Liposome-encapsulated Doxorubicin Targeted to CD44: A Strategy to Kill CD44-overexpressing Tumor Cells

Rom E. Eliaz and Francis C. Szoka, Jr.

Department of Biopharmaceutical Sciences and Pharmaceutical Chemistry, School of Pharmacy, University of California-San Francisco, San Francisco, California 94143-0446

ABSTRACT

Certain tumors, including many that are found in the lung, overexpress the CD44 cell-surface marker. CD44 is a receptor that binds to hyaluronan (HA), a carbohydrate consisting of β1,3 N-acetyl glucosaminyl-β1,4 glucuronic acid. We hypothesized that the incorporation of phosphatidylethanolamine lipid derivatives containing HA oligosaccharides (HA-PE) into liposomes could target drug-containing liposomes to tumor cells that express CD44. HA-PE containing palmitoyl oleoyl phosphatidylethanolamine or dipalmitoyl phosphatidylethanolamine (HA-P-PE) were incorporated into the lipid bilayer at various mole percentages of the total lipids; and the physicochemical properties (diameter, surface charge, and stability) of the resulting liposome preparations were characterized. HA-targeted liposomes (HALs) avidly bound to the CD44-high-expressing B16F10 murine melanoma cell line but not to the CV-1 African green monkey kidney cells, which express CD44 at low levels. Binding of the HALs to the B16F10 cells was rapid, concentration dependent, and saturated at a lipid concentration of about 250 μM. HAL binding to B16F10 was inhibited by HA with high Mₐ and by an HA-PE monoclonal antibody. Binding to the B16 melanoma cells occurred at a lipid composition that contained a ≥0.1 mol % of the HA-P-PE lipid. The bound liposomes were internalized by a temperature-dependent process. The IC₅₀ of doxorubicin (DOX) encapsulated in either HALs or nontargeted liposomes and of nonencapsulated DOX were compared in two protocols: continuous exposure of the cells to treatment for 24 h and transient exposure in which the treatment was applied for a 3-h period, and in which non-cell-associated drug was replaced with drug-free medium for the duration of the experiment. The IC₅₀ of free DOX, DOX-loaded nontargeted liposomes, and DOX-loaded HAL (HAL-DOX) for the transient exposure were 6.4 μM, >172 μM, and 0.78 μM, respectively. For the continuous exposure protocol, the IC₅₀ were 0.60 μM, 25.0 μM, and 0.14 μM, respectively. Thus, in both protocols, HAL-delivered DOX was significantly more potent than the nonencapsulated DOX in cells expressing high levels of CD44, which suggests that HALs may be a useful targeted drug carrier to treat CD44-expressing tumors.

INTRODUCTION

CD44 is found at low levels on epithelial, hemopoietic, and neuronal cells and at elevated levels in various carcinoma, melanoma, lymphoma, breast, colorectal, and lung tumor cells (1–9). This cell surface receptor binds to HA³ (hyaluronic acid) which is a high-Mₐ glycosaminoglycan polymer (Mₐ 1,1E6), composed of the repeating disaccharide β1,3 N-acetyl glucosaminyl-β1,4 glucuronic acid. HA is a major component of the extracellular matrix, and CD44 is implicated in the metabolism of solubilized HA. CD44 appears to regulate lymphocyte adhesion to cells of the high endothelial venules during lymphocyte migration (10, 11), a process that has many similarities to the metastatic dissemination of solid tumors (12–14). It is also implicated in the regulation of the proliferation of cancer cells (15). Birch et al. (16) suggest that CD44 may play a vital role in determining the fate of hematogenously disseminated melanoma cells. In the lung, squamous metaplasia, adenocarcinoma, and large cell lung cancer express CD44; indeed, it is a marker for these cell types of lung cancer (8, 9, 17). Although CD44 is expressed on a number of cell types in normal tissues, it turns out that these cell types are either not in direct contact with the blood or require activation before they bind to HA (4, 5).

Strategies that interfere with CD44-HA interaction, such as the administration of high Mₐ HA (18), an anti-CD44 mAb (19), or a CD44-receptor globulin (19, 20), reduce tumor formation in the lung for animal tumor models established from CD44-expressing tumor cell lines. Because the vascular system is leaky in many tumors (21) so that HA-liposomes would gain access to the tumor cells subsequent to extravasating into the tumor from the circulation, CD44 may be a suitable surface receptor for targeted chemotherapy of cancers that express this receptor. Indeed high Mₐ HA-drug conjugates have been devised for this purpose (22, 23).

The approach that we pursued in this research uses liposomes as a drug carrier into which lipid-linked oligomers of the HA repeat units are attached. The concept is that an appropriately designed ligand with a modest affinity for the receptor, incorporated into liposomes at a suitable surface density, will interact with a greater avidity with cells that have a high number of CD44 receptors on their surface than with cells with a low number of receptors. The liposome provides an ideal surface to test such a hypothesis because the surface density of the ligand in the lipid bilayer can be controlled; furthermore, the ligands are mobile in a fluid bilayer and can rearrange to minimize steric constraints in the interaction with multiple receptors in an adjacent surface (24).

In the present study, we incorporated a low Mₐ synthetic ligand into the liposome surface. We show that B16F10 cells expressing high levels of CD44 avidly bind and internalize HAL in a temperature-dependent manner, whereas cells expressing low levels of CD44 do not. An important finding in this work is that DOX encapsulated in HAL is more potent than the free DOX in both transient and continuous exposure conditions for periods up to 24 h. Thus, the HALs may provide an effective vehicle for delivering chemotherapeutic agents into CD44-expressing tumors in animals.

