Haloperidol-associated Stealth Liposomes
A POTENT CARRIER FOR DELIVERING GENES TO HUMAN BREAST CANCER CELLS*

Amarnath Mukherjee‡§, Tekkatte Krishnamurthy Prasad¶, Nalam Madhusudhana Rao¶, and Rajkumar Banerjee†‡||

From the ‡Division of Lipid Science & Technology, Indian Institute of Chemical Technology, Hyderabad 500 007, India and the §Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

Sigma receptors are membrane-bound proteins that are overexpressed in certain human malignancies including breast cancer. These receptors show very high affinity for various sigma ligands including neuroleptics like haloperidol. We hypothesized that in associating haloperidol-linked lipid into the cationic lipid-DNA complex, we can specifically target and deliver genes to breast cancer cells that overexpress sigma receptors. In the present study, haloperidol was chemically modified to conjugate at the distal end of the polyethylene glycol (PEG)-linked phospholipid, which was then incorporated into the cationic liposome known to condense and deliver genes inside cells. The resulting haloperidol-conjugated targeted lipoplex showed at least 10-fold higher (p < 0.001) reporter gene expression in MCF-7 cells than control lipoplex. The reporter gene expression of the targeted lipoplex was significantly blocked by haloperidol (p < 0.001) and by another sigma ligand, 1,3-ditolyguanidine (p < 0.001) in the majority of cationic lipid to DNA charge ratios (±). Spironolactone-mediated sigma receptor down-regulation enabled MCF-7 to show 10-fold lower transgene expression with targeted lipoplex compared with that obtained in spironolactone-untreated cells. The targeted lipoplex generated nonspecific gene expression in sigma receptor-nonexpressing human cancer cells such as Hela, KB, HepG2, and Chinese hamster ovary cells. Moreover, the transgene expression remained unabated in physiologically relevant serum concentrations. This is the first study to demonstrate that haloperidol-targeted gene delivery systems can mediate efficient targeting of genes to sigma receptor-overexpressing breast cancer cells, thereby becoming a novel class of therapeutics for the treatment of human cancers.

Haloperidol is a common neuroleptic drug that is subtype non-selective yet shows a strong affinity for sigma receptors. Haloperidol and other sigma ligands have been shown to elicit various physiological processes, which include triggering apoptosis in cells of neuronal origin and in rapidly proliferating cells (1–4). It is evident now that these physiological processes are exerted through haloperidol-sigma receptor interaction.

Sigma receptors are membrane-bound protein receptors that are expressed in normal tissues, such as liver, endocrine glands, kidneys, lungs, gonads, central nervous system, and ovaries at basal levels (5, 6). Although the physiological roles of these receptors in normal tissues are not yet clear, a diverse set of human tumors, such as melanoma, non-small cell lung carcinoma, breast tumors of neural origin, and prostate cancer overexpress sigma receptors (7–13). Structurally diverse sigma ligands have been shown previously to exhibit high affinity to these sigma receptor-expressing cells, suggesting the prospect of using these sigma receptor binding ligands for diagnosis as tumor-imaging agents and targeted therapy of a variety of tumors (12, 13).

Liposomes encapsulating biologically active molecules have long been used as a vehicle to target the payload in vivo. In cancer-targeting studies, specific targeting to tumor cells by liposomes has previously been demonstrated (14, 15). Also immunoliposomes associated with PEG1 have previously shown stealth property and tumor-targeting efficiency in vivo (16–18).

Recently, a non-immunogenic, small molecular weight sigma ligand carrying an anisamide moiety was used as a targeting ligand in a drug-carrying liposomal system to target anticancer drugs to sigma receptor-expressing prostate cancer cells in the xenograft tumor mice model (19).

The above-mentioned study propelled us to investigate the use of a readily available inexpensive generic drug, haloperidol, which shows very high affinity for sigma receptors, as a targeting ligand in a stealth liposomal system carrying genes to sigma receptor-overexpressing breast adenocarcinoma cells. Without potentially reducing targeting ability, the haloperidol is chemically conjugated to phospholipids with a PEG spacer in between. This ligand-conjugated PEG-lipid is included in 5 mol %, along with a known cationic lipid (20) and cholesterol, to form long-lived circulating and targeted cationic liposomes. These cationic liposomes pre-condense plasmid DNA containing the reporter or therapeutic gene. It is expected that haloperidol having high affinity for the sigma receptor, upon incorporation into the surface of the liposome, will avidly interact

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a doctoral research fellowship from the University Grants Commission, Govt. of India.
¶ Recipient of a Department of Science & Technology (DST), Government of India grant for research support provided under a Fast Track Proposal for Young Scientists. To whom correspondence should be addressed: Division of Lipid Science & Technology, Rm. 345, Indian Institute of Chemical Technology, Hyderabad 500 007, India. Tel.: 91-40-2719-3201; Fax: 91-40-2716-0757; E-mail: rkbanerjee@yahoo.com.

