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Lipid rafts-mediated endocytosis and physiology-based cell membrane traffic models of doxorubicin liposomes

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ABSTRACT

The clathrin-mediated endocytosis is likely a major mechanism of liposomes' internalization. A kinetic approach was used to assess the internalization mechanism of doxorubicin (Dox) loaded cationic liposomes and to establish physiology-based cell membrane traffic mathematic models. Lipid rafts-mediated endocytosis, including dynamin-dependent or -independent endocytosis of noncaveolar structure, was a dominant process. The mathematic models divided Dox loaded liposomes binding lipid rafts (*B*) into saturable binding (*SB*) and nonsaturable binding (*NSB*) followed by energy-driven endocytosis. The intracellular trafficking demonstrated early endosome-late endosome-lysosome or early/late endosome-cytoplasm-nucleus pathways. The three properties of liposome structures, *i.e.*, cationic lipid, fusogenic lipid, and pegylation, were investigated to compare their contributions to cell membrane and intracellular traffic. The results revealed great contribution of cationic lipid DOTAP and fusogenic lipid DOPE to cell membrane binding and internalization. The valid Dox in the nuclei of HepG2 and A375 cells treated with cationic liposomes containing 40 mol% of DOPE were 1.2-fold and 1.5-fold higher than that in the nuclei of HepG2 and A375 cells treated with liposomes containing 20 mol% of DOPE, respectively, suggesting the dependence of cell type. This tendency was proportional to the increase of cell-associated total liposomal Dox. The mathematic models would be useful to predict intracellular trafficking of liposomal Dox.

1. Introduction

Liposome, the drug deliver, may help small and large molecules to target the tumor tissue. However, the design of effective liposomes of anti-tumor drugs and gene therapeutics remains a major obstacle [1]. The liposomes comprising 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-DSPE) and cholesterol have shown promising utility [2,3], but the mechanisms of their cell process including cell membrane binding, internalization and intracellular trafficking remain unclear.

In the nonsaturable and/or saturable manners liposomes bind with tumor cell membrane [4]. An optimal nonsaturable binding requires calcium ions and the components on cell membrane are protease-sensitive [5], while a saturable binding is usually achieved by ligands/antibodies-linked liposomes targeting the membrane receptors or antigens [6]. The internalization mechanisms of liposomes include clathrin-mediated endocytosis, caveolae pathway and clathrin-/caveolae-independent endocytosis (*e.g.*, macropinocytosis, lipid rafts-dependent endocytosis) [7,8]. Clathrin-mediated endocytosis has been broadly studied as the major internalization mechanism of various nanomedicines' particle of ~150 nm in diameter [4], while lipid rafts are poorly understood. However, clathrin-mediated endocytosis is not always the leading pathway. Macropinocytosis may surpass clathrin-mediated endocytosis in some cases of cationic liposomes [9–11]. The endocytosed vesicle fuses with endosomes and moves to lysosomes where the liposomes may be degraded by the enzymes [12]. Some liposomes may escape from early endosomes and enter into the cytosol, in which liposomes may further bind with mitochondrion or enter into nucleus [13,14]. It is well known that siRNA and DNA act in cytosol and nucleus, respectively. Therefore, desirable cell membrane binding, endocytosis and selective distribution in cytosol and nucleus are essential for the design of effective liposomes.

Cationic liposomes facilitate the binding and internalization and are popular deliveries of drugs and siRNAs [15]. Pegylation benefits the passive tumor targeting of liposomes *via* the enhancing permeability and retention (EPR) effect but inhibits liposomes binding with cell surface due to the stealth effect [16]. Due to siRNA-mediated gene silencing been completely abolished in cationic liposomes containing 5 mol%, but not 1–2 mol%, of PEG-DSPE [17], the cationic liposomes containing 1 mol% of PEG-DSPE were adopted in current study to achieve surface

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pegylation [2]. The fusogenic lipid DOPE helps liposomes to efficiently enter into cytosol, but not endosomes, since it adopts a hexagonal inverted phase structure allowing liposome fusion with the endosomal membrane [18]. Due to the restrict effects of lipid structures, *i.e.*, cationic lipid, fusogenic lipid, and pegylation, on the ability of liposomes to target intracellular sites, the understanding of the quantitative relationships between these parameters and the determination of the kinetic processes of liposomes in intracellular compartments are critical for optimizing the formulation. The aim of this study is to assess whether clathrin-medicated endocytosis is the leading endocytic pathway and to establish the physiology-based pharmacokinetic (PBPK) models to reflect cell membrane trafficking events. Human epithelial carcinoma A375 cells and human hepatoma HepG2 cells were selected to avoid the uncertainty of single cell experiments. Doxorubicin hydrochloride (Dox), as the model drug in the study, targets nucleus, intercalates DNA and inhibits macromolecular biosynthesis [19]. Dox loaded cationic liposomes with various fractions of DOTAP (change surface charges), pegylation, and fusogenic lipid were studied. The trafficking mechanisms were examined. Based on previous studies, we further established the mathematic model to predict the effect of time and concentration on cell membrane binding and internalization of Dox loaded liposomes.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride (Dox) was obtained from Aladdin® Reagent (Shanghai, China). Didecyldimethylammonium bromide (DDAB), 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-DSPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesterol (CHOL) was purchased from Beijing Aoboxing Biotech Co., Ltd. (Beijng, China). Ammonium sulfate was purchased from Tianjin Fuchen Chemical Reagents Factory (Tianjin, China). Triton-X 100 was purchased from Beijing Probe Bioscience Co., Ltd. (Beijng, China). Wortmannin was purchased from Cayman Chemical Company. Chlorpromazine, genistein, methyl-β-cyclodextrin (MβCD) and Hoechst 33342 were purchased from Sigma-Aldrich. 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) was purchased from Roche. Lysotracker® Green was purchased from InVitrogen Corp. (Eugene, OR). Tween-80 was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). All the reagents and solvents were analytical or HPLC grade. All materials were used as received.