MATERIALS AND METHODS

Chemicals. All of the phospholipids were purchased from Avanti Polar Lipid (Birmingham, AL) or were synthesized in our laboratory. Chol, SRB, TCA, bee venom, human umbilical cord hyaluronic acid, sodium cyanoborohydride, tAT, ammonium sulfate and FITC-labeled affinity-purified antirat IgG (whole molecule) were purchased from Sigma Chemical Co. (St. Louis, MO). Dowex 50WX4 resin was purchased from Aldrich (Milwaukee, WI). DOX was...
purchased from Bedford Laboratories (Bedford, OH). Culture medium (MEM Eagle’s) with EBSS and DME H-21 (high glucose 4.5 g/liter) was obtained from UCSF Cell Culture Facility. Normal goat serum was obtained from Vector Laboratories Inc. (Burlingame, CA) and antimouse H-CAM (CD44) purified monoclonal. IM7.8.1 isotype rat IgG2b was purchased from Endogen (Woburn, MA). All of the other reagents were of analytical grade.

**Cell Culture Conditions.** B16F10 murine melanoma cell line was obtained from UCSC Cell Culture Facility. CV-1 African green monkey kidney cells were obtained from the American Type Culture Collection (Rockville, MD) and were *Mycoplasma* free. B16F10 cells were maintained in MEM Eagle’s with EBSS medium containing 10% fetal bovine serum, 1% MEM non-essential amino acids, 1% sodium pyruvate 11 mg/ml and 1% penicillin-streptomycin, and 0.1 μM of steroid filtered. CV-1 cells were maintained in DMEM H-21 (high glucose, 4.5 g/l) medium containing 10% fetal bovine serum, 0.01 MEM non-essential amino acids, 1% HEPES buffer (1 m) and 0.01 penicillin-streptomycin. 0.1 μM sterile filtered. Cells were cultured with complete medium at 37°C in a humidified atmosphere of 5% CO2 in air. For all of the experiments, cells were harvested from subconfluent cultures using trypsin and were resuspended in fresh complete medium before plating. Cells with >90% viability, as determined by trypsin blue exclusion, were used.

**Ligand Preparation.** Bee venom hydrolisis of human umbilical cord hyaluronic acid was used to degrade high M₆ HA into smaller fragments (2, 4, 6, and 8 saccharides). Fragments were separated on 11 x 265 mm column of the formate form of Bio-Rad AG-3 x 4A ion-exchange resin (eluted with 270-ml portions of 0.015, 0.05, 0.15, 0.30, 0.50, 0.80, and 1.00 mM formic acid). The oligomers attached to the ligand consisted of the following mole ratio of oligosaccharides: tetramer/hexamer/octamer, 0.6/0.3/0.1. This is a consequence of overlap in the peaks of oligosaccharides isolated during elution of the fragments from the ion exchange column. The phosphatidylethanolamine-HA conjugate was prepared by reductive amination of the HA oligomers to the terminal portion of a phosphatidylethanolamine lipid, using sodium cyanoborohydride as described previously (25) and modified. Two different acyl chain compositions were prepared: DPPE and POPE. The lipid derivatives were purified by silicic acid column chromatography eluted with chloroform/methanol and showed a single spot at Rf 0.45 on silica gel TLC runs in chloroform/methanol/water 65/25/4 (v/v). The spots were ninhydrin negative and phosphate and carbohydrate positive. Ligand concentration was quantified by phosphate assay.

**Liposome Preparation.** Chol was obtained from Sigma Co. (St. Louis) and recrystallized from methanol. Lipid films were prepared by drying 10 μmol of lipid including POPE-HA or DPPE-HA from solvent (butanol saturated with distilled water or chloroform/methanol 7:3, respectively) under vacuum using rotary evaporator at room temperature. Liposomes (composed of POPC:Chol:HA₅POE 40:40:30 or POPC:Chol:POPE 40:40:9) were prepared by rehydrating the lipid film with 1 ml of 10 mM HEPES, 5% glucose (pH 7.4), followed by mixing on a vortex mixer for 1 min, sonication for 15 min in a bath type sonicator (Laboratory Supplies Company Inc., Hicksville, NY) under argon and extrusion through 0.2 and 0.1 μm polycarbonate membranes (26). Liposomes were used within 1 day of preparation and stored at 4°C under argon. The hydrodynamic diameter of the liposomes was determined by dynamic light scattering (Malvern Instruments, Southborough, MA). The net surface potential was determined with a Malvern Zetasizer IV (Malvern Instruments). The ζ potential of liposomes containing 3 mol% HA₅-P was -9.9 mV.

**Liposome Uptake Assay.** Cells (2 x 10⁶), B16F10 melanoma cell line or CV-1 cell line, were placed in each well in a 24-well plate and grown overnight at 37°C and 5% CO₂ in medium. The cell monolayer was rinsed with PBS-free medium and medium containing liposomes was added. Liposomes containing trace amounts, circa 0.01 mol% of 125I-p-hydroxy-benzamide dihexadecylphosphatidylethanolamine (125I-BPE) (27), were diluted in serum-free antibiotic-free medium and incubated with cells for 3 h at 4°C or 37°C. At the end of the incubation, the medium was removed, and the cells were washed with three successive aliquots of 0.5 ml ice-cold PBS (PBS). The medium and washes were pooled and assayed for radioactivity. The cells were lysed and removed from the well with 1 ml of 0.5 N NaOH. The well was then washed two additional times with 1 ml PBS aliquots and the cell lysate and washes were pooled. Radioactivity associated with the cell lysate and washes was determined in a Beckmann gamma scintillation spectrometer (Irvine, CA).