‡‖ The abbreviations used are: PEG, polyethylene glycol; DODEAC, N,N-di-o-tetradecyl-N,N-(2-hydroxyethyl)ammonium chloride; DSPE-PEG(2000)-mal, distearoyl-sn-glycero-phosphatidylethanolamine-[omega-maleimido-polyethylene glycol(2000)]; DSPE-PEG-HP, distearoylglycerolphosphatidylethanolamine-polyethylene glycol[omega-carboxamido(2-O-haloperidyl)]ethyleneketablate; DTG, 1,3-o-ditolyl guanidine; HP, haloperidol; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; CHO, Chinese hamster ovary; N-Boc, N-butyloxycarbonyl; CMV, cytomegalovirus.

This paper is available on line at http://www.jbc.org. Printed in U.S.A.
with cells expressing the sigma receptor. This liposome-cell interaction eventually ferries the genetic cargo inside the cell, possibly through receptor-mediated uptake. As an added advantage, the liposome-DNA complex will remain stabilized because PEK is known to prolong the circulation times of the liposome in vivo (21, 22). Moreover, because of the low mol % of haloperidol-associated lipid, the lipoplex is expected to exert minimal agonistic effect, if any, to sigma receptor-expressing cells. To this end, we have incorporated haloperidol-conjugated PEK phospholipid in the cationic liposome formulation and condense reporter plasmid DNA. The targeted uptake followed by the efficiency of reporter gene expression in sigma receptor-overexpressing human breast carcinoma cell MCF-7 was studied in the presence of serum-containing medium and in the absence or presence of free haloperidol and ditolylguanidine.

MATERIALS AND METHODS

Chemicals and General Procedures—Phospholipids such as DSPE-P EG/2000-maleimide and DSPE-P EG/2000-COOH were purchased from Avanti Polar Lipids. Bovine serum albumin (BSA, trypsin, EDTA, pCytosine, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (MeSO), and haloperidol (HP) were purchased from Sigma. All the chemicals and organic solvents for synthesis were purchased from either Aldrich (Milwaukee, WI) or S.D. Fine Chem (Mumbai, India). They were used without further purification. Spiroonolactone was obtained from the drug alactone (RPG Life Sciences Ltd., Ankleshwar, India). Brieﬂy, aldehyde tablets (50 mg by spiroonolactone weight) were crushed and dissolved in 10 ml of water. The drug was extracted by dichloromethane (2 × 25 ml). Upon evaporation of the non-aqueous layer, the free drug was crystallized out in methanol at −20 °C. The purity and authenticity of the crystallized compound (white needle, 48 mg) was characterized by TLC, melting point analysis, and by its NMR spectrum. 1H NMR spectra were recorded on a Bruker FT 300 MHz and Varian FT 200 MHz and 400 MHz instrument.

Cell Culture—MCF-7, CHO, Hela, KB, and HepG2 cells were purchased from the National Center for Cell Sciences (Pune, India) and were mycoplasma-free. Cells were cultured in DMEM (ATCC) containing 10% fetal bovine serum (Sigma) and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO2 in air. Cultures of 85–90% confluency were used for all of the experiments. All cells, trypsinized, counted, and subcultured in 96-well plates for transfection experiments hereafter.

Synthesis of Ligands—The synthetic procedure for preparing the lipid DSPE-P EG-HP is depicted schematically in Fig. 1. Detailed experimental procedures are delineated in the Supporting Materials and Methods. Step a: Synthesis of N-Boc-β-Alanine-Haloperidol Conjugate (Compound I, Fig. 1)—A mixture of N-Boc-β-alanine (400 mg, 2.1 mmol), haloperidol (400 mg, 1 mmol), and N,N-dimethylaminopropionic acid (DAMPA, 20 mg, catalytic) were mixed in a 25-ml round bottom flask and stirred in 5 ml of dry DCM and stirred in ice for 0.5 h. To the mixture, EDC (240 mg, 1.2 mmol) was added, and the sample was stirred in an ice bath for 1 h. The reaction mixture was further stirred for 12 h at room temperature. The reaction mixture was dissolved in 20 ml of dichloromethane, washed with water (2 × 20 ml), and brine (1 × 20 ml), and dried with anhydrous Na2SO4. Column chromatographic purification (using 60–120 mesh silica gel and 2% methanol-chloroform as eluent) of the residue yielded compound I, a white solid (116 mg, 20% yield, Rf 0.6 in 5% methanol/chloroform).

