International Journal of Pharmaceutics 419 (2011) 347–354

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Targeted liposomes to deliver DNA to cells expressing 5-HT receptors

Vijaya Gopal, Jennifer Xavier, Gulam Hassan Dar, Md. Jafurulla, Amitabha Chattopadhyay, N. Madhusudhana Rao*

Centre for Cellular and Molecular Biology (A Council of Scientific and Industrial Research Laboratory), Uppal Road, Hyderabad 500007, India

ARTICLE INFO

Article history:
Received 17 June 2011
Received in revised form 30 July 2011
Accepted 2 August 2011
Available online 10 August 2011

Keywords:
5-HT
Serotonin receptor
Cell-targeting
Stealth liposomes
Cationic lipids
Transfection

ABSTRACT

Cell targeted delivery of drugs, including nucleic acids, is known to enhance the therapeutic potential of free drugs. We used serotonin (5-HT) as the targeting ligand to deliver plasmid DNA to cells specifically expressing 5-HT receptor. Our liposomal formulation includes the 5-HT conjugated targeting lipid, a cationic lipid and cholesterol. DNA-binding studies indicate that the targeting 5-HT-lipid binds DNA efficiently. The formulation was tested and found to efficiently deliver DNA into CHO cells stably expressing the human serotonin$_{1A}$ receptor (CHO-5-HT$_{1A}$R) compared to control CHO cells. Liposomes without the 5-HT moiety were less efficient in both cell lines. Similar enhancement in transfection efficiency was also observed in human neuroblastoma IMR32 and hepatocellular carcinoma (HepG2) cells. Cell uptake studies using CHO-5-HT$_{1A}$R cells by flow cytometry and confocal microscopy clearly indicated that the targeting liposomes through 5-HT moiety may have a direct role in increasing the cellular uptake of DNA–lipid complexes. To our knowledge this is the first report that demonstrates receptor-targeted nucleic acid delivery into cells expressing 5-HT receptor.

1. Introduction

Realization of the potential of nucleic acids as therapeutic molecules critically depends on the ability to efficiently deliver them into target tissues. Vehicles delivering nucleic acids should be able to protect the nucleic acids in the biological milieu, specifically transport them to the target tissues and facilitate uptake into the cells to realize the therapeutic potential. A variety of vehicles containing lipids (Niculescu-Duvaz et al., 2003), polymers (Boussif et al., 1995), dendrimers (Paleos et al., 2009) and nanoparticles (Li and Szoka, 2007) have been successfully tested for their *in vitro* and *in vivo* transfection properties. Systemic delivery of the nucleic acid formulations leads to extensive dilution and reduces the effective concentration of nucleic acid at the target site. To enhance the target specific localization, nucleic acid vehicles can be designed to carry target specific ligands. The unique biochemical properties of the target tissue are utilized in the design of the vehicles. In targeting the vehicles to the tumor tissues the enhanced expression of receptors of folate (Taniguchi et al., 2010), transferrin (Zhai et al., 2010), epidermal growth factor (Medina-Kauwe et al., 2001; Jeyarajan et al., 2010; Gopal and Guruprasad, 2010), sigma receptor (Mukherjee et al., 2005) have been exploited by attaching ligands to lipidic and non-lipidic vehicles as these impart receptor-specific interaction and are specifically taken up by receptor-mediated endocytosis. These specific interactions were shown to be important to load the vehicles into targeted cells and small-molecule targeted-ligand approach has been effective for *in vivo* situations leading to organ- or tumor-specific delivery of nucleic acids (Liu et al., 2010; Hood et al., 2002).

Serotonin (5-hydroxytryptamine (5-HT)) is an intrinsically fluorescent (Chattopadhyay et al., 1996), biogenic amine which acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous systems. Receptors for 5-HT are abundant in the central and peripheral nervous systems as well as in non-neural tissues such as gut, blood and cardiovascular system (Jacobs and Azmitia, 1992). 5-HT receptors are G-protein coupled receptors (Pierce et al., 2002) involved in the etiology of large number of neural diseases and are intensively investigated by academia and pharmacology industry. The role of 5-HT in tumor progression was demonstrated for the first time by Dizeyi et al. (2004) from the observation of the overexpression of 5-HT receptors in prostate cancer tissues and through ligand binding assays in prostate cancer cell lines. The authors proposed the role of 5-HT in tumor progression particularly in androgen-independent states and implicated the role of biogenic amines such as serotonin in the proliferation of prostate cancer. From their study, a potential treatment of cancer was also suggested through the use of serotonin-uptake inhibitors. In yet another study, nearly 15 years back, Merzak et al. investigated the effect of 5-HT on glioma and demonstrated that serotonin positively modulated cell proliferation, migration,

* Corresponding author. Tel.: +91 40 27160310; fax: +91 40 27160311.
E-mail address: madhu@ccmb.res.in (N.M. Rao).

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doi:10.1016/j.ijpharm.2011.08.004

and invasion *in vitro*. Overexpression of seven 5HT receptors in glioma cell lines and not in normal fetal astrocytes indicated the involvement of serotonin in modulating cell proliferation and migration of human glioma cells (Merzak et al., 1996). Together, these studies suggested the significant role of 5-HT in the control of the biological properties of human glioma and prostate cancer.

There are two reactive groups in the chemical structure of serotonin. These are the phenolic hydroxyl and the primary amine group. By employing site-directed mutagenesis on serotonin receptors, the hydroxyl group of serotonin has been shown to be directly involved in binding by interacting with serine, aspartate and threonine residues present in different transmembrane helices of the receptor (Ho et al., 1992). Interestingly, it has also been shown using mutational and modeling studies that the serotonin$_{1A}$ receptor prefers ligands with a hydrogen bond acceptor at a position corresponding to the hydroxyl group in serotonin (Sylte et al., 1996; Kuipers et al., 1997). We therefore decided to covalently conjugate serotonin to carboxylic acid 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy(polyethyleneglycol)-2000] [DSPE-PEG(2000)] through the reactive amine group, leaving the hydroxyl group intact in order to retain the binding properties of serotonin to the receptor.

