Tumor-Cell Targeted Epidermal Growth Factor Liposomes Loaded with Boronated Acridine: Uptake and Processing

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Purpose. The aim of this work was to investigate the cellular binding and processing of polyethylene glycol-stabilized epidermal growth factor (EGF) liposomes. The liposomes were actively loaded with water-soluble boronated acridine (WSA), primarily developed for boron neutron capture therapy.

Methods. The uptake, internalization, and retention of EGF-liposome conjugates were studied in two cultured monolayer cell-lines, A-431 and U-343, with regard to the nuclide-label on the targeting agent, the carrier, and the load. The subcellular localization of WSA was studied using confocal microscopy.

Results. We found that the liposome complex was internalized after specific binding to the EGF receptor. After internalization in the tumor cells, WSA was distributed mainly in the cytoplasm and was shown to have long cellular retention, with 80% of the boron remaining after 48 h.

Conclusions. The long retention of the compound and the cellular boron concentration reached makes these targeted liposomes interesting for further development toward boron neutron capture therapy.

KEY WORDS: EGF; liposome; BNCT; tumor targeting; cellular processing.

INTRODUCTION

Liposomes, i.e., phospholipid bilayer spheres, have been of interest as delivery vehicles for tumor therapy more than 25 years (1). The first liposomes used, i.e., conventional liposomes, were rapidly taken up by the immune system when injected in the bloodstream. About 10 years ago, sterically stabilized liposomes were first used (2) and have since gained much attention. The most widely used method for sterical stabilization is attachment of polyethylene glycol (PEG) chains on the surface of the liposome. This makes the liposome more stable and less prone to RES recognition (3,4). Liposomes have the clear therapeutic advantage of being able to deliver large amounts of drug and they are known to gather in sites of pathology even without a targeting ligand, mainly because of the leaky vasculature of tumors and inflammations (2,3,5).

We have chosen to further improve tumor uptake by attaching a targeting ligand on the distal end of the liposomal PEG chain (6). Because the epidermal growth factor (EGF) receptor is overexpressed in many tumor cells, including gliomas, breast cancers, colon, and prostate cancers, EGF is used as a tumor-seeking agent (7). It is also known that EGF undergoes receptor-mediated endocytosis, which can bring the ligand-receptor complex inside the cell. Although this makes EGF an ideal targeting agent for liposomes, few studies are made so far (8,9) and none using PEG-stabilized liposomes. A recent review on targeted liposomes inquires studies of EGF-targeted liposomes (10).

The use of liposomes for tumor targeting has mostly been studied with antibody-conjugated immunoliposomes to target, for example, HER-2 (11) and CD-19 (12). Other receptor structures have also been studied such as the folate receptor that can be successfully targeted by attaching folic acid to the liposome (13). Our goal is to develop tumor targeting one step further by applying a concept called two-step targeting. In this model, targeting is directed to known target structures on tumor cells in a first step. The nuclides should be released from the liposomes after receptor-mediated internalization and should also have a chemical structure that provides binding to chromosomal DNA of the targeted tumor cell in a second step. Calculations (14) have shown that treatment of cancer with nuclide therapy would be much more effective if the radiation can be localized within the cell nucleus of the tumor cell rather than to the cell membrane or the cytoplasm. To achieve such two-step targeting, DNA-binding compounds have been developed, and here we used a boronated acridine derivative, water-soluble boronated acridine (WSA; Fig. 1; 15).

WSA is a candidate drug for boron neutron capture therapy (BNCT), where the stable nuclide, ^{10}B, produces a helium ion and a lithium ion after neutron capture-induced fission. Healthy tissue can be saved because only the cells that take up the compound are affected by the ion particles (16). The two major problems with BNCT so far are specificity and intracellular concentration. Targeted liposomes might surmount these obstacles. With targeting against EGF receptor, better specificity could be obtained compared with the compounds used today, and because of the properties of liposomes, large amounts of boron can be loaded. Because WSA consists of a boron cage with 10 boron atoms and, because of its charge, can be actively loaded into liposomes (17), high intracellular concentrations could be achieved through receptor interactions. Furthermore, because WSA is an acridine analogue, the desired intracellular concentration is decreased if WSA target the DNA of the tumor cell.

The aim of this study was to investigate the cellular uptake and processing of EGF-liposomes loaded with WSA. The specificity of uptake, internalization, and retention of EGF-targeted liposomes in two different cell lines both overexpressing the EGF-receptor, U-343 MGa Cl 12:6, a glioma cell line and A-431, a squamous carcinoma cell line, were studied. We also tried to localize the boron compound after internalization and compared this with the localization of doxorubicin after uptake of EGF-targeted liposomal doxorubicin.

MATERIALS AND METHODS

Materials

Murine EGF, obtained from Chemicon International, was labeled with ^{125}I from Amersham Biosciences, Uppsala,

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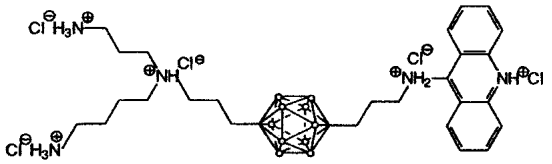

Fig. 1. Structure of water soluble boronated acridine.