1H NMR (200 MHz, CDCl3): δ = 1.2 [s, 9H, -(CH2)2-C(O-CONH)], 1.9 [t, 4H, -CH2-CH2-N-Chloro-CH3], 2.2–2.6 [8H, (CH2)2-N-Chloro-CH3], 2.7 [2H, -O-CO-CH2-CH2-NHBOC], 2.9 [2H, -CH2-COAR], 3.2 [2H, -CH2-NHBOC], 4.8–4.9 [bs, 1H, BOC-NH], 6.9–7.2 [3H, 6H, o- and m-CH3], 7.9 [2H, o-CH3-CO].

FABMS (LSIMS): m/z = 547 [M + Na]+ for CH14H32N2O2Cl.

Evaporation of the organic layer afforded compound II as a gummy material (160 mg, 70% yield. Rf 0.1 in 5% methanol/chloroform, active in ninhydrin staining). Because compound II was observed as ~95% pure (revealed by TLC), it was directly used for the final step.

Step b: Synthesis of DSPE-P EG-COOH—A mixture of DSPE-P EG-2000-COOH (50 mg, 0.018 mmol) and compound II (30 mg, 0.06 mmol) was put into a 10-ml round bottom flask in 3 ml of dry DCM and stirred over an ice bath. After 0.5 h, dicyclohexylcarbodiimide (DCC, 5 mg, 0.023 mmol) was added to the reaction, with continued stirring at room temperature for 12 h. The solvent was evaporated, and the crude product was purified three times by recrystallization using methanol/ether (1.15 v/v) as solvent. The purified compound obtained as a white gummy material (38 mg, 66% yield with respect to the PEG- lipid, Rf 0.1 at CHCl3/aceton/methanol 79:20:1, UV active).

2H NMR (400 MHz, CD,OD) of representative peaks: δ = 0.9 [t, 6H, O-CO-CH2-(CH2)2-CH2-CH2-CH2-CH2-CH2-CH2-CH2], 7.2 [2H, & o-CH2Cl], 7.4 [bs, 4H, o- and m-CH3], 8.2 [2H, o-C6H4-CO].

The integration of the protons in the aromatic moiety when attached to the high molecular weight PEK does not give accurate peak heights in contrast to other protons in NMR, as reported elsewhere (23). Electrospray mass spectra of the product revealed an inverted U shape mass spectral pattern with an increment of 44 (a characteristic of PEG-based oligomer) and 12 (a characteristic of cholesterol). The peak of the mass spectral pattern is observed at molecular weight 3149 (possibly M + 2). The starting material DSPE-P EG-COOH shows a pattern spanning between molecular weights 2630 and 2985, with a peak at molecular weight 2718. The peak for the product was considered 3147 [2718 + 429 (contributed from HP)] and was accepted as the molecular weight of the product for the molecular weight determination by SEC.

Liposome Preparations—The lipid films were prepared by drying the chloroform solution, from a total of 2.05 μmol of DODAC, cholesterol and DSPE-P EG-HP or DSPE-P EG-mal under a gentle stream of N2, under vacuum for at least 6 h. The lipid mixtures were composed of DODAC/Chol/DSPE-P EG-HP or DODAC/Chol/DSPE-P EG-mal in a molar ratio of 1:1:0.05. The mixture was hydrated with 1 ml of sterile water and then subjected to a low intensity bath sonication for 15 min at room temperature and then probe sonication for 2 min in ice using a constant duty cycle and output control magnitude of 2–3 in a Branson Sonifier 450.

DNA Binding Assay—The DNA binding ability of the targeted and non-targeted lipids containing DSPE-P EG-HP and DSPE-P EG-mal, respectively, was assessed by gel retardation assay on a 0.8%–agarose gel. The liposome-DNA (at a cationic lipid/DNA charge ratio 8:1, 4:1, 2:1, and 1:1) was mixed with the 0.2 μmol of dodecylamine in the presence of 0.01 M Tris and 0.025 M NaCl, 0.05% bromphenol blue, 0.05% xylene cyanol, and 1% Triton X-100. The solution was incubated at 60°C for 10 min in a water bath. The aqueous layer was extracted with 50 μl of dichloromethane to provide a clear lipid-DNA complex. The solution was loaded to the gel well. The bands were visualized by staining for 30 min with ethidium bromide solution followed by 30 min of destaining in water.