Current trends for effective gene therapy demand gene delivery systems that are versatile, highly efficient and specific. The present methodologies to deliver DNA into cells is challenging primarily due to various membrane barriers encountered during the course of entry into cells making the process toxic, non-specific and inefficient. Cell-specific introduction of therapeutic genes calls for the development of efficient vectors and formulations that can introduce nucleic acids in a non-toxic manner. In this study, we synthesized and characterized a 5-HT functionalized cell-targeting lipid that can be reconstituted with N,N-di-n-hexadecyl-N,N-dihydroxyethylammonium bromide (DHDEAB), an efficient cationic lipid (Banerjee et al., 1999) to deliver nucleic acids into cells expressing 5-HT receptor. We employed a CHO cell line stably expressing the 5-HT receptor (CHO-5-HT$_{1A}$R) (Banerjee et al., 1993) being an ideal system to test the transfection efficiency of reconstituted targeting liposomes.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy(polyethyleneglycol)-2000] (ammonium salt) {DSPE-PEG(2000) carboxylic acid}, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt) {DSPE-PEG(2000) maleimide} were purchased from Avanti Polar Lipids (Alabaster, AL, USA). DHDEAB (N,N-di-n-hexadecyl-N,N-dihydroxyethylammonium bromide) was synthesized in house as described (Banerjee et al., 1999). Receptor agonist [^{3}H] 8-OH-DPAT ([^{3}H]8-hydroxy-2(di-n-propylamino)) tetralin was purchased from DuPont New England Nuclear (Boston, MA, USA). Phenyl methyl sulphonyl fluoride (PMSF), serotonin (5-HT), sodium bicarbonate, polyethylenimine, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DMEM/F-12 (Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) (1:1)) and geneticin (G 418) were from Life Technologies (Grand Island, NY, USA). *FAM*-labeled DNA was obtained from Bioserve Technologies, Hyderabad, India. GF/B glass microfiber filters were from Whatman International (Kent, UK). Plasmid pCMVβ-gal and pEGFPN$_3$ plasmid DNA was purified using endotoxin free kit (Qiagen). All other reagents and chemicals used were of the highest purity available.

2.2. Synthesis and characterization of DSPE-PEG (2000)–5-HT: conjugation of 5-HT to DSPE-PEG (2000)–COOH

2.2.1. Crosslinking 5-HT to DSPE-PEG (2000)–COOH

DSPE-PEG (2000)–COOH (25 mg, 0.0088 mmol) crystals were dissolved in 800 μl of dry dichloromethane (DCM) taken in a flask. To this, serotonin hydrochloride (12.5 mg, .071 mmol) dissolved in 100 μl of DMF was added and kept stirring for ~15 min on an ice bath. After thirty minutes dicyclohexylcarbodiimide (DCC, 2.4 mg, 0.0116 mmol) solubilized in DMF was added and incubated with stirring at room temperature overnight. The solvent was evaporated and product purified three times by recrystallization using methanol/ether (1:15, v/v) as solvent under cold conditions. The purified compound obtained was dried by flushing the solid with a stream of nitrogen gas. The total yield of the pure compound was 6 mg, 24% yield with respect to the DSPE-PEG (2000)–lipid, Scheme 1. The presence of 5-HT on DSPE-PEG (2000) was confirmed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Suppl. S1). Ligand conjugation of DSPE-PEG (2000)–COOH to 5-HT was verified by Proton NMR spectroscopy (Suppl. S2) using Triple resonance probe of FTNMR Spectrometer (Model AV600 AVANCE 600 MHz).

2.3. Preparation of liposomes

Targeting liposomes were formulated by reconstituting chloroform stocks of DHDEAB and cholesterol at 1:1 mole ratio. The DSPE-PEG (2000)–5-HT and dried using a thin flow of nitrogen and dried lipid film was kept under vacuum for 4–5 h. Subsequently, deionized water was added to the dried lipid film for overnight rehydration. The vial was vortexed thoroughly at room temperature to produce multilamellar vesicles (MLVs) which was sonicated until clarity to obtain small unilamellar vesicles (SUV). DHDEAB:Chol was reconstituted similarly with either DSPE-PEG (2000)–Mal or DSPE-PEG-(2000)–COOH to serve as non-targeting lipid controls.

