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Nanoassemblies containing a fluorouracil/zidovudine glyceryl prodrug with phospholipase A_2-triggered drug release for cancer treatment

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ABSTRACT

Secretory phospholipase A_2 ($sPLA_2$), which is overexpressed in many tumors, cleaves ester bonds at the *sn*-2 position of phospholipids. A PLA_2-sensitive amphiphilic prodrug, 1-O-octadecyl-2-(5-fluorouracil)-N-acetyl-3-zidovudine-phosphorylglycerol (OFZG), was synthesized and used to prepare nanoassemblies through the injection of a mixture of OFZG/cholesterol/Tween 80 (2:1:0.1, mol:mol:mol) into water. Cholesterol and Tween 80 was incorporated into the OFZG monolayers at the air/water interface to yield nanoassemblies. The resulting nanoassemblies exhibited a narrow size distribution with a mean size of 77.8 nm and were stable due to their high surface charges. The *in vitro* experiments showed that PLA_2 degraded OFZG. The nanoassemblies exhibited higher anticancer activity than the parent drug 5-fluorouracil (5-FU) in COLO205, HT-28, and HCT-116 cells. The intravenous (i.v.) administration of the nanoassemblies into mice resulted in the rapid elimination of OFZG from the circulation and its distribution mainly in the liver, lung, spleen, and kidney. After their injection into tumor-bearing mice, the nanoassemblies exhibited anticancer efficiency comparable to that of 5-FU, even though the nanoassemblies contained concentrations of only 1/10 of the molar amount of 5-FU. The lessons learned from the study and methods for the design of PLA_2-sensitive amphiphilic prodrugs are also discussed. Enzyme-sensitive amphiphilic combinatorial prodrugs and prodrug-loaded nanoassemblies may represent a new strategy for anticancer drug design.

1. Introduction

The severe side effects of anticancer agents generally result from their wide distribution *in vivo*. Nanoparticles penetrate into tumor tissues based on the enhanced permeability and retention (EPR) effect [1]. However, ordinary or naked nanoparticles do not achieve tumor targeting due to opsonization [2]. Long-circulating vehicles are coated with long-chain hydrophilic groups and achieve tumor targeting based on the EPR effect [3]. Lipid derivatives of poly(ethylene glycol) (PEG) become long-circulating materials through insertion into lipid bilayers or cores [4].

Prodrugs are defined as derivatives of drugs that are metabolized or activated in the body to release or generate the active drug at the site of action if possible. Certain specific enzymes are overexpressed in tumor tissues and thus may be targeted in the design of prodrugs [5]. For example, capecitabine is activated in a series of reactions catalyzed by hepatic carboxylesterase, by cytidine deaminase in the liver or tumor, and by the tumor-associated enzyme thymidine phosphorylase to yield 5-fluoruracil (5-FU) [6].

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of phospholipids at the *sn*-2 position to generate fatty acids and lysophospholipids. This enzyme plays key roles in many inter- and intracellular signal transport and pathological pathways in human physiology. PLA_2 is subdivided into families, including Ca^{2+}-dependent secretory PLA_2 ($sPLA_2$), cytosolic Ca^{2+}-sensitive PLA_2 ($cPLA_2$), and Ca^{2+}-independent PLA_2 ($iPLA_2$). $sPLA_2$ is released to the outer matrix of cells and is overexpressed in many tumors, including colon, large and small intestine, breast, prostate, liver, lung, gastric, pancreatic, and ovarian cancers. High levels of $sPLA_2$ are correlated with tumor neovascularization, growth enhancement, and metastasis [7–11]. The $sPLA_2$ content in tumor effusions is high, *i.e.*, more than fivefold higher than that in blood, where its content is very low. The $sPLA_2$ mRNA is highly expressed in cells from effusions but not in blood cells [8]. The level of $sPLA_2$ in the peripheral region of tumors is twofold higher than that found in the central region [12]. Therefore, $sPLA_2$ is a good target for the design of anticancer drugs.