DNase I Sensitivity Assay—In a typical assay, 3 nmol of DNA (1 μg) was complexed with both targeted and non-targeted cationic lipids containing DSPE-P EG-HP and DSPE-P EG-mal, respectively, in a (±) charge ratio of 8:1, 4:1, 2:1, and 1:1. The mixture was incubated at room temperature for 30 min on a rotary shaker. Subsequently, the complex was treated with DNase I (at a final concentration of 10 ng/3 nmol of DNA), in the presence of 20 mM MgCl2. The volume was brought up to 50 μl with HEPEs buffer (pH 7.4) and incubated at room temperature for 30 min on a rotary shaker. Following this, 0.9 μl of a 20 mM MgCl2 stock solution was added to the mixture to reach a concentration of 20 mM MgCl2. The volume was brought up to 50 μl with HEPEs buffer (pH 7.4) and incubated at 37 °C for 0.5 h. To stop the hydrolysis reaction, EDTA was added to a final concentration of 20 mM, and the mixture was incubated at 60 °C for 10 min in a water bath. The aqueous layer was extracted with 50 μl of phenol/chloroform mixture (1:1, v/v) and subsequently centrifuged at 10 000 rpm for 5 min. The aqueous supernatant was separated, loaded (20 μl) on a 0.8% agarose gel, and electrophoresed at 80 V for 3 h. The bands were visualized after 45 min of ethidium bromide staining followed by 30 min of destaining in water.

Western Blot Analysis—MCF-7 and CHO cells were grown in 75-cm2 flasks until they reached a confluency of about 80%. Cells were detached from the flasks using 0.1% EDTA solution. Whole cell lysates were prepared by directly lysing the cells in SDS loading buffer. Total protein content in each sample was determined by amido black staining (24). Equal amounts of protein were separated and separated on a 15% SDS-PAGE. Proteins were transferred onto nitrocellulose membrane (Hybond-C extra, Amersham Biosciences) using
semidry blotting. Gels were stained with Coomassie Brilliant Blue R-250 to visualize equal loading of protein samples. The membrane was blocked overnight at 4 °C with 5% BLOTTO (Santa Cruz Biotechnology). The blot was then incubated with polyclonal antibody raised against full-length recombinant sigma receptor 1 of human origin in rabbit (Santa Cruz Biotechnology) at 1:5000 dilution for 1 h at room temperature. After washing, the membrane was incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) at 1:4000 dilution for 45 min. To confirm equal loading of protein samples, the blot was also probed for levels of β-actin using monoclonal antibody (Sigma) raised in the mouse against β-actin at 1:5000 dilution for 1 h. After incubation of the cell plates in 10% FBS-containing DMEM, the reporter gene assay was performed according to the above-mentioned procedure.

For studies with spironolactone-treated MCF-7 cells, the cells were treated with spironolactone at a final concentration of 10 μM in serum medium for 1 day. Microscopically, cells show no change in cellular morphology compared with spironolactone-untreated MCF-7 cells before plating. The cells upon becoming 80–90% confluent were plated in 96-well plates in the presence of spironolactone. The cells were kept in spironolactone-containing medium overnight. The medium was removed, and cells were washed with PBS (1 × 100 μl) before lipoplex was added to the cells. The transfection and assay were performed as mentioned before.

For the serum dependence study, upon incubation for 30 min in DMEM, 200 μl of either of the following were added to the lipoplex: (a) 10% FBS-containing DMEM, (b) 40% FBS-containing DMEM, (c) 80% FBS-containing DMEM, or (d) 100% FBS. 100 μl of each of the 300 μl of resulting complex solution were added to each of the wells of the triplicate experiments, and the cells were incubated for 4 h as described above. The final percentages of serum were 0, 26, 53, and 66, respectively, during incubation. After 4 h the medium was removed, and the cells were incubated in 10% FBS-containing DMEM and were incubated for another 48 h. The cellular assays were done as described previously.

The transfection values are reported as the average values of triplicate experiment performed in the same plate on the same day. To verify reproducibility, each transfection experiment in the MCF-7 cell was performed at least four times on four different days. Other cell line data were representative data from at least two transfection experiments. The day to day variations in transfection efficiency were mostly within 2–3-fold and were dependent on the condition of cells.

Cell Viability Assay—Cytotoxicity of the cationic lipids was assessed using the MTT reduction assay as described earlier (25). The cytotoxicity assay was performed in 96-well plates, keeping the ratio of number of cells to amount of cationic lipid constant as was maintained in previously described transfection experiments. Briefly, 4 h after the addition of lipoplexes, MTT (5 mg/ml PBS) was added to the cells and the viability assay was performed in 96-well plates, keeping the ratio of number of cells to amount of cationic lipid constant as was maintained in previously described transfection experiments. Briefly, 4 h after the addition of lipoplexes, MTT (5 mg/ml PBS) was added to the cells and the viability assay was performed in 96-well plates, keeping the ratio of number of cells to amount of cationic lipid constant as was maintained in previously described transfection experiments.
was incubated for 3–4 h at 37 °C. Results are expressed as shown in Equation 1.

\[
\text{Percent viability} = \frac{\text{A}_{550(\text{treated cells})} - \text{background}}{\text{A}_{550(\text{untreated cells})} - \text{background}} \times 100 \quad (\text{Eq. 1})
\]

**Statistical Analysis**—Data were expressed as mean ± S.D. and statistically analyzed by the two-tailed unpaired Student's *t* test using Microsoft Excel (Seattle, WA). Data were primarily considered significant if *p* < 0.001.