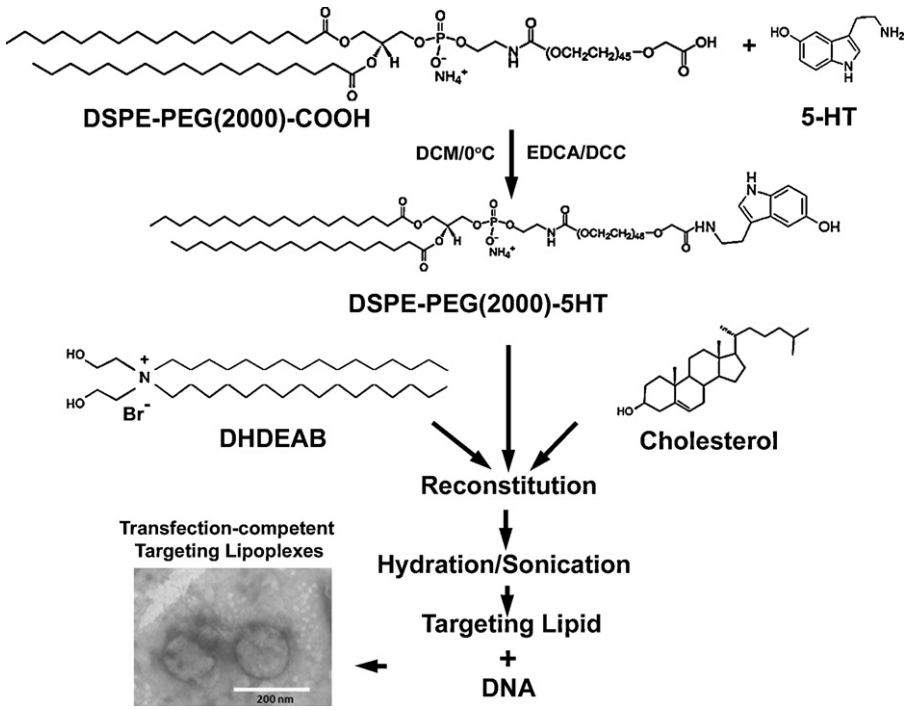
2.4. DNA-binding

2.4.1. Ethidium bromide (EtBr) displacement assay

The binding of DNA with the cationic liposomes was studied using EtBr, an intercalating agent, as the fluorescent probe that provides reproducible and efficient evaluation of lipoplex formation. The displacement of EtBr, upon lipid interaction, is reflected as a drop in the fluorescence signal, since unbound EtBr does not fluoresce. All measurements were carried out using a Hitachi F-4500 fluorescence spectrophotometer. The excitation wavelength, λ_{ex} was 516 nm and the emission wavelength was kept at 598 nm (slit width 5 nm × 5 nm). Briefly, 2.3 μg of pCMV-β-gal plasmid DNA was added to 500 μl of 20 mM Tris. HCl buffer (pH 7.4). EtBr (0.23 μg), from a diluted stock solution, was added to DNA and the baseline fluorescence was determined. The fluorescence intensity obtained upon each addition of lipid was normalized relative to the fluorescence signal of DNA–EtBr complex, in the absence of the lipid, which was taken as 100%. The binding of DNA was recorded after each addition at time intervals of 5 min.

2.4.2. The agarose gel-based retardation assay: protection of DNA from DNaseI digestion

DNA binding by targeting and non-targeting liposomes was observed in a gel binding assay where plasmid pEGFPN$_3$ DNA:lipid complexes (lipoplexes), at varying charge ratios, were incubated in 0.5× PBS for 30 min at room temperature prior to electrophoresis. Migration of complexes was observed as decreased mobility of DNA upon staining with ethidium bromide post electrophoresis. Complexes corresponding to a charge ratio of 3:1 lipid:DNA, was used



Scheme 1. Synthesis-schematic of targeting lipid outlining the conjugation of 5-HT to DSPE-PEG(2000)–COOH, for cell-specific targeting. A representative TEM image of the targeting liposome complexed with plasmid pCMVβ-gal DNA, denoted as targeting lipoplexes (<200 nm) is depicted.

for the binding assay. The ability of the lipid to protect DNA in a lipoplex was also investigated by incubating lipoplexes with DNaseI as described (Jeyarajan et al., 2010). Samples were treated with DNaseI at the indicated concentrations, processed by extraction with a mixture of phenol–chloroform, and subsequently analyzed by 1% agarose gel electrophoresis (Tris acetate buffer (TAE) pH 8.2). The gel was stained with EtBr post electrophoresis.

2.5. Uptake of DNA:lipid complexes

CHO and CHO-5-HT_{1A}R cells (20,000) were seeded on chambered cover glass and incubated with lipoplexes (with 3:1 Lipid/DNA charge ratio) prepared with targeting and non-targeting liposomes incubated with 5′-end FAM-labeled oligonucleotide in DMEM medium without serum. The FAM-labeled oligonucleotide was prepared by annealing to the complementary sequence to generate labeled, duplex DNA. After the incubation period of cells with the complexes for 3 h, DMEM was removed, and the cells were washed with PBS twice prior to analysis by confocal microscopy (Leica TCS S52). The emission collection wavelengths were set at 494 and 518 nm for FAM, 415–485 nm for Hoechst 33258.

2.6. Hydrodynamic radius of targeting and non-targeting liposomes

Lipoplexes were prepared with formulations of targeting and non-targeting liposomes complexed in 1× PBS with pCMVβ-gal DNA at 3:1 charge ratio and the hydrodynamic radius was measured on a Zetasizer 3000HSA (Malvern, UK). Zetasizer measures the particle size/molecular weight and zeta potential of particles of nanometer to microsize using Dynamic Light Scatter (DLS) and by laser Doppler anemometry, respectively. All the size measurements were done 10 times in triplicate with the zero field correction and values represented as the average of triplicate measurements.

2.7. Transmission electron microscopy (TEM) of targeting lipoplexes

TEM of the targeting lipoplex was carried out using 200 kV Transmission Cryo Electron Microscope (JEOL), Japan. The targeting lipoplexes composed of plasmid pCMVβ-gal DNA and DSPE-PEG-(2000)-5-HT (targeting liposome) was prepared in PBS buffer (pH 7.4) at the 1:2 charge ratio, incubated for 30 min at room temperature and stained with uranyl acetate prior to observation by TEM.

2.8. Transient transfection

CHO, CHO-5-HT_{1A}R, IMR32 and HepG2 were obtained from in-house laboratory stocks. Stably transfected CHO-5-HT_{1A}R cells heterologously expressing the human serotonin$_{1A}$ receptor (5-HT_{1A}R) were grown in DMEM/F-12 (1:1) as described (Kalipatnapu et al., 2004). 20,000 cells in 100 μl medium were seeded in each well of a 96-well culture plate and incubated at 37 °C overnight. The following day, cells were washed with DMEM prior to the addition of lipid–DNA complexes. Plasmid DNA pCMVβ-gal for transfection was purified by using the Qiagen Kit (endotoxin free) using the manufacturer's protocol. Based on charge ratios, lipoplexes were prepared in DMEM without serum and incubated for 30 min prior to the addition to cells. The medium was then replaced with DMEM containing 10% serum following an incubation period of 3 h. Reporter gene assay was performed 48 h after transfection following which cells were washed with PBS and lysed in 100 μl of lysis buffer (0.25 M Tris–HCl, pH 8.0, and 0.5% NP40). β-Galactosidase activity was estimated by adding 50 μl of 2× substrate (1.33 mg/ml ONPG, 0.2 M sodium phosphate, pH 7.15, and 2 mM magnesium chloride) to an equal volume of the lysate in a fresh 96-well plate and incubated at 37 °C. Absorption at 405 nm was converted to β-gal units and protein normalized using a standard curve generated with pure commercial enzyme.

