In Vitro Characterization of a Liposomal Formulation of Celecoxib Containing 1,2-Distearoyl-*sn*-Glycero-3-Phosphocholine, Cholesterol, and Polyethylene Glycol and its Functional Effects Against Colorectal Cancer Cell Lines

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ABSTRACT: Nanosized liposomal drug delivery systems are well suited for selective drug delivery at tumor sites. Celecoxib (CLX) is a highly hydrophobic cyclooxygenase-2 inhibitor that can reduce the incidence of colorectal polyps; however, the adverse cardiovascular effects limit its applicability. Here, we report a liposomal formulation of CLX using 1,2-*Distearoyl-sn-glycero-3-phosphocholine,* cholesterol, and polyethylene glycol. Encapsulation efficiency of the drug was greater than 70%; the release was slow and sustained with only 12%–20% of CLX released in the first 12 h. Flow cytometry and confocal microscopy studies using the colon cancer cell lines HCT-116 and SW620 showed significantly higher cellular association and internalization of the liposomes after incubation for 6 h when compared with 30 min. The liposomes did not colocalize with transferrin, but had a punctuate appearance, indicating vesicular localization. Cell proliferation was inhibited by 95% and 78%, respectively, in SW620 and HT29 cells after incubation with 600 μM liposomal CLX for 72 h. Moreover, cellular motility, as shown by a scratch wound healing assay, was also significantly ($p = 0.006$) inhibited when SW620 cells were incubated with 400 μM liposomal CLX. This is the first report of the successful encapsulation of CLX in a long-circulating liposomal formulation that could be effective against colorectal cancer. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:3666–3677, 2013

Keywords: liposome; celecoxib; colon; cancer; drug delivery systems; nanoparticles; pegylation

INTRODUCTION

Epidemiologic studies show that the use of aspirin and non-aspirin nonsteroidal anti-inflammatory drugs (NSAIDs) results in reduced risk of colon cancer.[1,2] Histological examination of patient samples suggests that cyclooxygenase-2 (COX-2) overexpression is a frequent event in carcinogenesis and is associated with poor prognosis[3]; therefore, at least part of the antitumor activity of NSAIDs stems from the inhibition of COX-2.

Celecoxib (CLX) (4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide) is a first-generation selective COX-2 inhibitor that serves as a chemopreventive agent in a variety of cancers including colorectal cancer.[4,5] COX-2-dependent effects of CLX can be summarized as induction of apoptosis, inhibition of angiogenesis, inhibition of invasiveness, modulation of inflammation, and immune suppression.[6] There are also COX-2-independent effects including inhibition of cell cycle progression, induction of apoptosis, inhibition of angiogenesis,[7] and reduction of membrane fluidity and metastatic potential of cancer cells in cell culture models.[8] The antiproliferative effect of CLX is unique in its family of compounds, which is one of the reasons why CLX is the only coxib that is approved for adjuvant treatment of patients with familial adenomatous polyposis.[9] Owing to its wide tissue distribution[10] and 97% plasma protein binding,[11] the recommended oral dose of CLX is high, which in turn raises concerns about serious possible side effects.[12] Therefore, there is growing interest in developing delivery systems for CLX at variety of compositions, sizes, and physicochemical properties.[13–16]

Liposomes for systemic applications can be designed to be small (100–200 nm) so that they can escape their uptake by the phagocytes, long circulating by the inclusion of polyethylene glycol (PEG), and stable by the inclusion of lipids with high melting temperature (T_m) and cholesterol. The leaky vasculature of the tumor environment and low lymphatic drainage in most solid tumors allows for the extravasation of the liposomes and their accumulation at the tumor site [enhanced permeability and retention (EPR) effect].[17] Small molecular weight drugs such as CLX, which usually have a wide tissue distribution and low tumor selectivity,[17] are good candidates for liposomal delivery exploiting the EPR effect. Contrary to the earlier liposomal formulations that had limited circulation time,[18] surface modification with hydrophilic PEG chains was shown to considerably enhance the circulation half-life of liposomes.[19]

In the present study, the highly hydrophobic-selective COX-2 inhibitor drug CLX was passively entrapped in nanosized large

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unilamellar vesicles (LUVs) composed of 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol, and PEG. The effects of variable cholesterol and PEG contents were analyzed in terms of particle size, drug loading, and *in vitro* drug release. One formulation was further tested in colorectal cancer cell lines with regard to qualitative and quantitative analyses of cellular binding and internalization, for inhibition of cellular proliferation and cell motility in a two-dimensional (2D) model system.

MATERIALS AND METHODS

Materials

Celecoxib was obtained from Ranbaxy Laboratories Limited (Mumbai, India). DSPC (18:0 PC), cholesterol (ovine wool, >98%), and mPEG(2000)–DSPE {1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N -[methoxy (polyethylene glycol)-2000]}were purchased from Avanti Polar Lipids (Alabaster, Alaska). SP-DiOC$_{18}$(3) [sodium salt of 3,3′-dioctadecyl-5,5′-di(4-sulfophenyl)oxacarbocyanine] was kindly provided by Dr. Ihsan Gursel from the Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey. Lissamine™ Rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rhodamine DHPE), and human Transferrin–AlexaFluor680 conjugate (Tr–AF680) were purchased from Invitrogen (Carlsbaad, California). Chloroform and methanol were obtained from Merck (Munchen, Germany). MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Invitrogen. Ultrafiltration device (VivaSpin2) with molecular weight cutoff (MWCO) 300 kDa membrane was purchased from Sartorius (Goettingen, Germany).