**RESULTS**

**Synthesis of DSPE-PEG-HP**—HP is a very potent sigma drug, which possesses excellent affinity toward both subtypes of sigma receptors. With the help of the β-alanine adduct, HP is suitably modified to conjugate it to the PEG-grafted phospholipid, wherein PEG (M, 2000) acts as spacer between the lipid and the targeting ligand. The inclusion of the PEG spacer is done to improve the targetability and to provide stealth property to the liposome containing the PEG-lipid. Fig. 1 shows the scheme for the synthesis of haloperidol-derivatized phospholipid. The overall yield for the reactions involved in the suitable modification of HP is 80%, and the final conjugation yield is 70%.

**Binding Study and DNase I Treatment Study of DNA**—The targeted and non-targeted lipoplexes showed practically no difference in their binding property (Fig. 2A). Both the liposomes exhibit comparable affinity and bind to DNA in a similar fashion. At and beyond the 4:1 (+) charge ratio, DNA remains completely complexed with PEG-associated cationic liposomes, whereas at and below the 2:1 (±) charge ratio, a majority of the DNA remains non-complexed. These binding properties are corroborated in the DNase I treatment study of lipoplex (Fig. 2B). The lipoplexes irrespective of the presence of targeted or non-targeted PEG-lipid save DNA from DNase I with similar avidity in a ± charge ratio of 4:1 or greater.

**Targeted Gene Transfection in MCF-7 Cells**—Fig. 3A shows the comparison between reporter gene expression in HP- and DTG-untreated sigma receptor-overexpressing breast adenocarcinoma MCF-7 cells treated with cationic lipoplex, which contain either targeted lipid DSPE-PEG-HP or non-targeted lipid DSPE-PEG-mal. The cells treated with targeted lipoplex express significantly more amounts of reporter gene than that of the non-targeted lipoplex-treated cells. The highest differences in expression (8–12-fold, *p* < 0.001) were at a cationic lipid/DNA charge ratio of 4:1 and 2:1. Fig. 3A also shows that Lipofectamine™, one of the most frequently used cationic lipid-based gene transfection reagent, has significantly less effect in transfecting DNA in MCF-7 cells; thereby, proving the utility of the DSPE-PEG-HP-containing targeted lipoplex to transfect MCF-7 cells.

Fig. 3B shows the comparison between reporter gene expression in MCF-7 cells treated with targeted lipoplex in the absence or presence of either HP or DTG. With a cationic lipid/DNA charge ratio of 4:1 the difference in reporter gene expression is maximum (*p* < 0.001). With a 2:1 charge ratio, the difference in transgene expression is significant in the case of HP (*p* < 0.001) and is nearly significant for DTG (*p* = 0.008). The result proves that the uptake of targeted lipoplex is indeed mediated through sigma receptors.

Fig. 3C shows a similar comparison in MCF-7 cells treated with non-targeted cationic lipoplexes in the presence or absence of either HP or DTG. The overall gene expression is severalfold lower compared with that obtained through tar-
targeted lipoplex. Simultaneously, there is no consistent evidence of targeted uptake and eventual gene expression in cells treated with non-targeted cationic lipoplex. The gene expression was nonspecific in all the charge ratios and remains uninhibited in the presence of either HP or DTG.

Gene Transfection in CHO Cells—CHO, a non-human cell, is known to possess very low levels of sigma receptors (26). It is one of the most transfectable cell lines by cationic lipids. Fig. 3D shows the relative comparison between the reporter gene expression in CHO cells treated with targeted cationic lipoplex in the presence or absence of HP. Fig. 3E shows the relative comparison of gene expression in CHO cells treated with non-targeted cationic lipoplex in the presence or absence of HP. The gene expression is relatively high because of known nonspecific cell surface interaction with transfecting cationic lipids. However, the difference in gene expression in cells pretreated or untreated with HP is shown to be mostly insignificant, and no specific comparable trend is observed between cells treated
Comparison of Sigma Receptor Levels in MCF-7 and CHO Cells—To correlate the difference in targeted transfection in MCF-7 cells compared with that obtained in CHO with the present levels of sigma receptors in respective cells, a Western blot analysis was undertaken for the cellular proteins of the two cell lines. Fig. 4 shows the Western blot analysis of MCF-7 and CHO for the detection of the sigma 1 receptor. Sigma 1 receptor polyclonal antibody raised against the full-length sigma 1 receptor protein (223 amino acids) was added to the blot. Upon chemiluminescence, secondary antibody stained a single band, corresponding to a molecular weight of ~15–18, only with MCF-7 cells. For reconfirmation of the molecular weight of the bands, the blot was also stained for β-actin. We believe that a sigma 1 splice variant, detectable by this polyclonal antibody, rather than a full-length sigma 1 receptor could be present in MCF-7 cells. However, CHO cellular proteins did not reveal any similar bands detectable by the sigma 1 receptor antibody.