2.2. Cell culture

Human epithelial carcinoma A375 cells and human hepatoma HepG2 cells were purchased from Beijing Cancer Hospital (Beijing, China). Cells were cultured in DMEM media (Hyclone®, Thermo Fisher Scientific, Beijing, China), supplemented with 100 IU/mL penicillin, 100 IU/mL streptomycin and 10% fetal bovine serum (FBS), and maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Preparation of Dox loaded cationic liposomes

Liposomes were prepared by thin-film hydration method and then loaded with Dox by $(NH_4)_2SO_4$-gradient driven remote loading [20]. The liposomes comprised cationic lipid (DDAB or DOTAP), neutral lipid (DPPC or DOPE) and CHOL. DDAB and DOTAP contribute positive surface charge of liposomes. DOPE promotes the fusion of liposomes and cell/endosome membranes, while DPPC promotes more stable laminar structures [18,21,22]. PEG-DSPE was used in some formulations to achieve surface pegylation. Table 1 shows the formulation of various blank liposomes, for which the lipids were dissolved in chloroform, dried into thin film under rotary evaporation and further dried overnight under vacuum, then hydrated with 180 mM ammonium sulfate solution, and then passed through a polycarbonate membrane (100 nm pore size) 11 times with an extruder (Avanti, Alabaster, AL) to produce liposomes with a particle size less than 200 nm. Diafiltration by 0.9% NaCl (pH = 7.4) was used to replace the outside $(NH_4)_2SO_4$. Dox solution was mixed with the blank liposomes at a drug-to-lipid ratio of 1:10 (w/w) and incubated at 40 °C for 30 min. Insignificant amounts of Tween-80 were added in 'C40' to avoid precipitation. Dox loaded liposomes were purified on a Sephadex G50 column to remove free Dox. The entrapped Dox was dissolved in 0.5% Triton X-100 solution for fluorescence measurement by absorption at Ex/Em = 543/594 nm on a RF-5301PC Fluorescence Spectrometer (Shimadzu Corp., Kyoto, Japan). The fluorescence intensity was converted to the amount of Dox in liposomes by using standard curve of Dox. The particle size, polydispersity index (PDI) and zeta potential (ZP) of Dox liposomes were determined on a ZetaPALS instrument (Brookhaven Instruments Corp., Worcestershire, NY).

2.4. Cell membrane traffic

A375 and HepG2 cells were seeded in six-well plates to reach 1×10^6 cells per well (~90% full). After washing and replacing the medium with a suspension of Dox liposomes, the plates were incubated at 37 °C and 4 °C for 0–6 h. At predetermined time points, culture medium containing cell-surface unbound Dox liposomes was removed, the attached cells were washed three times with ice-cold phosphate-buffered saline (PBS), suspended in 0.5 mL of 0.5% Triton-X 100 PBS, incubated at 37 °C for 30 min, diluted to 1 mL with PBS to measure the fluorescence intensities (FI) and then the amount of cell-associated liposomal Dox was calculated. We have proved that the concentration of cell-associated liposomes in cells treated at 37 °C expressed the sum of cell membrane-bound and intracellular liposomes (*TC*), the concentration at 4 °C expressed the membrane-bound liposomes (*B*), and the difference between *TC* and *B* expressed the intracellular concentration (*I*) [2].

The time courses of cell-associated liposomal Dox were measured for 0–6 h at 5 μg/mL of initial extracellular Dox to determine the time to reach equilibrium binding (T_{eq}). The area-under-concentration-time curve (AUC) was calculated using the trapezoidal rule. The concentration-dependent accumulations of cell-associated liposomal Dox were measured at T_{eq} from 0.5 μg/mL to 20 μg/mL of initial extracellular Dox concentration to further examine tumor cells selectivity of various formulations.

2.5. Endocytic pathway study with inhibitors

A375 and HepG2 cells were treated with chlorpromazine (10 μg/mL) or genistein (200 μM) or wortmannin (0.8 μM) or MβCD (5 mM) in serum-free culture medium for 1 h at 37 °C, into which Dox loaded liposomes C40 and C40-1-B (final concentration 5 μg/mL) were then added to incubate for additional 2 h [23–25]. The cells were subsequently washed with ice-cold PBS and harvested for flow cytometric analysis (LSRFortessa, BD Biosciences, USA).

Table 1
Formulation of blank cationic liposomes.

Blank liposomes	DDAB	DOTAP	DPPC	DOPE	CHOL	PEG-DSPE
	(mol%)					
$C20_{(DDAB)}$	20	0	30	0	50	0
C20	0	20	30	0	50	0
C40[a]	0	40	20	0	37	0
C40-1-0	0	40	20	0	39	1
C40-1-A	0	40	0	20	39	1
C40-1-B	0	40	0	40	19	1

[a] C40 contains 3 mol% of Tween-80.