2.9. Fluorescence activated cell sorting (FACS) of cellular uptake of liposomes

CHO and CHO-5-HT_{1A}R cells were grown as described in Section 2.5. Complexes of FAM-labeled DNA duplex and lipid with and without the targeting moiety were prepared at the ratios optimal for transfection and incubated for 3 h at 37 °C. Following incubation, cells were processed and examined for uptake by flow cytometry (10,000 gated events). Briefly, complexes were removed and cells washed with PBS three times and trypsinized, spun and resuspended with PBS. Samples were then transferred to tubes for FACS analysis using FACSCalibur (Becton Dickinson).

2.10. Radioligand binding assays for receptor expression

Agonist binding to CHO and CHO-5-HT_{1A}R cell membrane fractions was studied by preparing cell membranes as described earlier (Chattopadhyay et al., 2004). Briefly, confluent cells were harvested by treatment with ice-cold buffer containing 10 mM Tris, 5 mM EDTA, 0.1 mM PMSF, pH 7.4 and homogenized. The pellet obtained was resuspended in 50 mM Tris buffer, pH 7.4, flash frozen and stored at −70 °C till further use. Total protein concentration of isolated membranes was determined as described (Chattopadhyay et al., 2004). The final concentration of radiolabeled agonist [^{3}H] 8-OH-DPAT per assay tube was 0.29 nM. Receptor binding assays were carried out using [^{3}H] 8-OH-DPAT with ~40–50 μg of total protein ensuing from CHO cells harbouring the human 5-HT_{1A} receptors. Non-specific binding was determined by performing the assay in the presence of 10 μM 5-HT as described. Retained radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation counter as described (Kalipatnapu et al., 2004).

2.11. Assessment of cell viability

Cytotoxicity of the liposomes was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Complexes prepared with lipids, were evaluated for their toxicity to cells by performing the MTT assay as described (Hansen et al., 1989) in 96-well plates at charge ratios used in the transfection experiments. CHO, CHO-5-HT_{1A}R, HepG2 and IMR32 cells were incubated with transfection complexes for 3 h at 37 °C in serum-free DMEM. Soon after transfection, cells were washed with PBS and replaced with 75 μl DMEM + 25 μl MTT (2 mg/ml in PBS) for 3 h. The medium was removed and replaced with 100 μl DMSO:methanol 1:1 (v/v) and mixed to dissolve the formazan crystals. Absorbance was then measured at 540 nm with untreated cells serving as controls. Results were expressed as percent viability: [A_{540} (treated cells) − background/A_{540} (untreated cells) − background] × 100.

3. Results and discussion

3.1. Conjugation of 5-HT to DSPE-PEG (2000)

5-HT is a ligand to 5-HT receptors, a family of G-protein-coupled receptors localized in the central and peripheral nervous system and other tissues. The specific ligand–receptor interactions were utilized in this study to deliver nucleic acids in a receptor specific manner using 5-HT conjugated liposomes. Scheme 1 describes the synthesis of the targeting lipid wherein the targeting moiety, 5-HT, was conjugated to DSPE-PEG-(2000)-COOH, a PEG derivative of phosphatidylethanolamine and reconstituted with DHDEAB and cholesterol.

The purity of DSPE-PEG(2000)–5-HT was confirmed using mass spectrometry and also by NMR spectroscopy (Supplementary material S1 and S2) prior to its formulation at 5 mol% with DHDEAB and cholesterol as the colipid. It has been shown that PEG (MW = 2000) acts as a spacer arm, so that the targeting ligand is extended (Zalipsky, 1995). This may provide accessibility to the receptors while the cationic lipid shields the complexed DNA from degradation as seen in the subsequent section. The PEG spacer between the lipid and the targeting ligand may position the targeting moiety suitably and provide stealth property to the liposome DSPE-PEG(2000) reconstituted at 5 mole% in the formulation with DHDEAB:Chol.

PEG-coated liposomes also referred to as stealth liposomes, have a stabilizing effect and where the electrostatic interactions amongst macromolecules during systemic circulation are not favoured. PEG-derived lipids are popularly used as long-circulating liposomes (Zalipsky, 1995) particularly for systemic delivery of nucleic acids. The rationale for the covalent attachment of 5-HT to PEG was to maintain the useful properties of PEG such as low toxicity (Pang, 1993), solubility (Powell, 1980) and low immunogenicity (Dreborg and Akerblom, 1990) and at the same time retain its targeting ability when formulated with an efficient cationic lipid (Banerjee et al., 1999).

3.2. Interaction of targeting lipid with DNA

Cationic lipids electrostatically bind to DNA to form lipoplexes. DNA-binding being the prerequisite for transfecting cells, we studied the DNA-binding potential of targeting 5-HT-derived liposomes and compared with the non-targeting derivatives. The binding efficiency of lipids can be monitored in a fluorescence-based assay where efficiencies are correlated with displacement of EtBr from an EtBr:DNA complex. We investigated the DNA-binding potential of the targeting liposomes 5-HT-derived lipid formulation DHDEAB:Chol:5-HT-DSPE-PEG (2000) at 1:1:0.05 mole ratio by ethidium bromide displacement assays. The binding efficiency is reflected as a drop in the fluorescence intensity upon binding of the lipid that displaces EtBr. Non-targeting lipid DHDEAB:Chol:DSPE-PEG (2000)–Mal (1:1:0.05 mole ratio), at the identical mole ratio served as control. All the formulations bind DNA similarly with small variations. Maximum displacement was observed at 3:1 charge ratio of Lipid/DNA indicating comparable binding efficiencies, Fig. 1a. The efficiency with which DNA is condensed appears to be different in the case of the 5-HT derived liposome at 1:1 Lipid/DNA. However at higher charge ratios of 3:1, the DNA-binding affinity was found to be similar. The ability to bind and also protect DNA in a lipoplex was investigated by incubating the complexes with DNaseI, Fig. 1b. DNA binding and protection of DNA from nucleases was observed with targeting (lane 4) and non-targeting liposomes (lanes 6, 8) at 3:1 charge ratio. Protected DNA, after treatment with phenol–chloroform extraction, migrated normally as either relaxed or supercoiled DNA. It is thus apparent that the presence of DHDEAB in the formulation was sufficient for protection of plasmid DNA from degradation.