Sweden. The protein was modified with 2-iminothiolane (Traut's Reagent from Sigma, St. Louis, MO, USA) to obtain thiol groups for conjugation. The Sephadex G-25 columns (NAP-5 columns) and Sephadex G-150 and Sepharose CL-4B gels used for separation were purchased from Amersham Biosciences, Uppsala, Sweden. PEG (3400)-maleimide was purchased from Shearwater polymers (Huntsville, AL, USA) and 1,2-disteaoryl-sn-glycero-3-phosphatidylethanoleamine (DSPE) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). The lipids forming the liposomes, 1,2 disteaoryl-sn-glycero-3-phosphatidylcholine (DSPC), and 1,2-disteaoryl-sn-glycero-3-phosphatidylethanolamine-*N*[poly(ethylene glycol)-2000] (DSPE-PEG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol was obtained from Sigma Aldrich (Stockholm, Sweden). (^{3}H)cholesteryl hexadecyl ether was purchased from NEN, Perkin-Elmer Life Sciences (Boston, MA, USA). The DNA binding compound, 1,8-diamino-4-*N*-3-[12-(N-9-acridinyl-3-aminopropyl)-*p*-carborane-1-yl]propyl-4-azooctane hydrogen chloride (WSA derivative) was synthesized as described in (15).

Monolayer Cells

The cell lines used, U-343 MGaCl2:6 (a subclone of U-343MG, hereafter referred to as U-343; 18) and A-431 (American Type Culture Collection, CRL 1555, Rockville, MD, USA; 19) were grown in Ham's F-10 medium supplemented with 10% fetal calf serum and PEST (penicillin 100 IU/mL and streptomycin 100 μg/mL), L-glutamine (2 mM), and amphotericin B (2,5 μg/mL; all from Biochrom KG, Berlin, Germany). The cells were incubated at 37°C in humidified air containing 5% CO_2. For experiments the cells were grown in 3-cm dishes with 3–10 $\times 10^5$ cells each. Before all experiments, the cells were washed once with serum-free medium.

Methods

Preparation of Targeted Liposomes

Preparation of (^{125}I)EGF-liposome-WSA. Conjugates were prepared according to Bohl Kullberg *et al.* (20). Briefly: DSPE-PEG(3400)-maleimide lipids were diluted in HEPES buffer (20 mM with 150 mM NaCl pH 7.4) to form micelles. EGF (25 μg), radiolabeled with ^{125}I (20 MBq when boron determination and fluorescent imaging was intended, 50 MBq for all other preparations) using Chloramine-T (20), was modified by Traut's reagent. The modified (^{125}I)EGF-SH was mixed with the lipid micelles for 24 h at room temperature (1:30 EGF:phospholipid molar ratio, PL concentration approx. 0.2 mM). The resulting (^{125}I)EGF-PEG-DSPE (yield, approx. 30%) was purified by gel filtration (Sephadex G-150) and added to preformed liposomes (DSPC:cho:DSPE-PEG 57:40:3) prepared by freeze-thawing and extrusion and thereafter actively loaded with WSA (0.2:1 molar ratio) as described earlier (17). The EGF lipids were allowed to incorporate in the liposome membrane during 1 h at 60°C (1:33, EGF-PEG-DSPE:liposome molar ratio, liposome concentration 1mM). After incorporation, the (^{125}I)EGF-liposome-WSA conjugate (yield, approx. 30%) was purified by gel filtration (Sepharose CL-4B).

Preparation of (^{125}I)EGF-Liposome-WSA (Nonpegylated). To test the importance of the PEG in the liposome membrane, targeted liposomes without PEG was prepared. The (^{125}I)EGF was conjugated directly to modified DSPE lipids in the liposomes. Liposomes was composed of DSPC-:cho:DSPE-SPDP (55:40:5; 0.5 μmol, 1 mM) loaded with WSA (0.2:1). EGF was labeled and modified with Traut's reagent as described above. The modified (^{125}I)EGF-SH was conjugated to the liposomes at room temperature overnight and the conjugate was thereafter purified by gel-filtration (Sephadex G-150; yield 30%).

(^{125}I)EGF-Liposome-Dox. The preparation procedure for the doxorubicin-loaded liposome-conjugates was identical as for (^{125}I)EGF-liposome-WSA except that the liposomes were actively loaded with doxorubicin using an ammonium gradient instead of a pH gradient (21).

Preparation of EGF-(^{3}H)Liposome-WSA. The conjugate preparation procedure was the same as for (^{125}I)EGF-liposome-WSA except that no radioactive label was used on EGF. Instead, 0.5 MBq (^{3}H)-cholesteryl hexadecyl ether per μmol lipid was added during the liposome preparation. This gave a (^{3}H)-labeled liposome allowing the studies of the liposome part of the conjugate.

Displacement

Displacement studies were made to analyze the specificity of the EGF-liposome conjugate. A volume of 0.5 mL of (^{125}I)EGF-liposome-WSA (final concentration: PL, 20 nmol/mL, boron, 67 nmol/mL) was added to dishes with 0.5 mL of nonradioactive EGF at the concentration 0, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^2, 10^3, or 10^4 ng/mL. The cells were incubated with the conjugates for 4 h. The incubation media was then removed, cells were washed six times with cold serum-free medium, trypsinized (trypsin EDTA), and resuspended as single cells (total volume 1.5 mL/dish). A volume of 0.5 mL was used for cell counting and 1 mL was measured in a gamma counter (1480 Wizard 3, Wallac, Turku, Finland) to determine the amount of liposome conjugate bound to the cells.

Uptake Studies

The cellular binding of the conjugates during continuos incubation were analyzed in uptake studies. A volume of 0.5 mL of (^{125}I)EGF-liposome-WSA, EGF-(^{3}H)liposome-WSA or (^{125}I)EGF-liposome-WSA (nonpegylated; only U-343 cells) was added to cells (final concentration: PL, 20 nmol/mL, boron, 67 nmol/mL). Control samples where the receptors had been preblocked with 0.5 mL EGF (1 μg/mL) was also used. The cell dishes were incubated for 1, 2, 4, 8, 14, and 24 h with (^{125}I)EGF-liposome-WSA and (^{125}I)EGF-liposome-WSA(nonpegylated), and for 1, 4, 8, and 24 h with EGF-(^{3}H)liposome-WSA. After incubation the cells were washed six times with cold serum-free medium and treated with trypsin-EDTA as described above. For (^{125}I)EGF-liposome-WSA, the radioactivity was determined with gamma counter and for EGF-(^{3}H)liposome-WSA by use of a liquid scintilla-

tion counter (1214 RackBeta Excel, LKB-Wallac, Turku, Finland).