Gene Transfection in Hela, HepG2, and KB Cells—Cervical cancer (Hela), hepatic cancer (HepG2), and a contaminant variant of cervical cancer (KB) are human cells, which show differential levels of sigma receptor expression. To our knowledge, Hela, HepG2, and KB cells have not shown a precedence of overexpressing sigma receptors. These cells were chosen for transfection with the targeted cationic lipoplex in the presence or absence of HP to ascertain the targeting property of the targeted lipoplex toward other non-breast cancer, non-sigma receptor-expressing cancer cells. Fig. 5 shows a 5–10-fold decrease in transgene expression in respective human cancer cells in comparison to that obtained in MCF-7 cells (Fig. 3, A and B). The transgene expression remains mostly unabated in the presence of the sigma ligand HP. Hence, no targeted gene transfections mediated through sigma receptors is observed.

Gene Transfection in Spironolactone-treated MCF-7 Cells—Spironolactone is known to down-regulate the number of sigma receptors on the cell surface without diminishing the binding affinity of the sigma receptor subtype non-selective ligand, DTG (27). This selective depletion of sigma sites was used to ascertain the role of HP-associated lipoplex in targeting and delivering genes to cells through sigma sites only. Fig. 6A shows the comparative transfection efficiencies of targeted lipoplexes in the absence or presence of free HP and sigma receptor ligand DTG in MCF-7 cells pretreated with 10 μM spironolactone for 2 consecutive days. In comparison to spironolactone-untreated MCF-7 cells, the extent of transfection is in general low, and there was barely any statistical significance between the treated and untreated groups in spironolactone-pretreated cells. These results show that the HP-associated lipoplex does not show targeted delivery of genes to sigma receptor-depleted MCF-7 cells and hence, for targeting purposes the HP-associated lipoplex needs overexpression of sigma receptors.

Serum Stability of the Targeted Cationic Lipoplex—Fig. 6B shows that in MCF-7 cells there is no inhibition of the transfection property of the HP-associated lipoplex in the presence of varied serum concentrations, unlike what is usually seen in cationic lipid-mediated gene transfection. Barring an interesting increase in the transgene expression in the presence of 7% serum, the transfection efficiency practically remains at par or insignificantly increased in the varied serum concentrations compared with non-serum-containing medium. Therefore, at the most pertinent in vivo serum condition (i.e. at 53% serum concentration) the cationic lipoplex appears to remain perfectly covered by the PEG layer, and the targeting remains uninhibited because of the presence of the HP moiety above the layer of PEG in the targeted lipoplex.

Lipoplex-mediated Cellular Toxicity—The MCF-7 cells upon treatment with targeted and non-targeted cationic lipoplex over the range of charge ratio from 8:1 to 1:1 for 4 h revealed that there was no immediate lipid- or lipoplex-mediated cytotoxicity to the cells (Fig. 6C). The cells remained nearly 100% viable irrespective of the treated lipid.

DISCUSSION

The sigma receptors are membrane-bound proteins that are overexpressed in a variety of human tumors including breast cancer cells (3, 4, 9–13). This cell membrane property makes sigma ligands potentially important for targeting various bioactive molecules to such tumors. Many newer synthetic sigma ligands have been produced that are useful in imaging sigma receptor-overexpressing tumors, including breast cancer rodent models (8–13). Herein, we have demonstrated successfully for the first time that an inexpensive, potent, small molecule, neuroleptic drug, HP can target genes to breast adenocarcinoma, which avidly express sigma receptors. Although the link between cancer pathogenesis and sigma receptor overexpression is not clear, studies of targeting sigma receptors has shown the increased possibility of delivering therapeutic genes for targeting and killing sigma receptor-
overexpressing breast cancer cells. Haloperidol exhibits the following properties: it is a sigma receptor subtype non-selective neuroleptic drug showing very high affinity for the receptor, is of small molecular weight, and is non-immunogenic. HP chemically conjugates to a phospholipid and hence is easier to incorporate in the liposome formulation in low mol %. A low mol % of HP will induce a smaller agonistic effect, if any, to breast cancer cells or in vivo to the common tissues expressing sigma receptors at basal levels. However, with a PEG spacer between the lipid and HP, there is efficient ligand-mediated targeting by the liposome. Additionally, the PEG moiety also provides improved stability and an increased circulation half-life of the liposome in vivo (29, 30, 21, 22).