In order to observe the macroscopic structure of the targeting lipoplexes, we used TEM. In such a study, the lipoplexes were observed to form compact structures of <200 nm (inset of Scheme 1). We also characterized the particle sizes of targeting and non-targeting liposomes by measuring the hydrodynamic diameter of lipoplexes prepared at 3:1 charge ratio, with and without the targeting liposomes. The hydrodynamic diameter of lipoplexes prepared with and without the targeting liposome was 180 ± 5 nm and 165.8 nm, respectively. The sizes of the lipoplexes were of the size that is generally found to be suitable for cellular uptake. Although the correlation of size with high transfection efficiency is widely debated (Ma et al., 2007), it is known that the heterogeneity and size of the lipoplex increases with increasing charge ratio of lipid to DNA and depends on the condensing ability of monovalent lipids. Lipoplex size is considered important for determining the entry

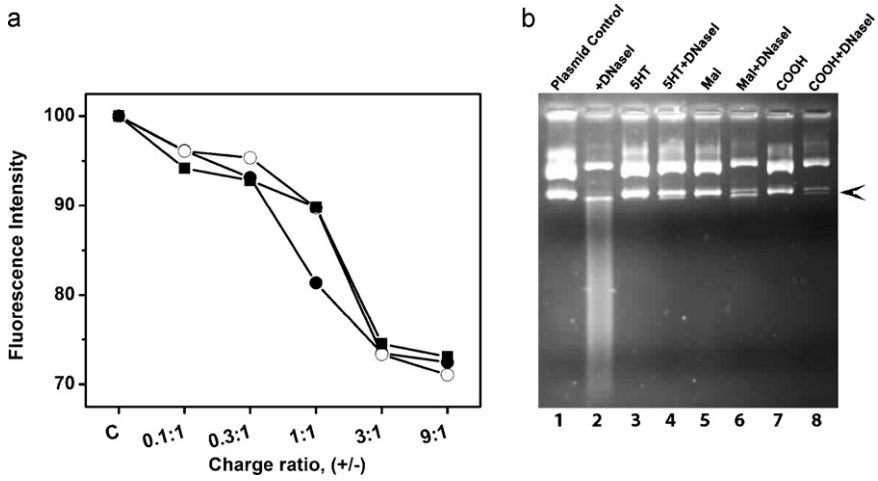


Fig. 1. (a) Binding of targeting and non-targeting lipids to DNA: fluorescence titration curve depicting quenching of fluorescence due to release of EtBr from lipoplexes prepared with $pEGFPN_3$ plasmid DNA: targeting lipid DHDEAB:Chol:DSPE-PEG (2000)–5-HT (1:1:0.05 mole ratio) (filled circle), non-targeting control lipid DHDEAB:Chol: 1:1 complex (filled square), and DHDEAB:Chol:DSPE-PEG (2000)–Mal (1:1:0.05 mole%) open circle. Details as in the experimental section. (b) DNA binding and DNaseI protection assay: plasmid $pEGFPN_3$ (0.2 μg) was incubated in a buffer (pH 7.4) with targeting lipid DSPE-PEG (2000)–5-HT denoted as 5-HT (lane 3) and control lipids DSPE-PEG (2000) Mal and DSPE-PEG (2000)–COOH and DHDEAB:Chol denoted as Mal and COOH (lanes 5, 7, respectively). Charge ratios for all the lipids were kept at 3:1 N/P. Samples were loaded along with untreated control plasmid DNA (lane 1) and in the presence of DNaseI (lane 2) which is a treated control. Lanes treated with DNaseI correspond to lanes 4, 6 and 8. Gels were stained with EtBr and visualized. Supercoiled DNA is marked by an arrow.

pathway, although this may not have a direct correlation on the transfection efficiency (Ilarduya et al., 2003; Kim et al., 2010).

3.3. Measurement of receptor levels in CHO vs. CHO-5-HT_{1A}R cells

To demonstrate specificity of 5-HT liposomes with regards to cellular uptake and transfection, we employed a CHO cell line that stably expresses the 5-HT_{1A} receptor (Banerjee et al., 1993) (CHO-5-HT_{1A}R). The relative expression levels of 5-HT_{1A} receptors in CHO and CHO-5-HT_{1A}R cell membranes were determined by radioligand binding assay using the selective agonist [^{3}H] 8-OH-DPAT as described earlier (Kalipatnapu et al., 2004). Non-specific binding was determined by performing the assay in the presence of 10 μM of 5-HT. Our data showed that the agonist [3H]-8-OH-DPAT binding to membranes isolated from CHO-5-HT_{1A}R cells was ~14-fold more (498 fmoles/mg) in comparison to membranes isolated from normal CHO cells (36 fmoles/mg). Ligand binding assays conclusively show elevated levels of the 5-HT_{1A} receptor in CHO-5-HT_{1A}R cells when compared to CHO cells that served as a control. Receptor specific uptake of 5-HT targeting liposomes was initially examined by fluorescent uptake into CHO cells at 1:1:0.05 mole ratio (DHDEAB:Chol:5-HT-targeting DSPE-PEG (2000) with labeled DNA at charge ratio 3:1 N/P 1:3 charge ratio). Cells were incubated for 3 h, processed and analyzed by FACS. As seen from Table 1 S3 cell associated relative fluorescence intensity in CHO-5-HT_{1A}R cells was nearly ~2-fold in target CHO-5-HT_{1A}R cells compared to CHO cells, indicating the role of 5-HT receptor for uptake and internalization of the lipoplexes. Cell associated fluorescence using plasmid alone was negligible. Significantly, the cell associated fluorescence with CHO and CHO-5-HT_{1A}R cells with non-targeting lipid formulations was similar further supporting the role of 5-HT with formulations for enhanced cell association/uptake of these particles.