Internalization Study

Internalization was analyzed by distinguishing between membrane-bound and internalized radioactivity during continuos incubation of the conjugates. A volume of 1 mL of (^{125}I)EGF-liposome-WSA or EGF-(^{3}H)liposome-WSA was added to cell-dishes (final concentration: PL, 20 nmol/mL, boron, 67 nmol/mL). Control samples with preblocked receptors were used. The cell dishes were incubated for 1, 2, 4, 8, and 24 h. The cells were washed six times with cold serum-free medium. To remove the membrane bound conjugate, 0.5 mL of ice-cold 0.1 M glycin–HCl (pH 2.5) was added to the cells and kept at 4°C for 6 min (22). The acid was removed and the dishes were rinsed once more with 0.5 mL of ice-cold glycin–HCl. To obtain the internalized conjugate, 0.5 mL of 1 M NaOH was added to the dishes and incubated for 1 h at 37°C. The solution was collected from the dishes, which were then rinsed once more with 0.5 mL of 1 M NaOH. The membrane-bound and internalized radioactivity was determined with a gamma counter (^{125}I) and liquid scintillation counter (^{3}H).

Retention

Retention of (^{125}I)EGF-liposome-WSA or EGF-(^{3}H)liposome-WSA was studied after 24 h of incubation with the cells. The retention of the ^{125}I-labeled on EGF, the ^{3}H-labeled cholesterol, and the boron in WSA were studied. After incubation with 1 mL of conjugate (final concentration: PL, 20 nmol/mL, boron, 67 nmol/mL), the cell dishes were washed six times with cold serum-free medium. Fresh culture medium without conjugate was added and the cells were further incubated for 1, 4, 8, 24, and 48 h. After incubation, the cells were washed another six times and trypsinized, and cell-bound radioactivity was determined with a gamma counter or in a liquid scintillation counter. The amount retained was determined by dividing by the radioactivity in the cell after the 24-h incubation.

For the boron determination experiments, cells were grown in 10-cm dishes to semiconfluence with approx. 10^7 cells per dish. They were incubated 24 h with 10 mL of (^{125}I)EGF-liposome-WSA (final concentration: PL, 4 nmol/mL, boron, 13 nmol/mL, WSA concentration 1 μg/mL) and then washed and further cultured as described above. After trypsinization, the cell suspension was transferred to a centrifuge tube and centrifuged for 5 min at 340*g* to obtain a cell pellet. The pellet was then resuspended and transferred to an Eppendorf tube and centrifuged at 7000*g* for 2 min. After thorough removal of the supernatant, the cell pellet was weighed, dissolved in nitric acid, and digested under heat and pressure (23). The samples were diluted in water and analyzed for its total boron content with inductively coupled plasma mass spectrometry.

Fluorescent Imaging

The natural fluorescence of WSA and DOX were used to analyze their cellular distribution. Cells grown on cover glasses in 3-cm dishes were incubated for 24 h with (^{125}I)EGF-liposome-WSA or (^{125}I)EGF-liposome-DOX (WSA and DOX concentration, 1 μg/mL and 2 μg/mL, respectively). Thereafter, the cells were washed six times with serum-free medium and once in phosphate-buffered saline. The cover glasses were mounted onto slides using fluorescent mounting medium (Fluorescent Mounting Medium; Dako, Carpinteria, CA, USA). Cells incubated with (^{125}I)EGF-liposome-WSA for 24 h and further incubated with fresh culture medium without conjugate for 6 days were also studied. After the 6-day incubation, the cells were washed and mounted as above. Ocular inspection of the cell-associated fluorescence was performed using laser confocal microscopy (Leica TCS-SP, Heidelberg, Germany). Fluorescence of both WSA and doxorubicin was studied using excitation at 488 nm and the captured images were projections of 20 slices through the cells.

To determine the DNA binding of free WSA, cells were grown as monolayer cultures on microscope slides, washed with serum-free medium, and incubated with WSA (1 μg/mL) for 24 h. Next, cells were thoroughly washed six times with complete culture medium and once in PBS. The slides were mounted as above and the DNA binding of WSA was analyzed by ocular inspection with a conventional fluorescence microscope (Leica DMRXE, excitation filter: 420–490 nm. Digital images were captured (ColourCoolView, Photonic Science).

RESULTS

Displacement

Displacement of (^{125}I)EGF-liposome-WSA binding with increasing amounts of nonradiolabeled EGF was possible for both cell-lines (Fig. 2). This was taken as evidence that the liposome conjugate binds to the EGF receptor. An unspecific binding of 10% remained for A-431 cells when the receptors were blocked, for U-343 the unspecific binding was slightly larger. The difference in background binding for the two cell lines could not be explained.

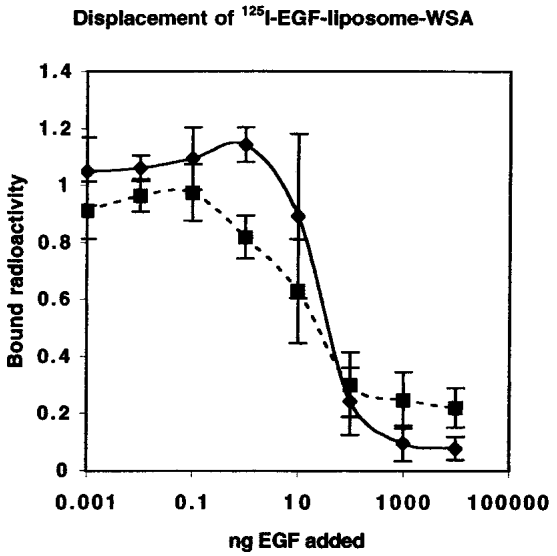

Fig. 2. Displacement of (^{125}I)EGF-liposome-WSA with epidermal growth factor. The incubation time was 4h. (♦) A-431 cells and (■) U-343 cells. The maximal uptake was set to 1. Error bars represent standard deviations. n = 6 for A-431 and 9 for U-343.