To assess the effect of HA-PE density on liposome uptake by target cells, 125I-labeled liposomes containing various amounts (0–12 mol%) of HA₅-P were incubated with B16F10 cells for 0.1–5 h. The effect of HA density on cell association was repeated three independent times with triplicate replications for each data point (n = 9). Each time course experiment was repeated two independent times with triplicate wells of cells (n = 6).

**Preparation of DOX-loaded Liposomes.** Liposomes were prepared by thin lipid film hydration followed by sonication and extrusion as described (28, 29) with minor modification. Briefly, the lipid components were weighed and dissolved in chloroform at the desired molar ratio (POPC:Chol:HA₅-PE:AT 60:40:30:1. POPC:Chol:POPG:AT: 60:40:9:0.1. HSPC:Chol:PEG:DSPE:AT: 60:60:10:1. HSPC:Chol:DPPE-T: 60:40:9:0.1. HSPC:Chol:HA₅-P, DPPE:AT: 60:40:3:0.1). A thin lipid film was formed by removing the solvent on a rotary evaporator under vacuum in a 10-ml glass screw-cap test tube. Each liposome batch consisted of 10 mol% phospholipid and was rehydrated in 250 mM ammonium sulfate. Hydration was done at room temperature for liposomes composed of POPC lipids and at 60°C for liposomes composed of HSPC lipids. For both types of liposomes, hydration consisted of vigorous vortexing, 30 min of shaking on an incubator shaker (New Brunswick Scientific Co., Inc., Edison, New Jersey) at a rate of 250 rpm and sonication for 15 min. Liposomes were repeatedly extruded (11 times) at room temperature for the POPC liposomes and at 60°C for the HSPC liposomes through polycarbonate membranes of gradually decreasing pore size (0.2 and 0.1 μm). Extruded liposomes were dialyzed extensively against a 100-fold volume of a 5% glucose solution (four changes over a 24-h period). DOX was actively transported into the liposome aqueous phase through the creation of ammonium sulfate gradient (28, 29). A 10-ng vial of DOX was dissolved in 5 ml of 5% glucose and then incubated for 2 h with the liposomes at 37°C for the POPC liposomes and at 65°C for the HSPC liposomes. Nonentrapped DOX was removed by passing the preparation through a column consisting of Dowex 50WX4 (28). Phospholipid concentration was determined by a phosphorous assay (30). DOX concentrations were determined by measurement of absorbance at 480 nm (using a molar extinction coefficient of 12,500 L/M) after solubilization of the liposomes in 90% isopropyl alcohol containing 0.075 m HCl as described (28). DOX encapsulation efficiency was usually greater than 90%, with drug: phospholipid ratio of approximately 100 μg/μmol. For both POPC and HSPC liposomes, mean vesicle diameter as measured by dynamic light scattering using the multimodal program was 110–140 nm (SD, <35% of the mean) with a monodisperse particle size distribution.

**Release of DOX from DOX-loaded Liposomes.** Release studies of DOX from DOX-loaded liposomes were done immediately after the DOX was loaded into the liposomes. A sample containing 100 μl of 10 μmol/ml liposomes containing 100 μg/μmol DOX were added into 10 ml of serum-free and antibiotic-free MEM Eagle’s with EBSS medium, vortexed, and incubated at 37°C. The suspension was mixed and a 100 μl sample was immediately removed and diluted into 10 ml of PBS. Measurements were done by two different methods to distinguish between DOX released from the liposomes and DOX remaining encapsulated in the liposomes. A 1-ml sample from the PBS solution was diluted in 9 ml of acidified isopropanol, and the total DOX content was measured fluorometrically using a Perkin-Elmer LS-50-B spectrophotofluorometer (excitation 480 nm, emission, 590 nm). Fluoresence intensity was translated to DOX concentration, using a standard curve prepared from DOX solutions. Another 1-ml sample from the PBS solution was passed through a column containing Dowex 50WX4 and then diluted in 9 ml acidified isopropanol, and the liposomal-entrapped DOX was measured fluorometrically as described above. These measurements were considered as time point zero. Samples of 100 μl of liposomes in medium were taken at 1, 2, 3, 5, 8 and 24 h post incubation and diluted in 10 ml of PBS as described above. Measurements were done in the same way as at time zero to determine the percent release of DOX relatively to the initial amount at time zero.

**FACS Analysis** B16F10 murine melanoma or CV-1 monkey fibroblasts were washed with PBS, detached from plates with 10 mM EDTA, washed once with PBS, centrifuged for 5 min at 800 rpm and the pellet was resuspended in 100 μl of normal goat serum and incubated for 10 min at 4°C, to reduce nonspecific binding of antibody to the cells. The cells were resuspended in PBS and incubated with rat anti-CD44 antibody (5 μg/10⁶ cells) for 20 min at 4°C. The cells were resuspended twice in PBS, and then incubated with...
FITC-labeled affinity-purified antirat IgG for 20 min at 4°C. The cells were resuspended twice in PBS and analyzed on a FACSscan (Becton Dickinson, Mountain View, CA) to determine the cell-associated fluorescence.

Analysis of rhodamine-labeled HAL binding to cells was performed as described above, but instead of preincubation with normal goat serum, cells were incubated with liposomes in serum-free and antibiotic-free medium (100 nmol total lipid/10⁵ cells) at 4°C for 2 h, then the cells were resuspended twice with PBS and analyzed by flow cytometry.

