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

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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,

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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