Uptake Studies

The time-dependent uptake is shown in Fig. 3. For (^{125}I)EGF-liposome-WSA, the ^{125}I uptake reached a plateau after 8–14 h incubation (Fig. 3A). Because the incubation medium is not depleted and the receptors not saturated, this plateau is probably caused by cellular degradation and excretion of low molecular weight ^{125}I. Excretion of radioactivity has previously been shown with EGF-conjugates to be low molecular degradation products (24). For EGF-(^{3}H)liposome-WSA (Fig. 3B), the uptake pattern was different; no plateau was reached even after 24 h of incubation. This difference indicates less excretion of radioactivity from the liposomes. The background with nontargeted liposomes was the same as for targeted liposomes with blocked receptors (data not shown). When using nonstabilized liposomes without PEG (Fig. 3C), the uptake was different; the unspecific background binding was over 50% after 24 h, which indicates that these liposomes also interact with the cell surface in some other way. Table I shows the level of liposomal PL uptake for the incubation with EGF-(^{3}H)liposome-WSA. After 24 h, the liposomes taken up by the cells corresponded to an intracellular PL concentration of 1.2 nmol/10^6 cells for A-431 and 1.9 nmol/10^6 cells for U-343. Table II shows the boron uptake corresponding to the incubation with EGF-liposome-WSA. This indicates continuous increase during the times studied. For U-343, the boron concentration reached 6 ppm after 24 h; and for A-431, 4.5 ppm.

Table I. Uptake of Phospholipid (PL) in Cells as a Function of Time

Incubation time (h)	U-343 nmol PL/10^6 cells	A-431 nmol PL/10^6 cells
1	0.16 ± 0.04	0.19 ± 0.02
4	0.55 ± 0.3	0.6 ± 0.3
8	0.9 ± 0.4	0.8 ± 0.4
24	1.9 ± 0.9	1.2 ± 0.4

Note: The uptake of PL was determined by measurements of (^{3}H)cholesteryl hexadecyl ether uptake.

Internalization Study

The results of the internalization study are shown in Fig. 4. For the cell line U-343, both conjugates were rapidly internalized (Fig. 4A and B). Even after the first hour, less than 20% was membrane-bound. For A-431, internalization was slower. Only after 24 h was most of the EGF-(^{3}H)liposome-

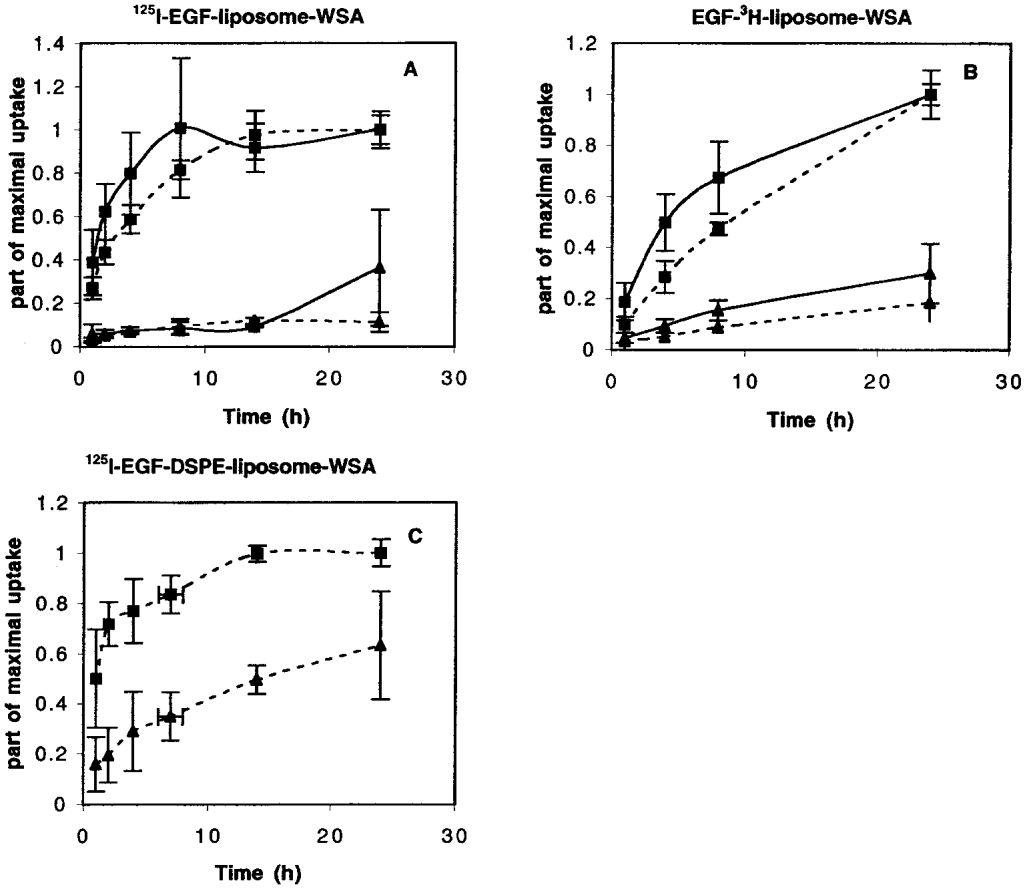

Fig. 3. Time dependent uptake of (A) (^{125}I)EGF-liposome-WSA, (B) EGF-(^{3}H)liposome-WSA, and (C) (^{125}I)EGF-liposome-WSA(nonpegylated). Solid lines refer to A-431 and dotted lines to U-343. (■)Specific uptake. (▲)Unspecific uptake is determined by targeted liposomes with blocked receptors. The uptake of nontargeted liposomes was studied as well but as the result did not differ from the results with blocked receptors, data are not shown. Maximal uptake after 24 h was set to 1. Error bars represent standard deviations. n = 6.