For assays designed to quantify the interference of liposome binding with anti-CD44 antibody, 10⁵ cells were preincubated with 100 nmol total lipid of HAL or POPG liposomes in serum-free and antibiotic-free medium, resuspended twice in PBS and incubated with anti-CD44 antibody (5 μg) and thereafter with FITC antirat IgG as described above. To quantify the ability of anti-CD44 antibody to block HAL binding, cells were preincubated with anti-CD44 antibody (5 μg/10⁵ cells) for 20 min at 4°C, then were resuspended twice with PBS and incubated with 100 nmol (total lipid) of rhodamine-labeled HAL for 2 h at 4°C. For the competition assay, 10⁵ cells were incubated simultaneously with 100 nmol (total lipid) of rhodamine-labeled HAL and 5 μg of anti-CD44 antibody for 2 h at 4°C. Rhodamine-DOX comprised 0.1 mol % of the total phospholipid.

**Chemotherapy Assay.** The cytotoxic effect of free DOX or liposome-encapsulated DOX on the cells was assayed colorimetrically by the SRB staining method (31), with slight modifications. Samples containing 16,000 B16F10 or 25,000 CV-1 cells (from an exponentially growing culture) in 100-μl aliquots were plated onto 96-well flat-bottomed microtiter plates. The culture plates were incubated for 24 h at 37°C and 5% CO₂, and then the medium in each well was replaced with 100 μl of serum-free and antibiotic-free medium containing various concentrations of free or liposome-encapsulated DOX. For each 10-fold increase in drug concentration, four drug concentration levels were tested. Each test was performed in triplicate wells and was repeated in an independent experiment at least once. The cells were incubated for 3 h (transient protocol) or 24 h (continuous protocol) at 37°C and 5% CO₂. For samples when the drug was removed at 3 h, complete medium that was lacking drug was added, and the incubation was continued for 24 h at 37°C and 5% CO₂ (transient protocol). At the end of the incubation period, the cells were washed once with complete (growth) medium, and 100 μl of complete drug-free medium was added to each well. The cultures were fixed by gently layering 25 μl of ice-cold 50% TCA (4°C) on top of the growth medium in each well to produce a final TCA concentration of 10%. The cultures were incubated at 4°C for 1 h and then washed and analyzed for SRB staining of the monolayers as described previously (31). The measurement of the absorbance of the SRB at 564 nm in the monolayers was determined by an Optimax microplate reader (Molecular Devices, Sunnyvale, CA). Each experiment was repeated twice in triplicate (n = 6).

**RESULTS**

**Measurements of CD44 Receptor Levels in Cultured Cells.** Many cells express the CD44 receptor at a low level. However, CD44 expression is often significantly increased in tumors. To mimic this situation in a cell culture system, we used the high CD44-expressing B16F10 (murine melanoma) tumor cell line (18), and CV-1 (monkey fibroblasts) cells as representative of low CD44-expressing normal fibroblasts) cells as representative of low CD44-expressing normal cells. The tumorigenic B16F10 murine melanoma cells express CD44H, a receptor that adheres to surface-bound HA (15) and also to HA oligomers (18). The relative receptor level on the two cell lines was evaluated by labeling the cells using rat anti-CD44 antibodies and fluorescence cell sorting (Fig. 1). The CD44 expression on the B16F10 cells is 39 times higher than CD44 expression on CV-1 cells.

**Liposome Characterization.** The structures of the lipids used to prepare the fluid targeted liposomes are illustrated in Fig. 2A. Unilamellar vesicles composed of POPC/Chol (6:4 mole ratio) containing various mole ratios of HA-POPE (HA₃-PE) were prepared and their physical properties were determined by standard methods. The extruded liposomes exhibited a Gaussian size distribution and HALs with 3-mol % HA₃-PE had a mean diameter of 110–140 ± 40 nm and a ζ potential of −9.9 mV.

**Chemoresistance Assay.** The cytotoxic effect of free DOX or liposome-encapsulated DOX on the cells was assayed colorimetrically by the SRB staining method (31), with slight modifications. Samples containing 16,000 B16F10 or 25,000 CV-1 cells (from an exponentially growing culture) in 100-μl aliquots were plated onto 96-well flat-bottomed microtiter plates. The culture plates were incubated for 24 h at 37°C and 5% CO₂, and then the medium in each well was replaced with 100 μl of serum-free and antibiotic-free medium containing various concentrations of free or liposome-encapsulated DOX. For each 10-fold increase in drug concentration, four drug concentration levels were tested. Each test was performed in triplicate wells and was repeated in an independent experiment at least once. The cells were incubated for 3 h (transient protocol) or 24 h (continuous protocol) at 37°C and 5% CO₂. For samples when the drug was removed at 3 h, complete medium that was lacking drug was added, and the incubation was continued for 24 h at 37°C and 5% CO₂ (transient protocol). At the end of the incubation period, the cells were washed once with complete (growth) medium, and 100 μl of complete drug-free medium was added to each well. The cultures were fixed by gently layering 25 μl of ice-cold 50% TCA (4°C) on top of the growth medium in each well to produce a final TCA concentration of 10%. The cultures were incubated at 4°C for 1 h and then washed and analyzed for SRB staining of the monolayers as described previously (31). The measurement of the absorbance of the SRB at 564 nm in the monolayers was determined by an Optimax microplate reader (Molecular Devices, Sunnyvale, CA). Each experiment was repeated twice in triplicate (n = 6).

**Liposomes containing POPG in place of the HA₃-PE at a mole ratio to confer similar physicochemical properties on the liposomes as those of the HALs were prepared and characterized (Table 1). Because of the additional negative charge on the HA₃-PE, it was necessary to include a 3-fold greater mole ratio of POPG than of the HA₃-PE in the lipid composition to obtain a similar surface charge (Table 1). Lipid films containing the HA₃-PE readily hydrated on exposure to the aqueous phase, and liposomes prepared from the films were stable. The HAL exhibited the physicochemical properties expected from lipid dispersions of this surface charge and composition.

