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Transporter occluded-state conformation-induced endocytosis: Amino acid transporter $ATB^{0,+}$-mediated tumor targeting of liposomes for docetaxel delivery for hepatocarcinoma therapy

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ABSTRACT

Rapidly proliferating tumor cells upregulate specific amino acid transporters, which hold great potential for tumor-selective drug delivery. Published reports have focused primarily on blocking these transporters as a means of starving the tumor cells of amino acids, but their potential in drug delivery remains understudied. In the present study, we developed liposomes functionalized with lysine and polyoxyethylene stearate conjugate (LPS) to interact with $ATB^{0,+}$, an amino acid transporter overexpressed in hepatocarcinoma and the liver cancer cell line HepG2. The LPS modified liposomes (LPS-Lips) were ~100 nm in size and exhibited high drug encapsulation efficiency as 94.7%. The uptake of LPS-Lips in HepG2 cells was dependent on Na^+ and Cl^-. Molecular dynamic simulation showed that a sustained occluded state of the transporter upon binding to co-transported ions was formed and LPS-Lips triggered the cellular internalization of liposomes. We loaded these LPS-Lips with docetaxel and evaluated the potential of $ATB^{0,+}$-mediated endocytosis of the drug-loaded LPS-Lips in HepG2 cells in vitro and in syngeneic mouse transplants in vivo. Compared with unmodified liposomes, which did not interact with $ATB^{0,+}$, LPS-Lips exhibited the ability to deliver docetaxel more efficiently into tumor cells with consequent greater antitumor efficacy and less systemic toxicity. These studies provide first evidences that $ATB^{0,+}$ can be used as a novel and effective target for drug delivery system in tumor cells using chemically modified liposomes for loading with chemotherapeutics and targeting them for the transporter-mediated endocytosis. As $ATB^{0,+}$ is highly upregulated in several cancers, this approach holds potential for tumor-selective delivery of drugs to treat these cancer types.

1. Introduction

Transporters belong to the class of integral membrane proteins localized on the cell surface and organelle membranes, which primarily contribute to the movement of ions and small molecules across biomembranes. When transporting substrates, transporters function via complex allosteric mechanisms as they bind their substrates at one side of the membrane in a specific conformation, undergo a conformational change through the occluded state and then release the substrates on the other side in a different conformation [1]. Transporters are classified into two major families, the ATP binding cassette (ABC) transporters and solute carriers (SLCs) [2]. SLCs contain facilitative transporters, which are energy-independent and equilibrative, and secondary active transporters (symporters and antiporters), which are energy-dependent and concentrative [3,4]. In many instances, SLCs play an important role in the uptake of nutrients; this is particular in rapidly proliferating cancer cells, which need increased nutrient flux to support the cell division [5–7]. Consistent with this, elevated expression of nutrient transporters is a typical characteristic of many malignant cancers; as such, these upregulated transporters could represent excellent targets not only for pharmacological blockade to reduce the supply of nutrients to cancer cells but also for tumor-selective transporter-mediated delivery of chemotherapeutics [8,9].

To date, several different transporters in the plasma membrane have been targeted for tumor-selective delivery of drugs via nanomaterials, including the glucose transporter GLUT1 (*SLC2A1*) [10–13], choline transporters (*SLC44* family members) [14], and the vitamin B6 (pyridoxine) transporter whose molecular identity remains unknown [15].

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For GLUT1, the surface of the nanoparticles has been modified with glucose, 2-deoxyglucose or dehydroascorbic acid, all of them being substrates for the transporter. A choline derivative with high affinity for the choline transporters has been utilized to target the nanoparticles selectively to these transporters. Similarly, vitamin B6 has been used to chemically modify the nanoparticles to target them to vitamin B6 transporter. Among these three transporters, GLUT1 and choline transporters are known to be upregulated in cancer cells, thus making them suitable for tumor-selective delivery of chemotherapeutic drugs. However, transporters represent a diverse group of proteins that differ in topology, substrate specificity as well as energy coupling mechanism [16]. Therefore, the innate nature of transporters, such as the conformation and energy changes during the mediation of cellular endocytosis needs to be taken into consideration. Moreover, the transporter activity induced by the exposure to the targeting nanoparticles (NPs) should be investigated.

In order to elucidate the individual properties of transporter-mediated endocytosis, amino acid transporter $ATB^{0,+}$ is selected as a targeting site of liver tumor in this study. $ATB^{0,+}$ (also known as *SLC6A14*) belongs to the SLC transporter and possesses a broad substrate specificity of 18 types of proteinogenic amino acids. As a secondary-active transporter, $ATB^{0,+}$ is driven by electrochemical ion gradients. The energetics of $ATB^{0,+}$ indicate that the transporter has the capacity to concentrate its substrates inside the cells against 1000-fold concentration gradient unidirectionally, in sharp contrast to the nature of the facilitative transporters [17]. Due to the excessive requirement for essential amino acids, $ATB^{0,+}$ highly expresses in tumor cells to support their rapid growth, while only maintains a physiological level in normal cells. [18–20].

In this study, we synthesized three targeting conjugates by covalently linking polyoxyethylene stearate to glycine (a neutral amino acid), aspartate (an acidic amino acid) and lysine (a basic amino acid). We found that the lysine-conjugated polyoxyethylene stearate (LPS) the most suitable for modification of the cell surface of liposomes to target them selectively to $ATB^{0,+}$. These liposomes also exhibited excellent encapsulation efficiency for the chemotherapeutic drug docetaxel. We were able to show that these LPS-modified and drug-loaded liposomes bound to *SLC6A14* on cancer cells, rendering the transporter to assume an occluded conformation state with the binding of the LPS-liposomes and the cotransported ions Na^+ and Cl^- and that the occluded state conformation triggered the transporter-mediated endocytosis of the drug-encapsulated liposomes in $ATB^{0,+}$-positive cancer cells. These results provide the proof-of-concept for exploiting $ATB^{0,+}$ for tumor-selective delivery of chemotherapeutic drugs in the form of nanomaterials.