Table II. Boron Determinations

Incubation time (h)	U-343 Boron concentration (ppm)	A-431 Boron concentration (ppm)
1	1.0 ± 0.03	1 ± 0.04
4	2.4 ± 0.3	1.4 ± 0.03
24	5.7 ± 1.7	4.5 ± 1.5

WSA internalized (Fig. 4D). By incubation on ice, this method was verified to work with the conjugates. This treatment lead to a shift in radioactivity from internalized fraction to membrane-bound fraction.

Retention

The retention in cells preincubated for 24 h with the various liposome conjugates was good (Fig. 5). The retention of the targeting agent clearly differed from that of the liposome and the boron. After 24 h, about 80% of the (^{3}H)liposomes and the boron remained in both cell lines. When the label on EGF was studied, 35–40% remained instead. The difference was even more pronounced after 48 h when 80% of the boron remained in both cell lines. The (^{3}H)liposome remained above 80% for the U-343 cells, compared to 30% for the ^{125}I on EGF.

Fluorescent Imaging

The internalization of the WSA-liposome conjugate was studied using laser confocal microscopy. The compound was clearly visualized within the cell but no nuclear staining could be seen (Fig. 6A). Even after 6 days, the compound taken up by the cells was clearly visible but did not reach the nucleus (Fig. 6B). EGF-targeted liposomes loaded with doxorubicin was used to identify whether the lack of nuclear staining with WSA was an effect of the EGF receptor-mediated endocytosis pathway or the properties of the compound WSA. Doxorubicin is known to enter the nucleus after liposomal tumor targeting (25). We also found that the nuclei of A-431 cells were stained with doxorubicin (Fig. 6C). To confirm that WSA is a DNA-binding compound we demonstrated that WSA *per se* bound to the cell nucleus (Fig. 6D).

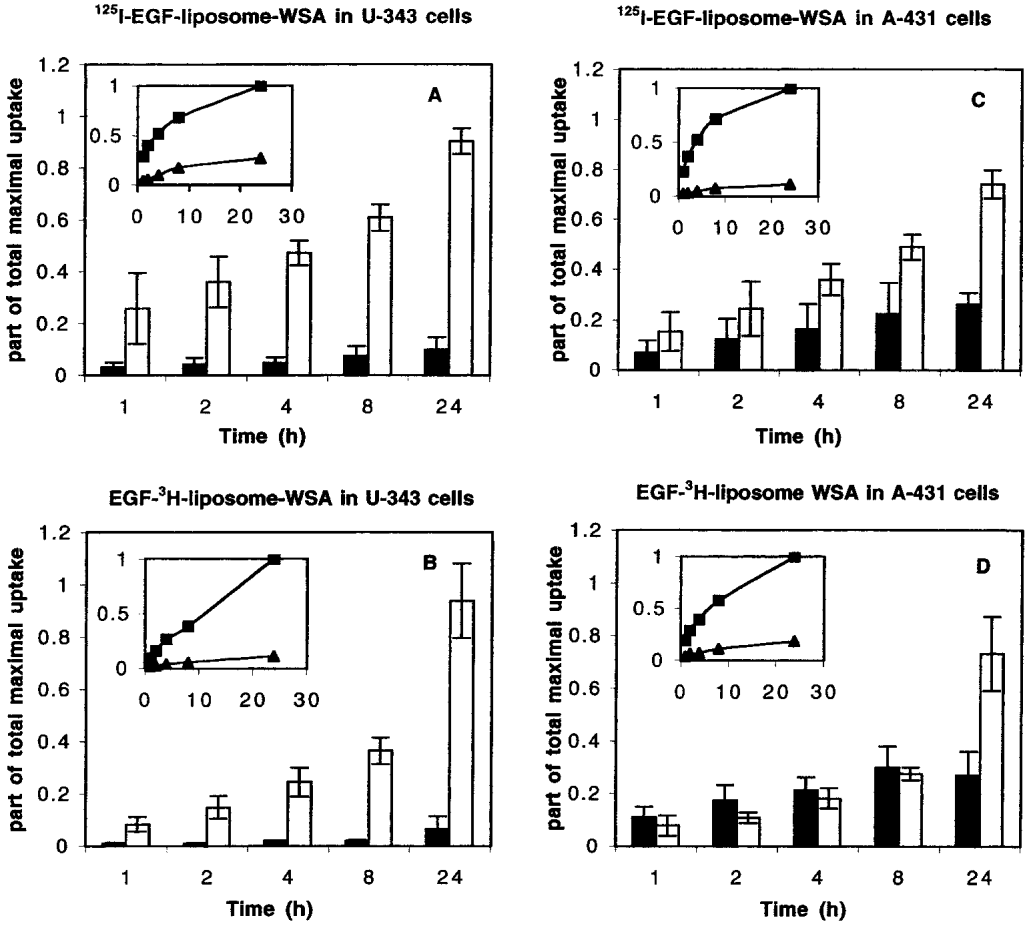

Fig. 4. Internalization of (^{125}I)EGF-liposome-WSA and EGF-(^{3}H)liposome WSA. (A) (^{125}I)EGF-liposome-WSA internalization in U-343 cells. (B) EGF-(^{3}H)liposome WSA internalization in U-343 cells. (C) Internalization of (^{125}I)EGF-liposome-WSA in A-431 cells. (D) EGF-(^{3}H)liposome WSA internalization in A-431 cells. Filled bars: membrane-bound, empty bars: internalized. The time-dependent uptake (internalized + membrane-bound) is inserted in each graph. Specific uptake is marked with (■) and unspecific uptake, blocked receptors, is marked with (▲). In all cases maximal uptake (internalized + membrane bound) was set to 1. Error bars represent standard deviations. n = 6.