**Analysis of the Interaction of Radiolabeled HAL with High CD44- and Low CD44-expressing Cells.** When B16F10 cells were incubated with HAL, cell-association of the unilamellar vesicles was found to depend critically on the density of the HA on the liposome surface. Little uptake was observed in the absence of HA conjugation (0 mol % HA₃-PE density; Fig. 2B). The amount of total cell-associated HALs increased with increasing density of liposome-conjugated HA. Even at a low density (0.1 mol %) of HA₃-PE, the liposomes showed an increased cell-association to the B16F10 cells compared with liposomes lacking the HA₃-PE. The extent of binding began to saturate when the HA₃-PE mole ratio was above 3 mol % in the lipid composition. No significant uptake was observed when the liposomes were incubated with control CV-1 cells, regardless of the HA₃-PE surface density (Fig. 2B).

The amount of HAL or POPG liposomes bound to B16F10 cells as a function of liposome concentration is shown in Fig. 2C. The binding/association of HAL to the cells exhibited a classical saturation binding profile characteristic of specific binding. Little binding was
Fig. 2. Structure and characterization of HALs. A. Structure of the components composed to produce HALs: HA-conjugated POPE (HA-POPE), POPC, and Chol (Cholesterol). B. Effect of HA oligosaccharide-POPE (HAₙ-PE) density on the cell association of HA-conjugated liposomes by B16F10 and CV-1 cells. HA-conjugated liposomes were prepared as described under “Materials and Methods.” 0 mol %, POPC:Chol (6:4 mole ratio) liposomes lacking HAₙ-PE. B16F10 (•) and CV-1 (○) cells were incubated for 3 h at 37°C with liposomes (200 μM) containing various mole percentages of HAₙ-PE labeled with ¹²⁵I-BPE. Values presented are the mean ± SD of nine replicates. C. Binding of liposomes to B16F10 cells as a function of lipid concentration. The amount (nmol total lipid bound per 400,000 cells) of liposomes containing (F) HA (HAₙ-PE, 3 mol %) or (E) POPG liposomes (POPG, 9 mol %) bound to B16F10 cells after 3 h of incubation at 37°C was measured as a function of lipid concentration. The liposomes were labeled with ¹²⁵I-BPE. Values presented are the mean ± SD of six replicates. D. Time dependence of liposome uptake by B16F10 cells. B16F10 cells were incubated with both (F) HA (HAₙ-PE, 3 mol %) or (E) POPG liposomes (POPG, 9 mol %) labeled with ¹²⁵I-BPE at 37°C for different lengths of time. The total amount of cell-associated liposomes was determined as described in the “Materials and Methods” section. Values presented are the mean ± SD of six replicates.
observed with liposomes lacking HA₄₆-PE but containing a negatively charged phospholipid, POPG, to provide a ζ potential that was the same as that of HA₄₆-PE liposomes (~9.9 mV). This result suggests that the high affinity of the HAL to the B16F10 cells is not attributable to a nonspecific electrostatic force/attraction between the HA₄₆-PE and the cell surface.

To study the kinetics of lipid uptake by the receptor-bearing cell, B16F10 cells were incubated with HALs containing 3 mol % HA₄₆-PE for various intervals. HAL uptake exhibited a slight lag phase over the first hour of incubation and was linear during the second hour of incubation, and then no further increase in cell-associated-radioactivity was observed by the 3-h time point (Fig. 2D). This decline in the rate of internalization is attributable to saturation of the uptake system rather than depletion of liposomes, because lipid concentration in the medium declined by only 30% over the course of incubation. Moreover, similar results were obtained by repeating the experiment with the same concentration of liposomes in one-half the volume of the medium or using one-half of the number of cells (data not shown). We, therefore, conclude that HALs bind to B16F10 cells via a saturable mechanism followed by internalization into intracellular compartments. In contrast, little cell-association was observed with POPG liposomes that lacked HA₄₆-PE but had a similar negative ζ potential as the HALs.

To distinguish surface-bound from internalized liposomes, B16F10 cells were incubated for different periods of time with 200 μM liposomes at either 4°C or 37°C and then washed with cold PBS to remove unattached liposomes. The kinetics of total association (uptake) of HALs with cells was significantly faster at 37°C than at 4°C. Cells incubated with HALs at 4°C reached a constant value by the first period examined. In contrast, at 37°C, the total cell-association of liposomes was greater than the uptake at 4°C, and a steady-state value was not obtained until after 2 h of incubation (Table 2). This suggests that a substantial fraction of the cell-associated liposomes were internalized. Because HA-receptor binding proceeds at both 4°C and 37°C, but endocytosis occurs only at the latter temperature, we interpret these data to suggest that the HA-conjugated liposomes are endocytosed only at the higher incubation temperature. Little cell-association occurred for liposomes lacking HA₄₆-PE at either temperature.

To evaluate the role of HA in the cellular uptake of HALs, B16F10 cells were treated with 200 μM HALs in serum-free and antibiotic-free medium containing increasing concentrations of non-liposomal-associated high M₉ HA polymer (M, 50,000; Fig. 3). The uptake of HAL was inhibited by the presence of free HA (Fig. 3). Free HA has a Kᵦ₅₀ of 0.1–10 nM for the HA receptor (32). In the absence of free HA, ~80 nmol of lipid were bound to the cells. However, when HA was added to the medium, 100 μM and 1 μM HA reduced cellular uptake of liposomes by 30 and 62%, respectively. When the cells were incubated, first with the inhibitor for 1 h and then with the HAL for 3 more h, 100 μM HA reduced cellular liposome uptake by only 42%. The lipid derivative used in these studies contained predominantly a single complete disaccharide unit (e.g., the disaccharide attached to the lipid had an open ring at the site of amination); therefore, the data are plotted as the hyaluronic acid disaccharide equivalent concentration in the polymer. A 50% reduction of lipidic binding occurred at about 10 nmol of disaccharide equivalent. Thus the HALs exhibit tight and selective binding to CD44-expressing cells. At high levels of HA, HALs binding to the cells were reduced to the value observed with liposomes lacking HA₄₆-PE (Fig. 2D).

