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Surface-modified liposomes for syndecan 2–targeted delivery of edelfosine

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ABSTRACT

Here, we report that the modification of liposome surfaces with AG73 peptides enhances delivery of the lipophilic anticancer drug, edelfosine, to tumor cells overexpressing the cell-surface receptor, syndecan 2. To test the effect of liposomal surface density of AG73 peptides on cellular uptake, we synthesized AG73 peptide-conjugated polyethylene glycol (MW 2000) lipid and incorporated it into fluorescence dye-labeled anionic liposomes with different ligand densities (1, 2, or 5 mol% of total lipids). Cellular uptake of AG73-peptide–modified liposomes gradually increased in proportion to the surface ligand density. The percentages of cells positive for AG73-modified, fluorescent-dye–labeled liposomes were 19.8 ± 2.0%, 23.1 ± 5.0%, and 99.2 ± 1.0%, for ligand mole percentages of 1, 2, and 5, respectively. The cell-targeting ability of AG73-modified liposomes was not significantly altered by the serum content of culture media. In keeping with the observed enhanced cellular uptake, AG73-peptide–modified liposomes entrapping edelfosine exhibited greater cancer cell-killing effects compared with unmodified liposomes. Following intravenous administration into tumor-bearing mice, AG73-peptide–modified liposomes showed 2.1-fold greater accumulation in tumors than unmodified liposomes. These results support the feasibility of using syndecan 2–directed liposomes for delivery of edelfosine.

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1. Introduction

Edelfosine, an alkylphospholipid analog anticancer agent, is known to induce apoptosis of cells in solid tumor through an endoplasmic reticulum stress response and interaction with mitochondria [1,2]. Although edelfosine has shown promising anticancer effects, the dose-dependent hemolysis of edelfosine and its toxicity toward normal tissues such as bone marrow cells [3] have limited its further use in clinical applications. Thus, tumor-targeted delivery would be helpful for reducing the side effects of edelfosine to normal tissues.
Although previous studies have described nanoparticle formulations of edelfosine that reduce toxicity [4,5], there has been little effort to formulate edelfosine in surface-modified liposomes with appropriate tumor-cell–targeting ligands.

Syndecans are transmembrane heparan sulfate proteoglycans that act as cell surface receptors for a variety of proteins, including growth factors [6], enzymes [7] and extracellular matrix proteins [8,9]. Recent studies have demonstrated that syndecans are overexpressed in malignant cells [7,10], where they promote cell adhesion, proliferation [7], and invasion [11]. The receptor, syndecan 2, is considered a prognostic marker of various malignant cells, including those of pancreatic adenocarcinomas [12], oral squamous cell carcinomas [13], and colon carcinomas [14]. It is known to be upregulated on the surface of cancer cells [10,15] and has been shown to play an important role in the progression of breast carcinoma [11,16].

Here, we tested the syndecan 2–targeting ability of peptide-ligand–modified liposomes for tumor-cell–targeted delivery of edelfosine. We demonstrated that syndecan 2–targeting liposomes with 5 mol% density of ligand enhanced the cellular delivery, tumor accumulation, and therapeutic effects of edelfosine.

2. Materials and methods

2.1. Synthesis of lipids tagged with a syndecan 2 ligand

The peptide sequence of the syndecan 2 ligand, AG73, is CCGRRKLQVQSIRT (Peptron Inc., Daejeon, South Korea). In this sequence, the two glycines and a cysteine act as a spacer and an attachment site for maleimide-functionalized lipids, respectively. AG73 peptide (11 μmol) was dissolved in 10 ml of dimethyl sulfoxide (DMSO), to which dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) was subsequently added at a final concentration of 1 mM. After adding 10 μmol of 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)]2000 (ammonium salt) (Avanti Lipids, Birmingham, AL, USA) dissolved in 10 ml of DMSO, the reaction mixture was stirred at 4 °C for 24 h, then dialyzed (MWCO 2000 Da; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) against 25% ethanol for 12 h and against deionized water for 24 h. The resulting product was confirmed by 1H NMR analysis.

