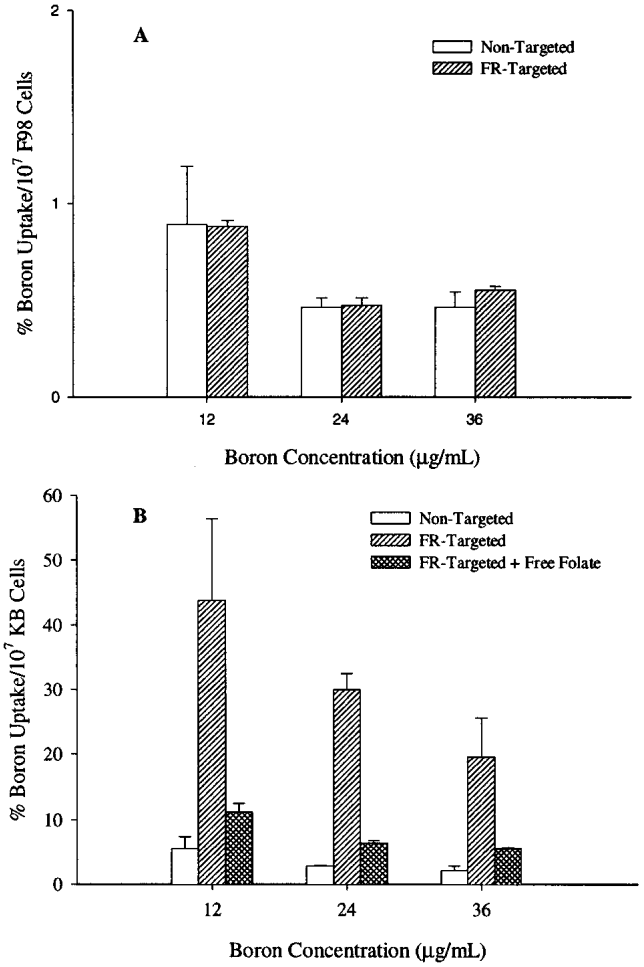


**Fig. 5.** The boron uptake efficiency by cultured (A) F98 cells and (B) KB cells after treatment with three different concentrations of boron-containing, folate receptor–targeted, or nontargeted liposomes. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

The overall boron uptake resulting from the delivery by nontargeted liposomes was shown to be much lower (42.9 μg $^{10}$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 nontargeted liposomes was undetectable in the cytosol/endosomes and the nuclear fractions. Overall, the boron uptake resulting from FR-targeted (587 μg $^{10}$B/10$^9$ KB cells) liposomal delivery exhibited much greater boron content in the subcellular fractions compared to nontargeted (42.9 μg $^{10}$B/10$^9$ KB cells) liposomes. These results demonstrate that FR-targeting specifically increased boron uptake by KB cells.

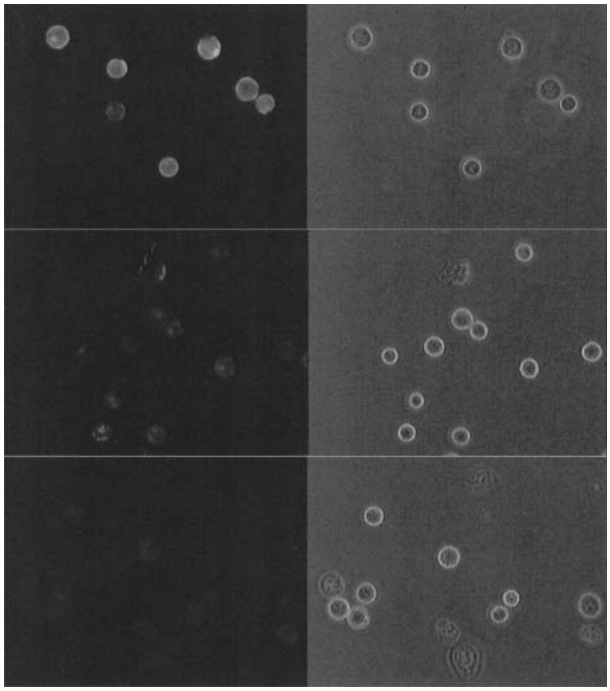

**Fig. 6.** Fluorescence micrographs of the selective uptake of folate receptor–targeted boron and calcein-containing liposomes by KB cells. A suspension of KB cells were incubated with folate receptor–targeted and nontargeted and boron (24 μg/mL)- and calcein (50 μM)-containing liposomes and were photographed in both the fluorescence (dark fields) and the phase contrast mode (bright fields) on a microscope as described in the Materials and Methods section. Top panels, cells treated with folate receptor–targeted liposomes; middle panels, cells treated with folate receptor–targeted liposomes + 1mM free folate; bottom panels, cells treated with nontargeted liposomes. Left panels: micrographs taken in the fluorescence mode; right panels: the same field viewed in the phase contrast mode.

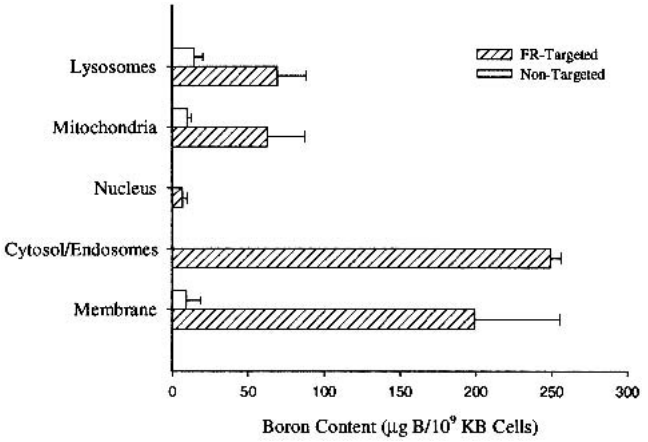


**Fig. 7.** Boron content of various subcellular fractions of KB cells measured by direct current plasma-absorption spectroscopy after exposure to either folate receptor–targeted or nontargeted, boron-containing liposomes for 2 h at 37°C, as described in the Materials and Methods section. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

## DISCUSSION

In this study, we have evaluated FR-targeted and nontargeted 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 nontargeted liposomes. Furthermore, 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 μg $^{10}B$/mL) for 2 h, after which boron concentrations were determined. The lipophilic boron compound showed remarkable intracellular accumulation in KB cells and was not removed by extensive washing. Because of 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 μg $^{10}B/10^9$ cells and 42.9 μg $^{10}B/10^9$ cells for FR-targeted and nontargeted boron-containing liposomes, respectively. Assuming that $10^6$ cells weigh ~1 mg and a boron level of 20–30 μg $^{10}B$/g of tissue would be sufficient for therapy, both FR-targeted and nontargeted 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. 3, the FR-targeted liposomes were not preferentially taken up by FR (−) F98 cells *in vitro*. However, FR-targeted liposomes exhibited over 10-fold greater boron uptake than the nontargeted 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. 6. Whereas the nontargeted liposomes only showed minimal fluorescence around the plasma membrane, which was indicative of nonspecific 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, is the target of choice (30). A boron concentration, which is 2.5-fold 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 (30). 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 (Figs. 6 and 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 nontargeted liposomes was the same (approximately 10-fold difference between FR targeted and nontargeted) at the same boron concentration.

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 used 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, because folate is endocytosed, targeted moieties can accumulate intracellularly because of receptor recycling. In addition, because of 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 use 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.

## ACKNOWLEDGMENTS

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