### Table 1

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<td>HAL (POPC:Chol:HA₄₆-PE, 60:40:3 mole ratio)</td>
<td>134 ± 39</td>
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<td>Nontargeted liposomes (POPC:Chol:POPG, 60:40:9 mole ratio)</td>
<td>127 ± 33</td>
<td>−9.8 ± 0.5</td>
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### Table 2

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suggesting a reduction in HAL uptake by the B16F10 cells in the presence of anti-CD44 mAb.

**In Vitro Cytotoxicity of Liposome-encapsulated DOX to B16F10 Cells.** The DOX dose dependence and time course of cytotoxicity have been determined after treatment of B16F10 cells with nonencapsulated DOX and HAL-DOX (Fig. 5). A transient-exposure and a continuous-exposure protocol were used in these experiments. In the transient-exposure protocol, the cytotoxicity at 24 h of HAL-DOX after a 3-h exposure followed by washing and replacement of the medium with drug-free medium was greater than for free DOX under the same conditions (Fig. 5A). The IC_{50} value for HAL-DOX (0.45 µg/ml, 0.78 µM) was 8- to 9-fold less than that for free DOX (3.7 µg/ml, 6.4 µM). In contrast, cells incubated for 3 h with POPG-DOX were not appreciably affected (Fig. 5A). Moreover, the HA nas-PE liposomes had no intrinsic cytotoxicity because the cytotoxicity of empty HALs plus free DOX is similar to that of free DOX alone (Fig. 5A).

After a 24-h incubation (Fig. 5B), the IC_{50} was significantly decreased for all of the treatments. The HAL-encapsulated DOX remained more potent than the nonencapsulated DOX. This is quite remarkable because we are unaware of any previous studies in which targeted liposomes containing DOX were more active than the free drug on continuous exposure to both treatments for 24 h (see “Discussion” below). Under these conditions, the nontargeted POPG liposomes had an IC_{50} of 14.5 µg/ml (25 µM). This is most likely attributable to the release of the DOX from the liposomes on incubation at 37°C during the course of the experiment. Under these incubation conditions, ~10% of the encapsulated drug was released by 3 h, and ~75% of the drug was released by 24 h (Table 3). Thus, we would expect to observe a cytotoxic effect from the nontargeted liposomes because free DOX would gradually accumulate in the medium as the experiment progressed. The rate of drug release would be a factor that would impact the observed cytotoxicity of the various formulations (Table 3).

To examine the specificity of the HALs, we treated CV-1 cells with the three formulations (Fig. 6). CV-1 cells express a very low level of CD44 (Fig. 1). The sensitivity of the CV-1 cells to DOX is significantly lower than that of B16F10 cells, and, unlike the case of the...
B16F10 cells, there was a reduction rather than an enhancement of cytotoxicity of DOX by encapsulating the drug in HALs (Fig. 6). Furthermore, the cytotoxicity of both the HALs and the POPG liposomes was similar, confirming the low interaction of the liposomes with CV-1 cells. A greater level of cytotoxicity was also observed in this cell line after 24-h incubation with the liposome formulations as a result of the release of DOX from the liposomes (Fig. 6).

To learn whether hyaluronic acid could effectively compete with the cytotoxicity observed using HALs, B16F10 cells were incubated for 1 h with $10^{-7}$ M free HA polymer ($M_\text{r}$ 50,000). Then the medium was removed, the cells were washed once with fresh medium, and the cells were exposed to various concentrations of HAL-DOX, POPG-DOX, or free drug in medium containing $10^{-7}$ M HA (Fig. 7). HA reduced the cytotoxicity of the HAL but had no effect on the toxicity of the free drug or the nontargeted liposomes (Fig. 7).

In recent years, evidence has been presented that long-circulating liposomes accumulate in animal and human tumors (33, 34) in vivo. In addition, there are data suggesting increased tumor localization and enhanced therapeutic efficacy of anthracyclines encapsulated in these long-circulating liposomes (35). For that purpose, we compared the effect of lipid composition on cytotoxicity. As can be seen in Table 3, liposomes varied in phospholipid composition but were of similar size. It was found that the cytotoxic activity of DOX-loaded liposomes is decreased 3- to 4-fold when liposomes were prepared from phospholipids of high phase transition temperature ($T_{\text{m}}$) such as HSPC. Drug-release experiments indicated that the cytotoxic effect is mediated by the release of drug from the liposomes in the case of nontargeted liposomes (continuous protocol). The data in Table 3 show that targeted liposomes containing DOX have the highest potency. The potency of the various treatments decreases in the following order: fluid targeted liposomes; solid targeted liposomes; free drug, fluid nontargeted liposomes; and solid nontargeted liposomes. Stealth liposomes (PEG-DSPE) had the same cytotoxicity as DPPG liposomes.