2.2. Preparation of liposomes

Liposomes were prepared as described previously [17] with slight modification. For preparation of liposomes, 2 μmol each of egg L-α-phosphatidylcholine (Avanti Lipids), egg L-α-phosphatidyl-DL-glycerol (Avanti Lipids), cholesterol (Sigma-Aldrich), and 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)]2000 (PEG2K–DSPE, Avanti Lipids) or AG73–PEG2K–DSPE were mixed at a molar ratio of 2:2:2:0.3 in organic solvents. In some experiments, fluorescent liposomes were prepared by adding 0.2 μmol of 18:1–12:0 Square-685 phosphatidylcholine (Avanti Polar Lipids) or N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycerol-3-phosphoethanolamine (triethylammonium salt) (NBD-PE; Molecular Probes, Inc., Eugene, OR, USA) to the previously described lipid mixtures. Liposomes loaded with edelfosine (Tocris Bioscience, Bristol, UK) were prepared by adding 2 μmol of edelfosine to the previously described lipid mixtures. For comparison with edelfosine-loaded liposomes, 1-stearoyl-2-hydroxy-sn-glyce-ro-3-phosphocholine (Lyso PC; Avanti Polar Lipids)–loaded liposomes were prepared by substituting 2 μmol of Lyso PC for edelfosine. Organic solvents were removed from lipid mixtures using a rotary evaporator. The resulting thin films were hydrated with 1 ml of HEPES-buffered saline (pH 7.4) and vortexed. The resulting multilamellar vesicles were extruded three times through 0.2-μm polycarbonate membrane filters (Millipore Corp., Billerica, MA, USA) and then incubated at 4 °C before use.

2.3. Syndecan 2 expression on cancer cell surfaces

Flow cytometry was used to evaluate the expression of syndecan 2 on the cell surface. After harvesting, BT20 cells were incubated for 1 h at 4 °C in cold phosphate-buffered saline (PBS) containing 3% bovine serum albumin. Next, cells were washed and incubated for 1 h at 4 °C with an allop-hocyanocyan-conjugated rat anti-human syndecan 2 monoclonal antibody (R&D Systems, Inc., Minneapolis, MN, USA), diluted 1:50 in cold PBS containing 2% fetal bovine serum (FBS). Antibody-stained cells were then analyzed by flow cytometry using a BD FACS Calibur system equipped with Cell Quest Pro software (BD Bioscience, San Jose, CA, USA).

2.4. Cellular uptake

Cellular uptake of liposomes containing NBD-PE was assessed using fluorescence microscopy and flow cytometry. For fluorescence microscopy, BT20 cells were seeded onto 24-well plates at a density of 5 × 10⁴ cells/well. In some experiments, the medium was replaced with medium containing different concentrations of FBS (10%, 20%, or 50%) or with 100% FBS. After 24 h, cells were treated with 20 μl of fluorescent liposomes for 30 min, then washed twice with PBS and observed under a fluorescence microscope (Leica DM II; Leica, Wetzlar, Germany). Fluorescent-liposome–treated cells were harvested, washed three times with cold PBS containing 2% FBS, and analyzed by flow cytometry.

2.5. Animals

Five-week-old female Balb/c athymic nude mice, obtained from Orient Bio. Inc. (Seungham, Kyonggi-do, South Korea; approved animal experimental protocol number SNU-150609-2), were used for in vivo experiments. Animals were raised under standard pathogen-free conditions at the Animal Center for Pharmaceutical Research, Seoul National University. All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Seoul National University.

2.6. Anticancer activity

The anticancer activity of liposomes containing edelfosine or Lyso PC was tested using a Cell Counting Kit (CCK) assay. BT20
cells were seeded onto a 48-well plate at a density of $2 \times 10^4$ cells per well, and treated with liposomes containing 5 nM edelfosine or Lyso PC. After 24 h, cell viability was quantified using a CCK according to the protocol provided by the manufacturer (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Cell viability was expressed as a percentage of that measured in control groups.