Preparation of CLX-Loaded PEGylated LUVs

Multilamellar vesicles (MLVs) were first prepared according to thin lipid film hydration method,[20] as described previously.[21] Briefly, DSPC, cholesterol, and CLX were dissolved in chloroform and mixed in 10:0, 10:1, and 5:1 ratios of DSPC–cholesterol and 18 mol % CLX in round-bottom polypropylene tubes. The lipid films were kept overnight under vacuum at 100 mbar to remove chloroform, and then flushed with argon and stored at 4°C. For hydration, the films were incubated in 1 mL phosphate-buffered saline (PBS) (0.1 M, pH 7.4), heated at 70°C and vortex mixed in 2 min cycles for a total duration of 1 h. Tubes were sonicated in a bath-type sonicator at 70°C for 15 min and allowed to reanneal at room temperature for at least 2 h. The MLVs were then subjected to 10 freeze–thaw cycles using liquid nitrogen to freeze and a 55°C water bath to thaw the samples. LUVs were obtained by extrusion through Whatman Nuclepore track-etched PC membranes with defined pore sizes (Whatman, Dassel, Germany). Extrusion was performed at 70°C–75°C by passing liposome suspensions five times through 800 nm, five times through 400 nm, and 15 times through 100 nm membranes using a mini-extruder set (Avanti Polar Lipids). The resulting clear suspensions were subjected to size-exclusion chromatography using Sephadex-G75 (GE Healthcare, Uppsala, Sweden) to separate unentrapped drug from LUVs. To prepare PEGylated liposomes, mPEG(2000)–DSPE molecules were incorporated to liposomes via the postinsertion method [22] at 0.5% and 2% of the DSPC content. Briefly, mPEG(2000)–DSPE lipid films were hydrated in PBS above the critical micelle concentration (>20 mM) at 60°C for 60 min. The micelles were then incubated with freshly extruded LUVs (prepared as described above) at 60°C for 1 h with intermittent mixing. The liposomes were then subjected to size-exclusion chromatography using Sephadex-G75 to separate unentrapped drug from LUVs. Empty liposomes were prepared in the same way as above, only with the exclusion of CLX. For cell culture studies, all liposomes were filtered through 0.45 μm Puradisc Polyether sulfone (PES) filters (Whatman). Sterility of the liposomes was ensured with the addition of antibiotics to the culture medium.

Quantification of CLX

After completely drying under vacuum, the liposome samples were dissolved in chloroform and analyzed by UV spectrophotometry at 260 nm. The CLX concentration of samples was calculated from a previously constructed CLX calibration curve (range: 10 –100 μg/mL).

To determine the CLX in the release medium, a modified HPLC method was used.[23] The samples were dried completely under vacuum, and then redissolved in pure methanol. A Shimadzu HPLC equipment (Shimadzu, Tokyo, Japan) and Inertsil ODS-3 C18 column (5 μm × 250 mm × 4.6 mm, Thermo Scientific, Rockford, Illinois) was used under ambient conditions with 85:15 (v/v) methanol–water as mobile phase at a flow rate of 0.8 mL/min. All samples were filtered through 0.45 μm filters before injection. Detection was performed at 254 and 260 nm wavelengths. The amount of CLX was calculated from previously constructed calibration curve in methanol.

Quantification of Phospholipids (DSPC)

1,2-Distearoyl-*sn*-glycero-3-phosphocholine was quantified by UV–visible spectrophotometry using the Stewart method.[24]

Characterization of Liposomes

Particle Size Analysis

The average hydrodynamic diameters of liposomes were determined by laser diffraction using Zetasizer after incubation at 25°C for 1 min (Nano ZS90, Malvern Instruments Malvern, Worcestershire, UK; METU Central Laboratory).

Morphological Characterization of Liposomes by Transmission Electron Microscopy

Liposomes were prepared for transmission electron microscopy (TEM) analysis by dilution (1:50) in PBS followed by negative staining with 2% uranyl acetate on 400 mesh formwar-coated copper grids. TEM images were obtained at 80 kV using a JEOL JEM 2100F microscope (JEOL, Tokyo, Japan; METU Central Laboratory).

Drug Encapsulation Efficiency and Percent Drug Loading

Aliquots (50 or 100 μL) of liposomes were dried completely under vacuum using HETO spin-vac system (HETO, Allerod, Denmark) and redissolved in chloroform by vortex mixing to disrupt the liposomes. CLX amount was determined as described above.

The drug encapsulation efficiency (EE) was calculated as:

$$\mathrm{EE}(\%) = \frac{\text{mg CLX in liposome}}{\text{mg CLX initially added}} \times 100$$