Uptake and localization of DNA in CHO and CHO-5-HT_{1A}R cells was also evaluated by confocal microscopy by treating cells with targeting and non-targeting lipoplexes. It is clearly evident that cell-associated particulate fluorescence with the targeting lipids 5-HT-DSPE-PEG (2000) was higher compared to the non-targeting lipid in CHO-5-HT_{1A}R cells, Fig. 2. An interesting observation is that particulate fluorescence was seen extensively in the cytoplasm and occasionally in the nucleus. The potential consequences of increased nuclear localization observed in CHO-5-HT_{1A}R cells may possibly lead to higher reporter gene expression after nuclear transcription. The fluorescence intensity observed with both CHO and CHO-5-HT_{1A}R cells treated with non-targeting liposomes DSPE-PEG (2000)–Mal was similar and lower than that observed with 5-HT conjugated lipid. These results further confirm the observations made by FACS that 5-HT conjugated liposomes causes specific uptake into CHO-5-HT_{1A}R cells.

3.4. Targeted transfection

Having characterized the potential for binding DNA and selective uptake into CHO-5-HT_{1A}R cells, stably expressing the 5-HT receptors, we evaluated the transfection efficiencies of targeting and non-targeting lipid formulations. Transient transfection assays were initially performed in a pair of cell lines i.e. CHO and CHO-5-HT_{1A}R using plasmid pCMVβ-gal:lipid at charge ratios ranging from 1:1 to 9:1 Lipid/DNA. Transfection mediated by targeting liposomes exhibited 3.5-fold higher reporter gene activity with targeting liposomes in CHO-5-HT_{1A}R compared to preparations of non-targeting lipids DSPE-PEG (2000)–Mal or DSPE-PEG (2000)–COOH, Fig. 3. Similar comparison of transfection efficiency of liposomes on normal CHO cells showed a 2-fold higher reporter gene activity.

This data indicated that the presence of 5-HT conjugated lipids at 5 mol% not only enhances transfection compared to non-targeting lipid but also enhances the β-galactosidase activity in a manner reflecting the density of 5-HT receptors in CHO-5-HT_{1A}R cells. As shown earlier, the receptor density, as determined by radioligand-binding assay, in CHO-5-HT_{1A}R cells, was ~14 times higher than CHO cells indicating that the presence of 5-HT on liposomes has a specific advantage. Specific receptor–ligand interactions and efficient internalization of the complexes into CHO-5-HT_{1A}R cells within the cytoplasm and the nucleus, as observed by confocal microscopy, possibly led to the observed increase in reporter gene expression.

Liposomal uptake is considered to be a critical rate-limiting step and has strong influence on the overall transfection efficiency. Many strategies including receptor mediated endocytosis (Banerjee et al., 1999), magnetofection (Huth et al., 2004), ligands for extra cellular matrices (Remy et al., 1995) were designed to increase the endosomal uptake of the lipid:DNA complexes. FACS

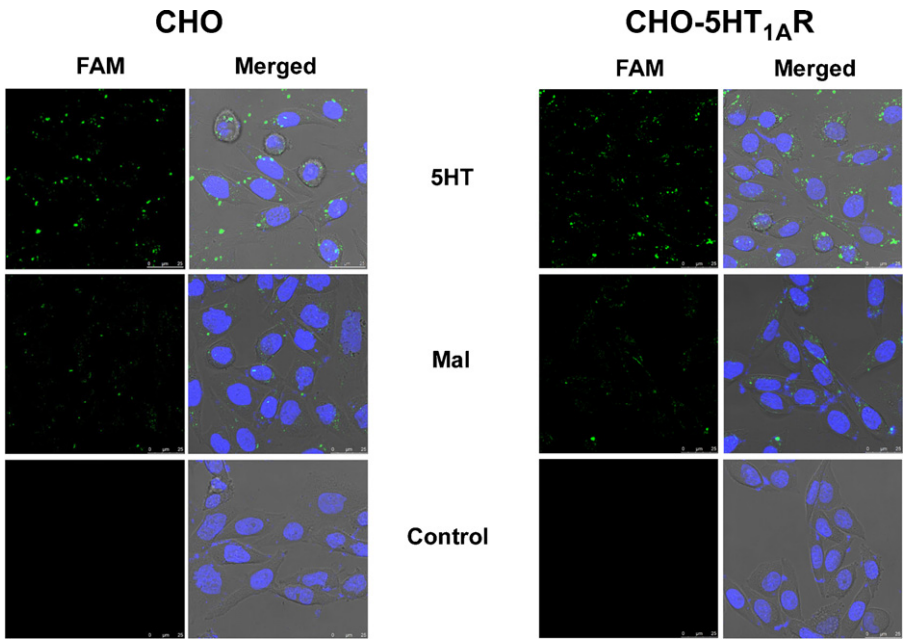


Fig. 2. Uptake of transfection-competent complexes into CHO-5-HT$_{1A}$R and CHO cells followed by confocal microscopy: FAM-labeled 18-mer DNA duplex was incubated with targeting lipid (5-HT, top), non-targeting lipid (Mal, middle) for 3 h and counterstained with Hoechst 33258. Relative uptake of labeled duplex DNA mediated by the targeting lipid and non-targeting lipid was compared with control DNA duplex (bottom). In each panel, 5 slices of 0.5 μm were combined. Merged panels depict nuclear staining with Hoechst 33258.

and confocal study of uptake of liposomes with and without 5-HT clearly indicated enhanced uptake (by approximately 2-fold) with 5-HT conjugated liposomes. The presence of 5-HT on liposomes has a positive effect on uptake and thereby on the transfection efficiency. Increasing the amount of DSPE-PEG (2000)–5-HT from 5 mole% to 10 mole% resulted in decreased transfection efficiency (data not shown).