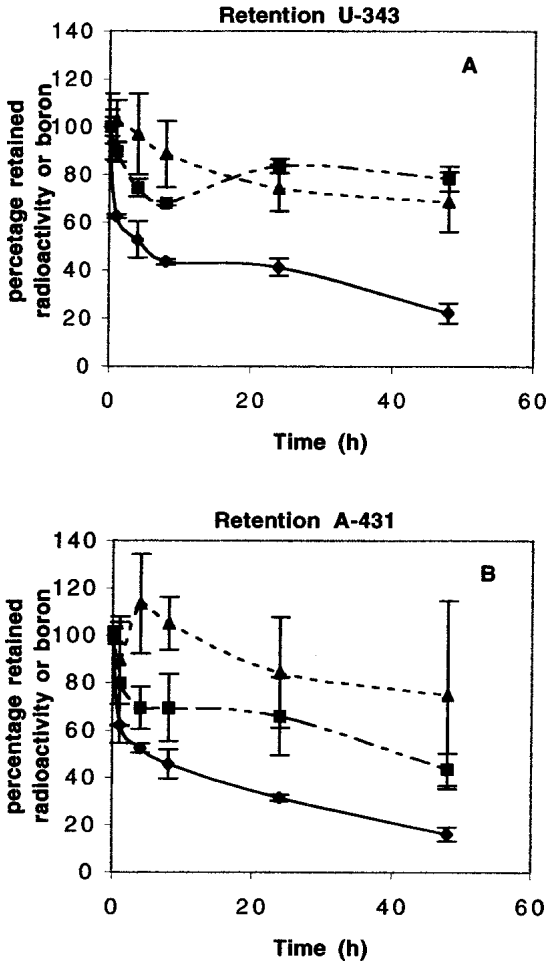

Fig. 5. Retention of (^{125}I)EGF-liposome-WSA, EGF-(^{3}H)liposome-WSA, and EGF-liposome-WSA (boron) after 24-h incubation. (**A)** U-343 cells and (**B)** A-431 cells. (♦) (^{125}I)EGF-liposome-WSA, (■) EGF-(^{3}H)liposome-WSA, and (▲) EGF-liposome-WSA (boron determinations). Error bars represent standard deviations. n = 6 for EGF-(^{3}H)liposome-WSA, n = 5 for EGF-liposome-WSA (boron), and n = 3 for (^{125}I)EGF-liposome-WSA.

DISCUSSION

This article describes the cellular uptake and processing of EGF-targeted liposomes loaded with the boronated acridine WSA. Cultured tumor cells showed a specific uptake of the liposome-conjugate with resulting boron concentrations of 6 ppm. The retention of this boron was high, 80% remained in the cells after 2 days, allowing long time for clearance of non-targeted conjugate in an *in vivo* situation. The liposomes specifically targeted the EGF receptor, as was shown by the competition assay. No difference in the unspecific binding of nontargeted liposomes and targeted was seen when an excess of EGF blocked the cell receptors, demonstrating that adding a targeting ligand improves the tumor-cell specificity. It is known that the stability of liposomes is greatly improved by adding PEG and this was also shown to improve the specificity. When using nonstabilized liposomes without PEG the unspecific uptake was as high as 50%, indicating that an uptake mechanism other than receptor-mediated endocytosis occurs. The uptake level of PL presented was above 1 nmol/10^{6} cells after 24 h incubation for both cell lines. This level of

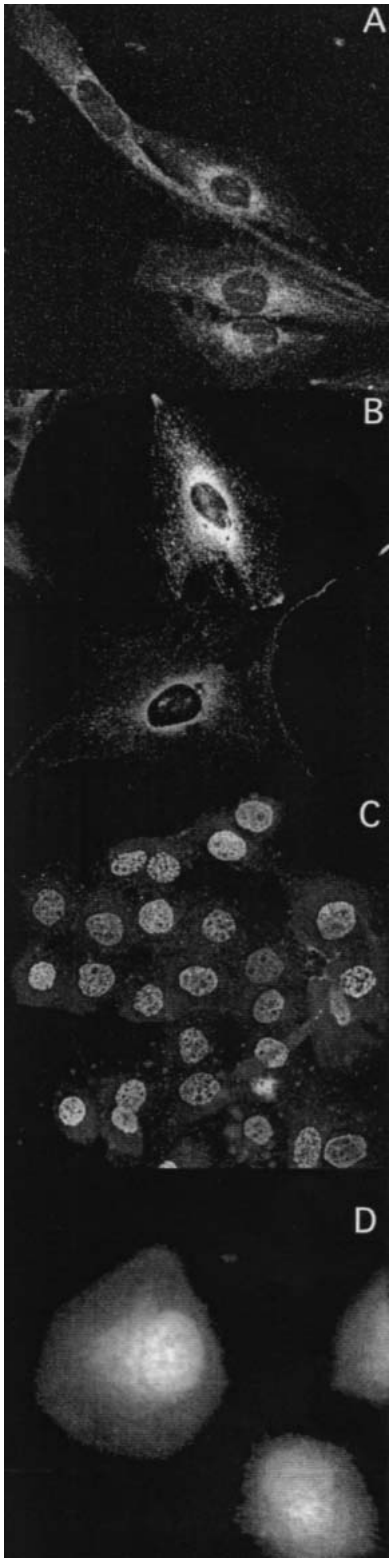

Fig. 6. Fluorescent images of (A) EGF-liposome-WSA in live cells after 24-h incubation, (B) EGF-liposome-WSA in live cells after 24-h incubation with conjugate and 6 days further incubation with fresh culture medium without conjugate, (C) EGF-liposome-doxorubicin in live cells after 24-h incubation, and (D) WSA in live cells after 24 h incubation. WSA concentration 1 μg/mL and doxorubicin concentration 2 μg/mL

uptake is approx. the same as has been reported with folate or anti-CD19 targeted liposomes, but here we used a lower concentration of conjugates (13,26), which is promising for EGF-receptor targeted delivery.