The following study demonstrated that a properly designed HP-conjugated PEG-lipid, which when associated with a cationic lipid, could offer sigma receptor-targeted enhanced gene transfection in breast adenocarcinoma MCF-7 cells. The chemistry of synthesizing targeted PEG-lipid is well known, and the final yield is high (Fig. 1). The non-targeted lipid (control PEG-lipid) is a PEG-lipid with no targeting ligand; this is included in the study to show the effect of the HP moiety in targeting sigma receptor-expressing MCF-7 cells. The targeting and non-targeting liposomes show no specific difference in binding affinity with DNA in the DNA binding and DNase I study (Fig. 2, A and B).

An optimization of the amount of targeted lipid varying from 0.05 mol % to 5 mol % is also done in which maximum observable targeting with minimum nonspecific gene transfection is obtained for 5 mol % only (data not shown). With this optimized condition, the basic cationic lipid (DOPEAC/cholesterol gene delivery formulation (20) attains a property of specifically targeting MCF-7 cells. The targeted lipoplex shows 10-fold excess targetability (p < 0.001) at ≥ charge ratio of 4:1 and 2:1 compared with non-targeted lipoplex (Fig. 3A). The targeting is specific, hence the gene expression by targeted lipoplex is significantly (p < 0.001) inhibited in HP-, and DTG- (another sigma subtype non-selective ligand) pretreated MCF-7 cells (Fig. 3B). All the transfection experiments are done in the presence of serum medium. The non-targeted lipoplex shows no specific inhibition in gene transfection in the presence of both HP and DTG (Fig. 3C). Upon simultaneous transfection with Lipofectamine™, it is revealed that this cell-nonspecific, widely used cationic transfection lipid is less efficient in transfecting MCF-7 cells than the HP-associated lipoplex, probably because of its minimal sigma receptor targeting ability.

CHO is a highly transfectable, and has a low level of sigma receptor (26), non-human cell line. The gene transfection by both targeted and non-targeted liposome is very high in MCF-7 cells. Yet, the gene transfection is nonspecific and therefore no specific trend is observed in both sets of experiments: treatment with targeted and non-targeted lipoplexes in the presence or absence of HP (Fig. 3, D and E).

The specific targeting to MCF-7 cells compared with that in CHO cell is ascertained by Western blot analysis of cellular sigma 1 receptor proteins. MCF-7 cells originally consist of very low amounts of sigma receptor subtype-1 as imaged by the

Fig. 5. Gene transfection in HeLa (A), HepG2 (B), and KB (C) cell lines. HeLa/HepG2/KB cells were transfected with 0.3 μg of pCMV-β-gal complexed in HP-associated targeted cationic liposome DOPEAC/cholesterol/DSPE-PEG-HP (1:1:0.05) or DOPEAC/cholesterol/DSPE-PEG-mal (1:1:0.05). The cells were untreated (white bar, HP-) or pretreated with 20 μM haloperidol (black bar, HP+) before treatment of the lipoplex. The transgene expression obtained for each data point was acquired from triplicate treatments done in a single day.
sigma 1-specific drug pentazocine (7). However, it is also evident from the excessive binding of sigma receptor subtype non-selective ligands such as HP and DTG to breast adenocarcinomas that these cells may overexpress sigma 2 receptors albeit there is a low expression level of sigma 1 receptors (4, 7). Experimentally, the binding constant of HP is about 3-fold lower than that of pentazocine to the sigma 1 site (28). This shows that HP can sense the residual sigma 1 sites, which remain undetected by pentazocine in MCF-7 cells. Fig. 4 shows the presence of a possible variant of the sigma 1 receptor in MCF-7 cells. However, sigma 1 or its variants remain undetected in CHO cells under the experimental conditions. Previous studies have also corroborated the presence of sigma 1 receptor splice variants in different cells (29). Therefore, the assumed sigma receptor splice variant protein as detected by Western blot may contain the ligand binding domain. The sigma ligand binding domain and the overexpressing sigma 2 sites together help the targeted lipoplex synergistically in targeting MCF-7 cells. CHO probably has an undetectable level of sigma 1 or its variant, and hence targeted gene delivery remains unnoticed in this case. Therefore, primarily the HP-associated lipoplex can target its genetic payload to MCF-7 cells in a target-specific manner by the virtue of the presence of a residual ligand-binding splice variant of sigma 1 sites and abundant sigma 2 sites in these cells.