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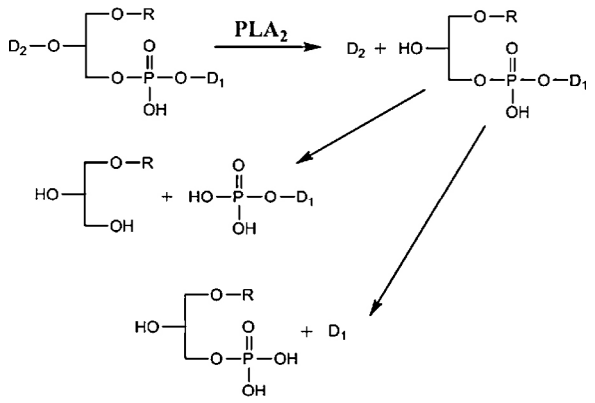

Fig. 1. Mechanism of $sPLA_2$-triggerred drug release.

The Andresen group has focused on the construction of a novel liposomal drug delivery system that takes advantage of the $sPLA_2$-triggered release of entrapped drugs [13–17]. An *sn*-1-ether-*sn*-2-ester phospholipid was synthesized and used to prepare liposomes loaded with anticancer drugs. Once the liposomes reached the tumor site, the *sn*-2 ester would undergo hydrolysis mediated by $sPLA_2$ to generate the *sn*-1-ether lysophospholipid, which is known as a potent membrane disruptor that can be used to kill cancer cells. The entrapped anticancer drugs are then released concomitantly due to dissociation of the liposomes. Thus, the cancer cells received a two-pronged attack from the lysophospholipids and drugs.

Combinatorial chemotherapy is widely applied to overcome the resistance of cancer cells to a single chemotherapeutic agent. However, the combinatorial agents do not necessarily enter a given cell simultaneously because each drug has a specific pharmacokinetic profile. We have designed a novel amphiphilic prodrug (Fig. 1) that combines two drugs in one molecule. The first drug (D_1) is conjugated at the *sn*-3-phosphoryl position of glycerol, and the second (D_2) is conjugated at the *sn*-2 position. Our aim was to prepare a nanoassembly that contains the prodrug targeted to tumor cells to achieve the release of both drugs (or active intermediates) simultaneously at the targeted sites. A phosphoryl group naturally occurs in the structure of phospholipid molecules. Therefore, an anticancer nucleoside analog is the optimal choice for D_1 because phosphoryl D_1 may be released and is more active than D_1 itself. A byproduct of PLA_2-triggered D_2 release may be *sn*-1-ether glyceryl, which can kill cancer cells through the mechanism described above. Therefore, cancer cells may be simultaneously attacked by three forces: ether-lysophospholipid, D_2, and phosphoryl D_1. In this study, the novel prodrug and nanoassemblies were prepared, and their behaviors *in vitro* and *in vivo* were subsequently explored.

2. Materials and methods

2.1. Materials

5-Fluorouracil (5-FU) was purchased from Shangdong Boyuan Pharmaceutical Co., Ltd., China. Zidovudine (AZT) was obtained from Zhang Jiang Desano Science and Technology Co. Ltd. (Shanghai, China). 1-O-octadecylglycerol (batyl alcohol) was purchased from TCI, Japan. Cholesteryl succinyl poly(ethylene glycol) 1500 (CHS-PEG_{1500}) was synthesized in our laboratory according to a previously published protocol [18]. Poloxamer 188 (P188, MW, ~8400, Shenyang Jiqi Pharmaceutical Co., Ltd., China) is a triblock copolymer with a hydrophobic middle block (poly(propylene oxide), PPO) and hydrophilic end blocks (poly(ethylene oxide), PEO); this copolymer is also written as PEO-PPO-PEO. Brij 58 (MW, ~1124, Sigma, USA) refers to polyoxyethylene (20) cetyl ether.