The conjugates were mostly internalized, especially in the U-343 cells, as shown with the acid wash experiment. Notably, the level of internalization between cell lines differed. This difference might be because the EGF-receptors recycle in A-431 cells (27). After internalization, the receptor binding might be so strong that the liposome conjugate is transported out to the cell surface again, giving rise to a higher membrane-bound signal. This has also been seen earlier for EGF-dextran conjugate when targeted against cultured A-431 cells (24).

The tumor cell retention of radioactivity from (^{125}I)EGF-liposome-WSA and EGF-(^{3}H)liposome-WSA clearly differed. When the ^{125}I label on the targeting ligand was studied, the excretion was rapid at first, probably corresponding to the degradation of EGF. This rapid excretion was not seen for boron or ^{3}H-labeled liposomes. Although (^{3}H)cholesteryl-ether is commonly used to study liposome-uptake in cells (13,26) it is not clear whether it reflects the degradation of liposomes. Retention of boron is the most important characteristic for a clinical application and the results shown here are promising because approx. 80% of the boron remained in both cell lines after 48 h. Furthermore, the result from laser confocal microscopy, showing clear WSA presence in cytoplasm 6 days after interrupted incubation, indicates that the retention of boron within the cells is high even for longer periods.

The goal of two-step targeting is to deliver nuclide-labeled compounds to the tumor cell nuclei. Proof of principle with two-step targeting was achieved when liposomes were loaded with doxorubicin; the first step by the specific binding and internalization of the EGF- targeted liposome, the second by observing the nuclear staining of doxorubicin. With WSA-loaded liposomes, the first step worked well but the second did not. The lack of nuclear staining even after long time study (6 days) indicates that some barrier has to be overcome to reach the nucleus. However, nuclear staining was observed when cells were incubated with WSA *per se,* ensuring that the compound really was DNA binding. Whether the lack of DNA binding when delivered with EGF-liposomes was caused by inability of WSA to enter the nucleus or inability to leave the lysosomes is not known yet. Another explanation could be that the liposomes were still intact within the lysosomes and the WSA was unable to leak out of the liposomes. It is known that delivery of actively loaded compounds can be problematic (28) and have been extensively addressed by proposing different liposomal formulations to make the liposomes release their content more readily after internalization (29,30). However, our preliminary tests using liposomes containing DOPE, a pH-sensitive phospholipid, did not yield a nuclear delivery with EGF-liposome-WSA (data not shown). Doxorubicin seems to be an exception to this problem. Several other reports have shown nuclear staining of liposomal doxorubicin (25,26) but the mechanism of drug delivery from the liposome to the nucleus is not yet known.

Because high cellular uptake and a good retention of the boronated compound WSA could be seen, the nuclear uptake might not be necessary for a cell-killing effect. It is known that about 20 ppm ^{10}B is needed for a therapeutic effect and by further development higher intracellular boron concentrations might be reached. The loading procedure and the number of EGF/liposome can be optimized for maximal uptake, thereby possibly elevating the amount of boron delivered to each cell.

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REFERENCES

1. G. Gregoriadis, E. J. Wills, C. P. Swain, and A. S. Tavill. Drug-carrier potential of liposomes in cancer chemotherapy. *Lancet* **1**:1313–1316 (1974).
2. D. Papahadjopoulos, T. M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S. K. Huang, K. D. Lee, M. C. Woodle, D. D. Lasic, C. Redemann, and F. J. Martin. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. USA* **88**:11460–11464 (1991).
3. A. A. Gabizon. Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. *Cancer Res.* **52**:891–896 (1992).
4. A. L. Klibanov, K. Maruyama, V. P. Torchilin, and L. Huang. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett.* **268**:235–237 (1990).
5. I. A. Bakker-Woudenberg, A. F. Lokerse, M. T. ten Kate, J. W. Mouton, M. C. Woodle, and G. Storm. Liposomes with prolonged blood circulation and selective localization in Klebsiella pneumoniae-infected lung tissue. *J. Infect. Dis.* **168**:164–171 (1993).
6. T. M. Allen, E. Brandeis, C. B. Hansen, G. Y. Kao, and S. Zalipsky. A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. *Biochim. Biophys. Acta* **1237**:99–108 (1995).
7. J. Carlsson, L. Gedda, C. Gronvik, T. Hartman, A. Lindstrom, P. Lindstrom, H. Lundqvist, A. Lovqvist, J. Malmqvist, P. Olsson, S. Mayfield, J. P. Vaughn, T. E. Kute, W. A. Denny, R. L. Gutman, G. Peacock, and D. R. Lu. Strategy for boron neutron capture therapy against tumor cells with over-expression of the epidermal growth factor-receptor. *Int. J. Radiat. Oncol. Biol. Phys.* **30**:105–115 (1994).
8. Y. Ishii, Y. Aramaki, T. Hara, S. Tsuchiya, and T. Fuwa. Preparation of EGF labeled liposomes and their uptake by hepatocytes. *Biochem. Biophys. Res. Commun.* **160**:732–736 (1989).
9. A. Kikuchi, S. Sugaya, H. Ueda, K. Tanaka, Y. Aramaki, T. Hara, H. Arima, S. Tsuchiya, and T. Fuwa. Efficient gene transfer to EGF receptor overexpressing cancer cells by means of EGF-labeled cationic liposomes. *Biochem. Biophys. Res. Commun.* **227**: 666–671 (1996).
10. D. C. Drummond, K. Hong, J. W. Park, C. C. Benz, and D. B. Kirpotin. Liposome targeting to tumors using vitamin and growth factor receptors. *Vitam. Horm.* **60**:285–332 (2000).
11. J. W. Park, D. B. Kirpotin, K. Hong, R. Shalaby, Y. Shao, U. B. Nielsen, J. D. Marks, D. Papahadjopoulos, and C. C. Benz. Tumor targeting using anti-her2 immunoliposomes. *J. Control. Release* **74**:95–113 (2001).
12. D. E. Lopes de Menezes, M. J. Kirchmeier, J.-F. Gagne, L. M. Pilarski, and T. M. Allen. Cellular trafficking and cytotoxicity of anti- CD19-targeted liposomal doxorubicin in B lymphoma cells. *J. Liposome Res.* **9**:199–228 (1999).
13. A. Gabizon, A. T. Horowitz, D. Goren, D. Tzemach, F. Mandelbaum-Shavit, M. M. Qazen, and S. Zalipsky. Targeting folate receptor with folate linked to extremities of poly(ethylene gly-