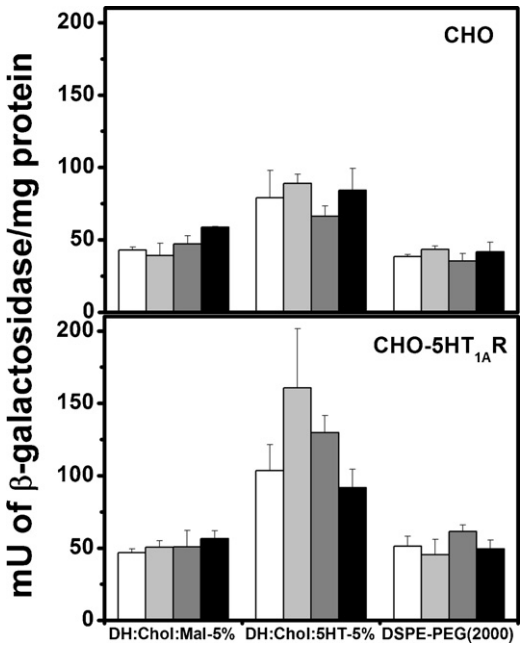


Fig. 3. Transient transfection in CHO-5-HT$_{1A}$R cells. Plasmid DNA pCMVβ-gal was complexed with targeting (DHDEAB:Chol:5-HT-5%) and non-targeting liposome formulations (DHDEAB:Chol:Mal-5% or DHDEAB:Chol:DSPE-PEG-(2000)) at varying charge ratios 1:1 (white), 2:1 (light gray), 3:1 (dark gray), 9:1 (black). β-Galactosidase activity was normalized per mg protein.

Transient transfection was also carried out in IMR32, a human neuroblastoma cell line and hepatocellular carcinoma cells, HepG2. The receptor density of IMR32 cells is reported to be similar to human or rat brain (Pranzatelli and Balletti, 1992). The involvement of serotonin in tumor growth of hepatocellular carcinoma was recently demonstrated (Soll et al., 2010). The enhancement in transfection efficiencies was more dramatic i.e. >20-fold higher with the targeting liposome than the non-targeting control DHDEAB:Chol:Mal that displayed very low activity at all charge ratios, Fig. 4. Activity with the targeting liposomes at 5 mole% 5-HT-conjugated lipid was quite significant at the charge ratios tested. In the case of HepG2, Fig. 5, maximal activity was observed at 1:1 Lipid/DNA, with the targeting lipid. This was ~4 fold greater when compared to a non-targeting control liposomal formulation. It may be possible, as seen from this study, that the cationic lipid formulation reconstituted with a targeting phospholipid may have mediated efficient uptake via the serotonin receptor.

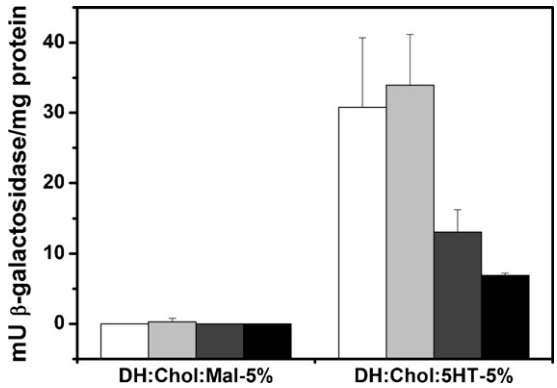


Fig. 4. Transient transfection in IMR32 cells. Plasmid DNA pCMVβ-gal was complexed with targeting (DHDEAB:Chol:5-HT-5%) and non-targeting liposome formulation DHDEAB:Chol:Mal-5% and DSPE-PEG(2000) at varying charge ratios 1:1 (white), 2:1 (light gray), 3:1 (dark gray), 9:1 (black). β-Galactosidase activity was normalized.

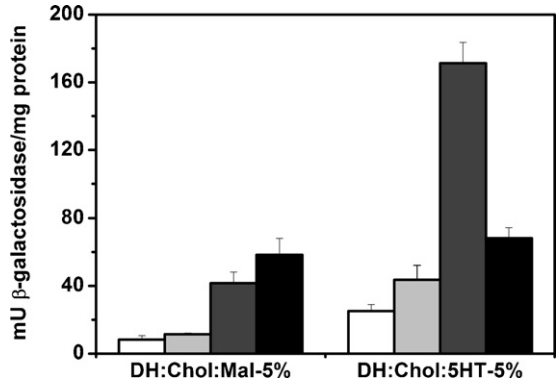


Fig. 5. Transient transfection in HepG2 cells. Plasmid DNA pCMVβ-gal was complexed with targeting (DHDEAB:Chol:5-HT-5%) and non-targeting liposome formulation DHDEAB:Chol:Mal-5% at varying charge ratios 0.1:1 (white), 0.5:1 (light gray), 1:1 (dark gray), 3:1 (black). β-Galactosidase activity was normalized.

To examine if the targeting liposomes have any effect on the cells, we assessed its through an MTT assay in CHO and CHO-5-HT_{1A}R, Fig. 6a. Lipids exhibited limited toxicity (<20% cell death) at charge ratio of 3:1 N/P. At 9:1 charge ratio ~60% of the cells were viable. The MTT assay was also performed in HepG2 and IMR32 (Fig. 6b). In HepG2, at charge ratios 3:1 N/P, the viability is greater than 90% while it is more than 70% for IMR 32 cells at the corresponding charge ratio of targeting liposomes. At higher charge ratios i.e. at 9:1, the viability of IMR32 and HepG2 cells was found to be ~70%.