2.7. In vivo molecular imaging

Molecular imaging was used to trace the distribution of PEG- and AG73-PEG–modified liposomes (PEG2K Lipo and AG73-PEG2K Lipo, respectively). Mice were subcutaneously inoculated in the dorsal right side with $4 \times 10^6$ BT20 cells. After tumors had grown to approximately 100–150 mm$^3$, 200 μl of Square-685 lipid-loaded fluorescent liposomes were intravenously injected. At 2 h post-dose, Square-685 fluorescence was imaged using an eXplore Optix system (Advanced Research Technologies Inc., Montreal, Canada). A 670-nm pulsed-laser diode was used to excite Square-685 molecules.

2.8. Statistics

Analysis of variance (ANOVA) with post hoc Student–Newman–Keuls test was used for statistical evaluation of experimental data. All statistical analyses were done using SigmaStat software (version 3.5; Systat Software, Richmond, CA, USA). A $P$-value <0.05 was considered statistically significant.

3. Results and discussion

3.1. Construction of AG73-PEG2K Lipo for syndecan 2 targeting

The premise of these studies is that syndecan 2 overexpressing cancer cells can be targeted by AG73-PEG2K Lipo through receptor-mediated cellular delivery, as shown in Fig. 1. To this end, we grafted the AG73 peptide, utilized as a ligand for the receptor, syndecan 2, onto liposomes as described in section 2; liposomes prepared without AG73 (PEG2K Lipo) were used as controls. There was no significant difference in particle sizes (Fig. 2A) or zeta potentials (Fig. 2B) between liposomes. AG73 is a biologically active peptide originating from the cell-adhesion protein laminin $\alpha_1$ [18,19] that is known to promote cell attachment [20], angiogenesis [21], tumor growth, and metastasis [22] in various malignant cells through binding to syndecans. Yamada and colleagues [23] have utilized AG73 as a cell-adhesion ligand to modify hyaluronic acid hydrogels for tissue engineering, and Negishi and colleagues [24] have used ultrasound imaging of AG73-modified bubble liposomes for tumor diagnosis.

3.2. Cellular delivery of AG73-PEG2K Lipo with various densities of ligand

AG73 peptide tagging affected the efficiency of cellular uptake of liposomes in syndecan 2–expressing cancer cells. Surface expression of syndecan 2 on BT20 cancer cells was confirmed by flow cytometry (Fig. 3A). BT20 cells treated with AG73-PEG2K Lipo, labeled with the fluorescent dye, NBD-PE, to allow fluorescence monitoring, showed a gradual increase in cellular uptake efficiency as a function of AG73 content on the liposome surface (Fig. 3E,F,G), whereas PEG2K Lipo was not taken up by cells regardless of PEG2K content (Fig. 3B,C,D). AG73-PEG2K Lipo modified with 5 mol% of added ligand achieved the greatest cellular delivery (Figs. 3 and 4), with 99.2 ± 1.0% of cells treated with this preparation exhibiting fluorescence (Fig. 4C,D) compared with 19.8 ± 2.0% and 23.1 ± 5.0% of cells prepared with 1 mol% (Fig. 4A,D) and 2 mol% (Fig. 4B,D) of ligand. Ligand modification of PEGylated nanoparticles has been suggested as an effective solution to the so-called “PEG dilemma” [25], which reflects inhibition of cellular uptake of nanoparticles by the PEG stabilizer. As an example of the latter effect, PEGylated nanoparticles with 5 mol% PEG achieved a significantly higher concentration in blood, but lower cellular delivery of cargo, than PEG-unmodified nanoparticles after systemic injection [26]. The AG73 ligand modification was able to reverse the effects of PEG on cellular delivery. This ability of AG73 modification to effectively overcome the PEG dilemma is important because PEGylation serves valuable functions, providing a hydrophilic, flexible spacer between ligand molecules and liposomes to improve functionality of the ligand and protecting liposomes from interaction with biological fluids [27].
3.3. Cellular delivery of AG73-PEG<sub>2k</sub>Lipo in biological fluids

The cellular-uptake efficiency of AG73-PEG<sub>2k</sub>Lipo was not influenced by serum-containing conditions that mimic biological fluids (Fig. 5). Likewise, the cancer cell-targeting ability of AG73-PEG<sub>2k</sub>Lipo was similarly unaffected by increased serum conditions. Only the 100% serum condition slightly decreased cellular uptake of AG73-PEG<sub>2k</sub>Lipo (to 82.5% ± 5.0%). On the basis of these results, AG73 modification would not be expected to alter the pharmacokinetic profile of PEG<sub>2k</sub>Lipo. Indeed, several groups have reported that modification of liposomes with ligands targeting epithelial cell adhesion molecule [28], epidermal growth factor receptor [29], or Her2/neu [30] did not increase clearance of liposomes, but did increase tumor accumulation compared with unmodified liposomes.