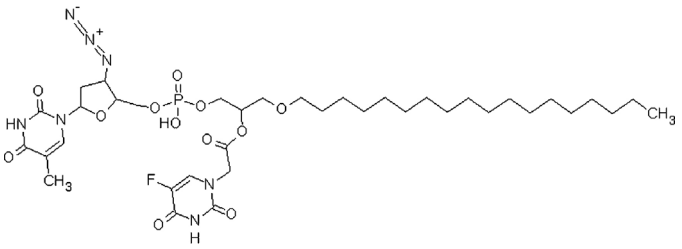

Fig. 2. Structure of OFZG.

Tween 80 (polysorbate 80, Shanghai Dazhong Pharmaceutical Co., Ltd., China) refers to polyoxyethylene (20) sorbitan monooleate. The organic solvents used in this study were of analytical grade, and all of the other chemicals were of reagent grade. Purified water was prepared with a Heal Force Super NW Water System (Shanghai Canrex Analytic Instrument Co. Ltd., China) and was used in all of the aqueous experiments. The ultraviolet (UV) spectra, infrared (IR) spectra, ^1H nuclear magnetic resonance (NMR) (400 MHz), and ^{13}C NMR (100 MHz) spectra were recorded on an ultraviolet-visible spectrophotometer (TU-1901, Beijing Purkinje General Instrument Co., Ltd., China), a Bio-Rad FTS-65A infrared ray (IR) spectrometer, and a JNM-ECA-400 NMR spectrometer, respectively. The ESI–MS were recorded on a Thermo LCQ Advantage mass spectrometer.

2.2. Animals

Female Kunming mice from the Laboratory Animal Center of the Beijing Institute of Radiation Medicine (BIRM) were used for the *in vivo* experiments. All of the animal handling and surgical procedures were conducted strictly according to the Guiding Principles for the Use of Laboratory Animals. This study was approved by the Animal Care Committee of BIRM. The mice were sacrificed to obtain the tissues. The mouse tissue homogenates used in the tissue distribution experiment were prepared in tissue/water (1:1, w/w). All of the studies were conducted in accordance with the Declaration of Helsinki.

2.3. Synthesis of the prodrug

The prodrug, which was denoted 1-O-octadecyl-2-(5-fluorouracil)-N-acetyl-3-zidovudine-phosphorylglycerol (OFZG, Fig. 2), was synthesized through sequential hydroxyl protection, esterification, de-protection, and phosphorylation (Fig. 3). The synthesis details are included below.

1-Carboxylmethyl-5-fluorouracil (**1**, $C_6H_5FN_2O_4$) was synthesized according to previous reports to obtain a powder at a yield of 70% [19,20]. 1-O-octadecyl-3-triphenylmethylglycerol (**2**, $C_{40}H_{58}O_3$) was synthesized according to previous reports [21]. Simply, 1-O-octadecylglycerol (3.40 g, 10 mmol), triphenylmethyl chloride (TrCl, 3.30 g, 12 mmol), and N,N′-dimethylaminopyridine (DMAP, 0.122 g, 1 mmol) reacted in a mixture of dichloromethane and pyridine at room temperature overnight followed by removal of solvent to yield a white viscous solid. The solid was then dissolved in DCM (50 ml) and washed twice with citric acid solution (0.05 M). The dried organic phase was evaporated to yield a white viscous solid. The solid was purified through silica gel column chromatography, eluted with petroleum ether:ethyl acetate (from 10:1 to 5:1, v/v), and dried to obtain compound **2** as a white wax at a yield of 87%.

1-O-octadecyl-2-(5-fluorouracil)-N-acetyl-3-triphenylmethylglycerol (**3**, $C_{46}H_{61}FN_2O_6$) was prepared through the following procedure. Compound **1** (1.11 g, 5.9 mmol), compound **2** (2.03 g, 3.48 mmol), DMAP (0.042 g. 0.59 mmol), and