**DISCUSSION**

The overexpression of the HA receptor, CD44, on a variety of tumors makes HA a potentially interesting ligand for targeted therapy of such tumors (1–7). Indeed, high $M_\text{r}$ HA has been used to inhibit tumor growth in vivo (18). Zeng et al. (18) showed that HA oligomers could inhibit B16F10 melanoma growth and s.c. tumor formation. Karasawa et al. (36) also showed that injection of high $M_\text{r}$ HA polymer ($M_\text{r}$ 10,000) hyaluronic acid dipalmitoylphosphatidylethanolamine (HA-PE) inhibited the metastasis of melanoma (although the inhibition by hyaluronic acid-PE was far lower than that by chondroitin sulfate-PE). They suggested that HA-PE immobilized onto the subendothelial matrix

![Image](https://example.com/image.jpg)
might prevent melanoma cells from adhering to the subendothelial substrata of lung capillaries and inhibit the subsequent invasion process of metastasis. These studies emphasize the ability of HA to interact with the CD44 receptor on tumors, and the role of the CD44 receptor in tumor spread. Other strategies that interfere with CD44-HA interaction, such as the administration of an anti-CD44 mAb (19) or a CD44-receptor globulin (19, 20) inhibited and blocked tumor metastasis in animal tumor models established from CD44-expressing tumor cell lines.

HA carbohydrate polymer is a potential ligand to target to tumor cells overexpressing the CD44 receptor. Recently HA polymer drug conjugates have been used to deliver drugs to CD44 expressing cells (22, 23). An obstacle in using high $M_r$ HA to target tumors is presented by its quick removal from circulation by cells in the liver (23). Akima et al. (23) point out that HA-drug conjugates would not be useful for systemic tumor targeting because of their rapid hepatic elimination. Instead, Akima et al. used the HA-drug conjugates for local injection into the lymphatic circulation. Liver sinusoidal endothelial cells express receptors that recognize and internalize HA. Moreover, high $M_r$ HA has been attached to liposomes to create a bioadhesive liposome system that is designed to adhere to the extracellular matrix (41, 42) rather than to circulate through the blood stream.

We have enzymatically degraded the HA polymer into low $M_r$ fragments and attached oligosaccharides to phosphatidylethano-
LIPOSOMES TARGETED TO CD44

Table 4 Methods designed to target DOX to cancerous cells

<table>
<thead>
<tr>
<th>Method</th>
<th>Cell line in use</th>
<th>Exposure duration (h)</th>
<th>IC_{50} (µM DOX)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR96 antibody conjugate to DOX(^a)</td>
<td>RCA human carcinoma</td>
<td>2</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>BN7005-colon carcinoma</td>
<td></td>
<td>0.7</td>
<td>7.0</td>
</tr>
<tr>
<td>HPMA copolymers bound to DOX(^b)</td>
<td>Mouse spleen T-cells</td>
<td>72</td>
<td>0.1 µg/ml</td>
<td>7.3 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Human ovarian carcinoma cells</td>
<td>0.5</td>
<td>19 ± 6</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Anti-p185HER2 immunoliposomes(^c)</td>
<td>SK-BR-3-breast cancer cells</td>
<td>1</td>
<td>0.52</td>
<td>0.34 and 1.72</td>
</tr>
<tr>
<td>174H.64 antibody immunoliposomes(^d)</td>
<td>KLN 205-murine squamous lung cancer</td>
<td>1</td>
<td>52</td>
<td>5 and 10</td>
</tr>
<tr>
<td>174H.64 antibody sterically stabilized immunoliposomes(^e)</td>
<td>KLN 205-murine squamous lung cancer</td>
<td>1</td>
<td>160.3</td>
<td>72.4</td>
</tr>
<tr>
<td>Anti-CD19 sterically stabilized immunoliposomes(^f)</td>
<td>Namalwa CD-19(^f) B cells</td>
<td>24</td>
<td>1.3 ± 0.6</td>
<td>13.5 ± 9.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24.0 ± 0.1</td>
<td>1.4 ± 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.07 ± 0.01</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>H9 CD-19(^f) T lymphoma cells</td>
<td>24</td>
<td>3.2 ± 1.0</td>
<td>38.5 ± 22.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.76 ± 0.6</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>Folate-PEG-liposomal DOX(^g)</td>
<td>KB human nasopharyngeal epidermal carcinoma</td>
<td>2</td>
<td>0.83</td>
<td>0.31</td>
</tr>
</tbody>
</table>

\(^a\) Cells were incubated for 2 h with the drug and then were washed and incubated for an additional 48 h with drug-free medium (transient protocol).

\(^b\) Cells were incubated for 3 days with medium containing different formulations of DOX. On the 3rd day, [³H]Thymidine was added, and after 6 h, the amount of incorporated radioactivity was determined. Free drug (0.1 µg/ml) was needed to inhibit 60% [³H]Thymidine incorporation in cells, and 7.3 µg/ml polymer-bound DOX induced 60% inhibition of cells proliferation.

\(^c\) Cells were incubated for 30 min with the drug and then were washed and incubated for an additional 72 h with drug-free medium.

\(^d\) Cells were incubated for 1 h with the drug and then washed and incubated for an additional 72 h with drug-free medium. DOX-loaded anti-p185HER2 immunoliposomes showed comparable dose-dependent cytotoxicity, with an IC_{50} of 0.2 µg/ml (0.34 µM) for conventional anti-p185HER2 immunoliposomes and 1.0 µg/ml (1.72 µM) for anti-p185HER2 immunoliposomes containing 2 mol % polyethylene glycol-modified phosphatidylethanolamine.

\(^e\) Cells were incubated for 1 h with the drug and then were washed and incubated for an additional 24 h with drug-free medium. DOX-loaded immunoliposomes showed IC_{50} of 5 µM for conventional immunoliposomes and 10 µM for immunoliposomes containing 5 mol % PEG-DSPE.

\(^f\) Cells were incubated for 1 or 24 h and then were washed and incubated for a total of 48 h with drug-free media. The IC_{50} (48) were given in µg of DOX/ml and translated here to µM.