The targeted lipoplex upon testing other heavily multiplying human cancer cells such as Hela, KB, and HepG2 shows 5–10-fold less transfection compared with that obtained in MCF-7 cells (Fig. 5). The transfections largely remain unabated when pre-exposed to HP. The transfection results with the currently used non-MCF-7 cells can essentially predict that the HP-associated lipoplex may not target human cancer or non-human cells that are not correlated with overexpressing sigma receptors.

To test the transfection efficiency of HP-associated lipoplex in sigma receptor-depleted cells, the sigma receptor expression would have to be down-regulated in MCF-7 cells. Because of the lack of known sigma receptor endogenous ligand the natural down-regulation of sigma receptor in cells by starving the cells with the ligand cannot be observed. Previously, the spironolactone drug was shown to down-regulate sigma recognition sites in brain and liver cells obtained from guinea pigs by exposing the animals to it (27). Following radiolabeled DTG saturation analysis to sigma sites revealed a depleted binding of DTG without any change in affinity. In the present study, we chose to treat the MCF-7 cells with spironolactone. This should reflect any change in the relative transfection efficiencies of targeted lipoplexes in the presence or absence of known sigma ligands HP and DTG. The cells upon treatment with 10 μM spironolactone, which show minimal toxicity to the cells yet
demonstrate possible down-regulation of sigma sites, exhibits decreased transfection with targeted lipoplexes (Fig. 6A) compared with that obtained in native MCF-7 cells (Fig. 3B). Additionally, in the presence or absence of HP and DTG, the transfections remain the same (Fig. 6A). This proves that the targeting efficiency of HP-associated lipoplexes is nullified in the sigma receptor down-regulated state of MCF-7 cells.

To extend the current study toward targeting breast cancer in tumor-bearing mice we chose to include DODEAC as the base cationic lipid, which has a proven in vivo biodistribution profile (30). The idea of including polyethylene glycol in cationic lipid-mediated gene transfection in vivo is to suppress the usual nonspecific lung affinity by increasing the longevity of the liposome in the body. During the long circulation, the liposomal system will utilize the sigma ligand to seek and bind to the sigma receptors overexpressed on the cancer cells. In conjunction, the targeted lipoplex was also tested for its stability using varied amounts of serum in the transfection medium. The MCF-7 cells were treated with targeted lipoplex in the absence or presence of varied amounts of serum-containing medium (Fig. 6B). Importantly, the transgene expression in the presence of serum remains unabated at the serum concentration relevant for in vivo conditions. The study indicated that the targeting formulation is serum stable. Additionally, there was no nonspecific lipid-mediated toxicity to the cells during the direct incubation of the lipoplexes to the cells (Fig. 6C).

In conclusion, the present study suggests that haloperidol with its active derivation shows new promise in targeting various types of anticancer genes to sigma receptor-positive cancers. The new system is advantageous over other known liposomal targeting systems such as immunoliposomes. This small molecule drug has no proven immunogenicity, and it has an excellent safety profile at the concentration at which it is associated in the liposome. Also the system should be versatile because of the fact that various types of tumors overexpress sigma receptors. In breast cancer alone there are several subtypes of sigma receptor-expressing carcinomas e.g. ductal carcinoma, and other adenocarcinomas, which can be targeted through this gene-targeting lipoplex. Other carcinomas like lung, renal, colon, prostate, and melanomas that overexpress sigma receptors are also likely to be targeted efficiently by this system (for a review see Ref. 31). Thus, haloperidol-targeted gene delivery systems may become a novel class of therapeutics for the treatment of human cancers. This gene delivery method can also find its utility in assessing the therapeutic effect of a candidate gene in this special type of tumor. Studies utilizing therapeutic genes for the treatment of sigma receptor-positive tumors of different origin are currently under progress in our laboratory.

Acknowledgment—We sincerely thank Dr. J. S. Yadav, Director, 1. I. C. T, Hyderabad, for his keen interest in the project.

REFERENCES
Haloperidol-associated Stealth Liposomes: A POTENT CARRIER FOR DELIVERING GENES TO HUMAN BREAST CANCER CELLS
Amarnath Mukherjee, Tekkatte Krishnamurthy Prasad, Nalam Madhusudhana Rao and Rajkumar Banerjee


Access the most updated version of this article at doi: 10.1074/jbc.M409723200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 11 of which can be accessed free at http://www.jbc.org/content/280/16/15619.full.html#ref-list-1