col)-grafted liposomes: in vitro studies. *Bioconjug. Chem.* **10**:289–298 (1999).
14. T. Hartman, H. Lundqvist, J. E. Westlin, and J. Carlsson. Radiation doses to the cell nucleus in single cells and cells in micrometastases in targeted therapy with (^{131})I labeled ligands or antibodies. *Int. J. Radiat. Oncol. Biol. Phys.* **46**:1025–1036 (2000).
15. H. Ghaneolhosseini, W. Tjarks, and S. Sjöberg. Synthesis of novel boronated acridines- and spermidines as possible agents for BNCT. *Tetrahedon* **54**:3877–3884 (1998).
16. R. F. Barth, A. H. Soloway, and R. M. Brugger. Boron neutron capture therapy of brain tumors: past history, current status and future potential. *Cancer Invest.* **14**:534–550 (1996).
17. M. Johnsson, N. Bergstrand, and K. Edwards. Optimization of drug loading procedures and characterization of liposomal formulations of two novel agents intended for boron neutron capture therapy (BNCT). *J. Liposome Res.* **9**:53–79 (1999).
18. B. Westermark, A. Magnusson, and C.-H. Heldin. Effect of epidermal growth factor on membrane motility and cell locomotion in cultures of human clonal glioma cells. *J. Neurol. Res.* **8**:491–507 (1982).
19. H. Haigler, J. F. Ash, S. J. Singer, S. Cohen, F. M. Yakes, W. Chinratanalab, C. A. Ritter, W. King, S. Seelig, and C. L. Arteaga. Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. *Proc. Natl. Acad. Sci. USA* **75**:3317–3321 (1978).
20. E. Bohl Kullberg, N. Bergstrand, J. Carlsson, K. Edwards, M. Johnsson, S. Sjoberg, and L. Gedda. Development of EGF-conjugated liposomes for targeted delivery of boronated DNA-binding agents. *Bioconjug. Chem.* **13**:737–743 (2002).
21. D. D. Lasic, B. Ceh, M. C. Stuart, L. Guo, P. M. Frederik, and Y. Barenholz. Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. *Biochim. Biophys. Acta* **1239**:145–156 (1995).
22. H. T. Haigler, F. R. Maxfield, M. C. Willingham, and I. Pastan. Dansylcadaverine inhibits internalization of 125I-Epidermal Growth Factor in BALB 3T3 cells. *J. Biol. Chem.* **255**:1239–1241 (1980).
23. P. Lindström, P. Olsson, J. Malmqvist, J. Pettersson, P. Lemmen, B. Werner, S. Sjöberg, Å. Olin, and J. Carlsson. New carborane-based compounds for boron neutron capture therapy: binding and toxicity of ANC-1, DAC-1 and B-Et-11-OMe in cultured human glioma and mouse melanoma cells. *Anticancer Drugs* **5**: 43–52 (1994).
24. P. Olsson, A. Lindstrom, and J. Carlsson. Internalization and excretion of epidermal growth factor-dextran-associated radioactivity in cultured human squamous-carcinoma cells. *Int. J. Cancer* **56**:529–537 (1994).
25. D. Goren, A. T. Horowitz, D. Tzemach, M. Tarshish, S. Zalipsky, and A. Gabizon. Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump. *Clin. Cancer Res.* **6**:1949–1957 (2000).
26. D. E. Lopes de Menezes, L. M. Pilarski, A. R. Belch, and T. M. Allen. Selective targeting of immunoliposomal doxorubicin against human multiple myeloma in vitro and ex vivo. *Biochim. Biophys. Acta* **1466**:205–220 (2000).
27. A. Sorkin and C. M. Waters. Endocytosis of growth factor receptors. *Bioessays* **15**:375–382 (1993).
28. E. Mastrobattista, G. A. Koning, and G. Storm. Immunoliposomes for the targeted delivery of antitumor drugs. *Adv. Drug Deliv. Rev.* **40**:103–127 (1999).
29. V. A. Slepushkin, S. Simoes, P. Dazin, M. S. Newman, L. S. Guo, M. C. Pedroso de Lima, and N. Duzgunes. Sterically stabilized pH-sensitive liposomes. Intracellular delivery of aqueous contents and prolonged circulation in vivo. *J. Biol. Chem.* **272**:2382–2388 (1997).
30. D. C. Drummond, M. Zignani, and J. Leroux. Current status of pH-sensitive liposomes in drug delivery. *Prog. Lipid Res.* **39**:409–460 (2000).