\(^g\) Cells were incubated for 1 or 24 h and then were washed and incubated for a total of 48 h with drug-free media.

\(^h\) Cells were incubated for 2 h with the drug and then were washed and incubated for an additional 48 h with drug-free medium.

nolamines. Hexameric fragments of HA have been reported to constitute the minimal sequence capable of binding to cell surface CD44 receptors (12), because CD44 preferentially binds to a six-sugar sequence of hyaluronic acid and has a weak affinity for the CD44 receptor, but, by incorporating multiple copies into a liposome bilayer, the modified liposome can preferentially bind to, and be taken up by, cells with a high density of receptors (Figs. 2 and 3). Because high-affinity receptors for hyaluronic acid are greatly enriched on certain cancer cells, it was reasoned that HALs could be used to target neoplastic tissues.

Indeed, the results demonstrated that HA facilitates the recognition of liposomes by B16F10 melanoma cells in culture and that, after cell surface binding, the liposomes are internalized into the targeted cells by a temperature-dependent process. Binding of the HALs to the B16F10 cells was rapid, dose dependent, and showed saturation kinetics. HALs binding to B16F10 cells was inhibited by HA and mAbs directed against the CD44 receptor. Thus, most of the HAL binding to B16F10 cells is attributable to interaction with the cell surface CD44 receptors. The B16F10 cell line has a high density of CD44, whereas the control CV-1 cells have a low CD44 density, similar to the CD44 density found on many normal cells in the body. CV-1 cells showed little uptake, a result that indicates that HAL had high affinity to the B16F10 but not to CV-1 cells.

The chemotherapeutic results demonstrate that both liposomes and the associated drug accumulated at the tumor cells that express high levels of CD44. HAL-DOX was 8.2-fold more potent than free DOX against the B16F10 cells in a transient-exposure assay. Most importantly, HAL-DOX exhibited 4.4-fold increase in potency compared with the free drug even under continuous-exposure conditions. Moreover, HAL decreased the cytotoxicity of DOX to cells expressing low levels of the CD44 receptor. The increased potency of HAL-DOX to CD44 high-expressing cells under continuous conditions is remarkable in comparison with those obtained by other methods designed to target DOX (Table 4). Sjögren et al. (43) conjugated the internalizing mAb BR96 to DOX. The conjugate, termed BR96-DOX, bound to a tumor-associated Lewis\(^a\) antigen that is abundantly expressed on the surface of human carcinoma cells, but it was less potent than free DOX. Rihova et al. (44) conjugated DOX to N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers but observed a substantial decrease in potency of the polymer-bound drug. Other groups have used immunoliposomes to target DOX to cancer cells in vitro (45–49). One group observed a decrease in the potency of the encapsulated DOX (45). Park et al. (46) observed a modest 1.5-fold increase in potency of the immunoliposomes-DOX compared with free drug, which was lost when PEG lipids were included in the formulation. Ahmad and Allen (47) obtained a 5- to 10-fold increase in potency of the immunoliposomes-DOX compared with free DOX under this transient condition. Allen et al. (48) observed a 2.2-fold increase in the potency of the immunoliposomes-DOX compared with free DOX in transient exposure of 1 h. After continuous exposure for 24 h, the immunoliposomes-DOX were 2.1-fold less active than the free drug. Lopez de Menezes et al. (49) from the same group, obtained a 10.4-fold and 5-fold decrease in potency of immunoliposomes-DOX compared with free DOX in exposures both of 1 and of 24 h, respectively. Finally, Lee and Low (50) observed a modest increase in potency for DOX delivered in folate-targeted liposomes. As can be seen in Table 4, few of the targeted systems are more potent than free DOX in vitro even under the transient-exposure condition. Thus, in vitro results with HAL-DOX under both transient- and continuous-exposure conditions are better than other DOX delivery systems reported to date. Our results also indicated that the potency of the liposomes to target to cells expressing high levels of CD44 was retained by using a variety of phospholipids with HA as the leading group (Table 3).

In this study, we have developed a strategy for targeting liposomal DOX to tumor cells using low M\(_{\text{n}}\) β1,3 N-acetyl glucosaminyl-β1,4 glucuronic acid oligosaccharides. In cases in which a tumor expresses the surface CD44 receptor and is accessible to HALs, the methodology should allow selective delivery of the drug to the cancerous tissue. We have demonstrated specific binding by a melanoma cell line in vitro of novel ligand-targeted liposomes and have shown significantly enhanced cytotoxicity by liposome-entrapped DOX in the presence of the ligand. These experiments are the first to show specific binding of HALs bearing low M\(_{\text{n}}\) HA oligosaccharides. The results showed higher potency of HAL-DOX to CD44-overexpressing cell line in comparison with free DOX and much less toxicity than the free drug.
to cells with low levels of CD44 on their surface. Experiments currently under way to evaluate in vivo activity of targeted liposomes in mice, in which the B16F10 cells spontaneously metastasize to the lung, indicate that HALs containing entrapped DOX have a subsequently improve efficacy in vivo with nontargeted liposomal DOX or free drug.4

This targeting approach using low M\(_{\text{o}}\) oligosaccharides of HA should complement efforts of others (22, 23) using higher M\(_{\text{o}}\) HA polymers to target drugs to CD44-expressing cancer cells. Whether or not such approaches will improve cancer drug therapy will require much additional preclinical testing in relevant animal tumor models.

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Liposome-encapsulated Doxorubicin Targeted to CD44: A Strategy to Kill CD44-overexpressing Tumor Cells

Rom E. Eliaz and Francis C. Szoka, Jr.

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