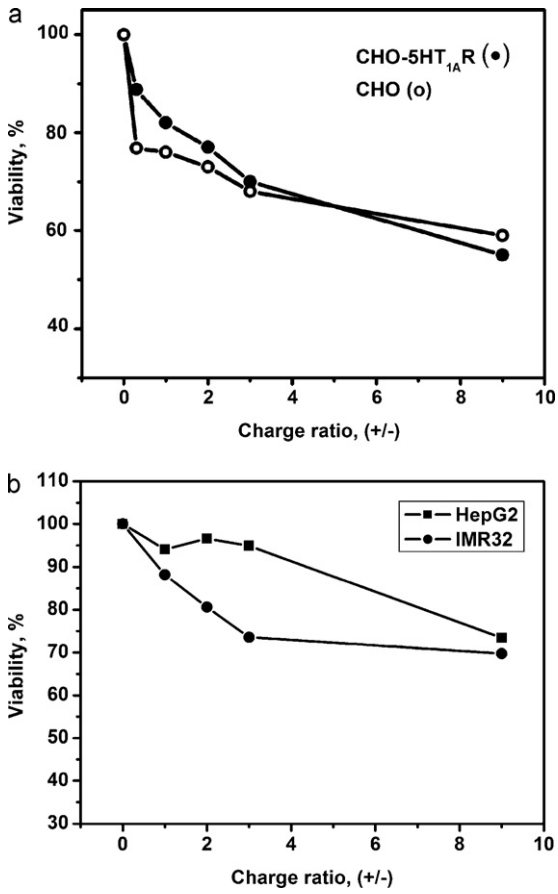


Fig. 6. MTT assay: determination of cell viability upon treatment of cells with lipoplexes prepared with the targeting liposomes formulated with DHDEAB:Chol:5-HT at 5 mol% at the indicated charge ratios in (a) CHO (open circle) and CHO-5-HT_{1A}R (solid circle) and (b) HepG2 (solid square) and IMR32 (solid circle) cells. Percent viability represents an average of an experiment performed in triplicate.

Success of therapies based on nucleic acids as drugs critically depend on the delivery efficiency. Approaches to enhance delivery of lipoplexes to specific cell types employ cell specific ligands as targeting molecules. A number of small molecules such as folic acid (Müller and Schibli, 2011), transferrin (Cardoso et al., 2009), haloperidol (Mukherjee et al., 2005), hyaluronic acid (Park et al., 2010), short peptides (Mäe et al., 2009) have been successfully used for transfection. The strategy of using antibodies has also been popular for drug and nucleic acid delivery (Chen et al., 2010). These strategies rely on abundance of the specific receptors for these ligands on tumor cells and occurrence of efficient receptor mediated endocytosis of these ligand–receptor complexes. 5-HT, an important neurotransmitter, has not been used as a ligand for targeting cells that specifically harbour 5-HT receptors. 5-HT has high affinity for 5-HT receptors (Kalipatnapu et al., 2004). 5-HT a derivative of tryptophan has a simple structure and the structural studies revealed that conjugation of amine of 5-HT does not compromise its properties. A large number of central and peripheral neural tissues and non-neural tissue express 5-HT receptors, thus providing an opportunity to use 5-HT as a ligand to deliver drugs into these cells. Interestingly, it has been previously reported that $serotonin_{1A}$ receptor levels in CHO cells could increase upon induction of stress (Singh et al., 1996). In addition, upregulation of hippocampal $serotonin_{1A}$ receptors has recently been shown in patients with amnesic mild cognitive impairment (aMCI) using positron emission tomograpy (PET) (Truchot et al., 2007). Our present approach of using serotonin as a ligand for targeted delivery could be relevant in such diseased conditions. 5-HT acts as a growth factor for several types of cancerous and non-cancerous cells (Soll et al., 2010). 5-HT antagonists were implicated in the progression of several kinds of carcinomas including lung, prostate and colon (Siddiqui et al., 2005). Fanburg and Lee (1997) demonstrated that 5-HT induces proliferation in smooth muscle cells and that serotonin plays a role through the signalling pathways through the serotonin receptor. A 1998 study demonstrated the involvement of serotonin and its receptor subtype in the induction of hepatocyte DNA synthesis, where increased DNA synthesis was observed in the presence of serotonin possibly dependent on the serotonin receptor on hepatocytes, which was considered crucial for liver regeneration (Balasubramanian and Paulose, 1998). The role of 5-HT in liver regeneration has also been investigated due to its contribution to the biology of hepatocellular carcinoma (HCC) (Soll et al., 2010).

We have used a conventional design of liposomes where attachment of 5-HT to DSPE-PEG (2000)–COOH provides the advantage of soft chemistry to conjugate the ligand and where the presence of PEG may provide protective advantage to the lipoplexes. Initially we found that both targeting and non-targeting lipids, bind plasmid DNA equally well. To demonstrate the specific role of 5-HT for plasmid delivery we have used a cell line CHO-5-HT_{1A}R cells that stably expresses the 5-HT_{1A} receptor. These cells have ~14 times higher receptor density than the normal CHO cells. Targeting 5-HT liposomes have distinct advantage when tested with the appropriate cell lines as assessed by flow cytometry and confocal microscopy. The 2-fold enhancement in cell associated liposomes has translated into higher transfection ~3.5 fold with CHO-5-HT_{1A}R cells when compared to CHO. With a neuroblastoma cell line IMR32, and hepatocellular carcinoma HepG2, cells, the targeting liposomes have remarkable enhancement of transfection. In our opinion this is the first study reporting the synthesis and use of 5-HT as a unique lipid conjugate for DNA delivery through specific targeting of cells that express different subtypes of receptors. Moreover, the targeting lipid formulations were found to be safe and non-toxic. We also propose that the formulation can be used to target serotonin receptors, observed in certain pathological states

particularly prostate cancer and glioma for drug or nucleic acid delivery.

4. Conclusion

Our studies demonstrate for the first time that lipids conjugated with 5-HT have the ability to deliver plasmid DNA to CHO-5-HT_{1A}R, IMR32 and HepG2 cells. The serotonin receptor route represents a new strategy to deliver therapeutic nucleic acids into cells specifically through the 5-HT moiety presented in a unique formulation with minimal toxicity.

Acknowledgements

We gratefully acknowledge funding from a CSIR grant NWP0035. The authors wish to acknowledge Dr. Anant Patel for help with NMR spectroscopy, Srinath Govindarajan and Sivakumar Jeyarajan for help with the experiments. Nandini Rangarajan for help with the confocal analysis and Shashi Singh for the TEM images.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.08.004.

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