3.4. Antitumor effect of edelfosine delivered by AG73-PEG<sub>2k</sub>Lipo

Edelfosine-encapsulated AG73-PEG<sub>2k</sub>Lipo showed enhanced cancer cell-killing effects (Fig. 6). CCK assays revealed that treatment with edelfosine-loaded liposomes with a PEG<sub>2k</sub> or AG73-PEG<sub>2k</sub> content of 1 or 2 mol% had no significant effect on BT20 cell viability. In contrast, treatment with edelfosine-loaded liposomes containing 5 mol% PEG<sub>2k</sub> or AG73-PEG<sub>2k</sub> content exerted tumor cell-killing effects, reducing cell viability to 64.2% ± 2.5% and 46.1% ± 9.2%, respectively. Edelfosine acts as an anticancer agent by inducing apoptosis in malignant cells, including breast cell carcinoma [3,31]. However, severe side effects of edelfosine have been observed after systemic administration [32]. Recent reports have reported that edelfosine can be formulated into lipid bilayers of liposomes owing to its lipid-like structure [5,33,34]. Liposomal edelfosine formulations have been shown to prevent against the toxicity of free edelfosine, demonstrating much higher safety than the free drug [5] and an improved therapeutic index as a result of their enhanced toxicity toward resting cancer cells [34].

3.5. Tumor accumulation of AG73-PEG<sub>2k</sub>Lipo

Systemically injected AG73-PEG<sub>2k</sub>Lipo nanoparticles with a 5 mol% AG73-PEG<sub>2k</sub> content were mainly distributed to tumor tissues of mice xenografted with syndecan 2-overexpressing BT20 cells (Fig. 7A). The fluorescence intensity in tumor regions...
of AG73-PEG<sub>2K</sub>Lipo–treated mice was 2.1-fold higher than that of PEG<sub>2K</sub>Lipo–treated mice (Fig. 7B). PEG<sub>2K</sub>Lipo, lacking the AG73 ligand, also showed some distribution to tumor sites. Such distribution to tumors in the absence of a targeting ligand frequently occurs with nanoparticulate delivery systems owing to the enhanced retention and permeability effect [35]. However, tumor-targeting ligands are still needed for improved intracellular delivery to tumor cells [36]. Indeed, whereas PEG<sub>2K</sub>Lipo–treated mice showed higher distributions to organs other than tumor tissue, AG73-PEG<sub>2K</sub>Lipo nanoparticles were mainly distributed in tumor tissue (Fig. 7).

4. Conclusions

We synthesized AG73-peptide–conjugated PEG-lipid and formulated AG73-grafted liposomes. AG73-PEG<sub>2K</sub>Lipo nanoparticles exhibited ligand density-dependent, enhanced cellular delivery in syndecan 2–overexpressing cancer cells in vitro, and showed higher tumor accumulation than ligand-unmodified liposomes after systemic administration in vivo. In addition, edelfosine-loaded liposomes significantly decreased tumor cell viability. These results provide evidence that AG73-PEG<sub>2K</sub>Lipo could serve as a receptor-specific nanovehicle for delivery of edelfosine to syndecan 2–overexpressing cancer cells.

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Fig. 7 – In vivo distribution of AG73-PEG<sub>2K</sub>Lipo. Square-685–labeled PEG<sub>2K</sub>Lipo or AG73-PEG<sub>2K</sub>Lipo was intravenously injected into BT20 tumor-bearing mice. (A) After 2 h, the in vivo distribution of liposomes was visualized using a molecular imaging system. (B) Average photon counts at tumor sites were quantified. *P < 0.05 vs. the PEG<sub>2K</sub>Lipo group (Student’s t-test).

REFERENCES