2. Materials and methods

2.1. Materials

Docetaxel (purity, >99%) was purchased from Gabriel Power Biochemical Technology Co., Ltd. (China). Polyoxyethylene 40 stearate (PS), coumarin-6 and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). N-α-Carbobenzyloxy-L-Lysine (Cbz-Lys), N-Carbobenzyloxy-L-asparatae α-benzyl ester (Cbz-Asp-Obzl) and N-Carbobenzyloxy-glycine acid (Cbz-Gly) were from GL Biochem Co., Ltd. (China). Soybean phospholipids were purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Tianjin Bodi Chemical Co., Ltd. (China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was obtained from Zhejiang Pukang Pharm Co., Ltd. (China). 4-Dimethylaminopyridine (DMAP) was purchased from Shanghai Medpep Co., Ltd. (China). RPMI-1640 and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD, USA). Antibodies to poly-(ADP-ribose)-polymerase (PARP), caspase-3, caspase-8, caspase-9 and $ATB^{0,+}$ (*SLC6A14*) were obtained from BD Biosciences (San Diego, CA, USA). TRITC-labeled goat anti-rabbit antibody IgG was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). BCA protein assay kit was obtained from Pierce (Rockford, IL, USA). DiR was purchased from Fanbo Biochemicals (China). All chemicals and reagents applied in this work were HPLC grade.

2.2. Synthesis of amino acid-conjugated polyoxyethylene stearate

Amino acid-modified polyoxyethylene stearate with glycine, aspartate and lysine (GPS, APS and LPS) was prepared by conjugating the hydroxyl group of polyoxyethylene stearate (PS) with carboxyl group of Cbz-Gly, Cbz-Asp-Obzl and Cbz-Lys respectively in the presence of EDC and DMAP. Two-step reaction involved an esterification using EDC/DMAP as the catalysts and the removal of protecting group of Cbz and Obzl by Pd/C (10%) in H_2 at 30 °C. The resultant copolymers were purified by silica column chromatography. The structures of the copolymers were confirmed by ^1H NMR (Bruker, AV-400, Switzerland) with d-DMSO as a solvent.

2.3. Preparation and characterization of liposomes

Docetaxel-loaded liposomes (PS-Lips/DTX, GPS-Lips/DTX, APS-Lips/DTX and LPS-Lips/DTX) were prepared by the lipid film hydration and probe-ultrasonic method. A mixture of SPC:cholesterol:DTX:amino acid conjugate (120:30:3:12, by weight) was dissolved in 2 mL dichloromethane and dried into a film with a rotary evaporator followed by hydration with 3 mL of phosphate-buffered saline (PBS, pH 7.4). The crude liposomes were sonicated for 5 min at 400 W in an ice water bath and then passed through 0.22-μm filter membrane to remove the untrapped DTX. Similarly, the unmodified liposomes were prepared using the same procedure by replacing amino acid conjugate with PS. For the construction of C6- or DiR-loaded liposomes, the aforementioned lipid formulations, together with C6 or DiR (SPC:cholesterol:C6/DiR:amino acid conjugate = 120:30:3/0.3:10, by weight), were first dissolved in dichloromethane and then evaporated to form a thin lipid film. Subsequently, the lipid film was hydrated with PBS (pH 7.4) and sonicated at 400 W in an ice water and then passed through 0.22-μm filter membrane to remove the untrapped C6 or DiR.

The structure of LPS-Lips was investigated by scanning electron microscopy (SEM, JEOL USA Inc., Peabody, MA). The particle size and zeta potential of liposomes were measured by dynamic light scattering (DLS) through a zeta potential/particle size analyzer (Malvern Instruments, Malvern, UK) at 25 °C.

EE and DL were analyzed by mini-column centrifugation method. The unloaded DTX was removed by the gel filtration over a Sephadex G-50 column (2.5 cm × 0.8 cm). Briefly, 0.2 mL of liposome suspension was added onto the column and centrifuged (400 ×*g*, 2 min) followed by rinsing with 0.2 mL of distilled water for several times. The eluant was collected and the amount of encapsulated DTX was determined by HPLC at 228 nm.

The colloidal stability of liposomes was estimated by measuring the time-dependent changes of the particle size. Briefly, 100 μL of liposomes were incubated with 1 mL rat plasma at 37 °C for 24 h. At various time points, 0.5 mL aliquots of the sample was withdrawn, centrifuged at 600 ×*g* for 10 min and measured by DLS.

The dialysis bag diffusion method was applied to investigate the in vitro release of DTX from different formulations. 1 mL of each DTX formulation was placed into a pretreated dialysis bag (MWCO: 8–10 kDa) and immersed in 50 mL of PBS 7.4 containing 0.5% Tween-80 (w/v) at 37 °C with gentle shaking. At predetermined time points, 100 μL aliquots of the release medium was withdrawn and replaced with an equivalent volume of fresh medium. The samples were centrifuged at 2000 ×*g* for 10 min. The released DTX was determined by HPLC at 228 nm.

2.4. Cell culture

Human hepatoma (HepG2), fibroblasts (L929) and murine hepatoma (H_{22}) cells were obtained from American Type Culture Collection