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Rapamycin encapsulated in dual-responsive micelles for cancer therapy

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ABSTRACT

Rapamycin has been developed as a potential anticancer drug for treatment in rapamycin-sensitive cancer models, but its poor water solubility greatly hampers the application to cancer therapy. This study investigated the preparation, release profiles, uptake and *in vitro*/*in vivo* study of a dual-responsive micellar formulation of rapamycin. Rapamycin-loaded micelles (rapa-micelles) measured approximately ca. 150 nm with narrow size distribution and high stability in bovine serum albumin solution. It was shown that rapamycin could be loaded efficiently in mixed micelles up to a concentration of 1.8 mg/mL by a hot shock protocol. Rapamycin release kinetic studies demonstrated that this type of micellar system could be applied in physiological conditions under varied pH environments. Confocal and pH-topography imaging revealed a clear distribution of rapa-micelles, and visible intracellular pH changes which induced encapsulated rapamycin to be released and then induced autophagolysosome formation. *In vivo* tumor growth inhibition showed that rapa-micelles exhibited excellent antitumor activity and a high rate of apoptosis in HCT116 cancer cells. These results indicated that dual-responsive mixed micelles provided a suitable delivery system for the parenteral administration of drugs with poor water solubility, such as rapamycin, in cancer therapy.

1. Introduction

Rapamycin is a carboxylic lactone-lactam macrolide derived from the bacterium *Streptomyces hygroscopicus* [1]. Rapamycin was originally used as an antifungal agent [1] and was later developed as an approved immunosuppressant [2] and potential anticancer drug [3]. Preclinical data show inhibition of tumor growth in a number of cell lines, including lung [4], cervix [5], colon [6] and breast [7] cell carcinomas. In addition, rapamycin is the most commonly used chemical to induce autophagy. Autophagy is a lysosome-based pathway that describes the degradation and recycling of proteins and intracellular components for maintaining cellular homeostasis. During autophagy, cytoplasm sequestered and then formed double-membraned vesicles which is termed as autophagosomes. Autophagosomes then fuse with lysosomes, containing acid hydrolases, which is called an autophagolysosome [8]. However, autophagy also protects some cancer cells against anticancer treatments by blocking the apoptotic pathway [9]. For cancer therapy, autophagy is associated with functions primarily in tumor suppression by removing possibly growth factors and reduces chromosome instability. Rapamycin is one of the few mammalian Target of Rapamycin (mTOR) inhibitors that specifically target an autophagy-regulatory protein. Autophagy could be a therapeutic target for rapamycin treatment of cancer. A growing volume of evidence supports the hypothesis that mTOR is a kinase that functions as a master switch between catabolic and anabolic metabolism [10]. Consequently, the mTOR pathway determines whether cells—particularly tumor cells—will grow and proliferate. Inhibitor of mTOR, rapamycin, induces cell cycle arrest at the transition from G1-S phase [11,12]. Recent studies have reported a sensitivity of cancer cell lines to the mTOR signaling pathway [5,10,13]. The mechanism behind this anticancer effect is based on the anti-angiogenic properties of rapamycin, which relate to blocking vascular endothelial growth factor (VEGF) production and stimulation of endothelial cells [14,15].

Despite the potency of rapamycin demonstrated in the above studies, the systemic delivery of rapamycin to tumor sites constitutes a major challenge in the field of cancer therapy. Delivery is impeded by rapamycin's poor solubility in water (2.6 μg/mL) [16], and by its low bioavailability [17] and dose-limiting toxicity. To fully exploit the therapeutic potential of rapamycin in systemic administration, the drug carriers should provide a suitable intravenous

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(iv) formulation, and should be non-cytotoxic, stimuli-responsive, and stable in the circulation. Various investigations have demonstrated that an efficient delivery system stabilizes rapamycin and achieves controlled drug distribution, despite its poor water-solubility. Approaches like successful local drug delivery systems of rapamycin have been used on immunosuppression of the immune system [18,19]. Anticancer therapy using rapamycin has been made possible by advances in the delivery system designed to control the release rate for drugs administered either by injection [20,21] or orally [22,23]. For iv injection, Forrest et al. reported a formulation of rapamycin prepared using poly(ethylene glycol)-*b*-poly(ε-caprolactone) micelles [20]. Lu et al. demonstrated that poly(ethylene glycol)-*b*-poly(2-methyl-2-benzoxycarbonyl-propylene carbonate) copolymer could be used to prepare rapamycin-loaded micelles [21]. However, the carriers did not optimize for the iv formulations of rapamycin because the encapsulated drug was rapidly or sustained released in a neutral environment. After iv injection, a long-circulating drug carrier (10–200 nm) demonstrated significant selective targeting of tumors of a specific size range, and the particle accumulated in solid tumors because of an enhanced permeability and retention effect (EPR effect) [24]. For tumor-specific drug release, carriers can be designed to stabilize under physiological condition and to selectively respond to stimuli near tumor sites, such as minor increases in local temperature [25] or decreases in extracellular pH [26]. Thus, for most anti-cancer drug particulate carriers, the triggering mechanism must occur in the tumor extracellular microenvironment and/or acidic organelles to release the drug into the cytoplasm. The properties of stimuli response for carriers of rapamycin could be designed to favor extracellular or intracellular pH-triggered drug release.

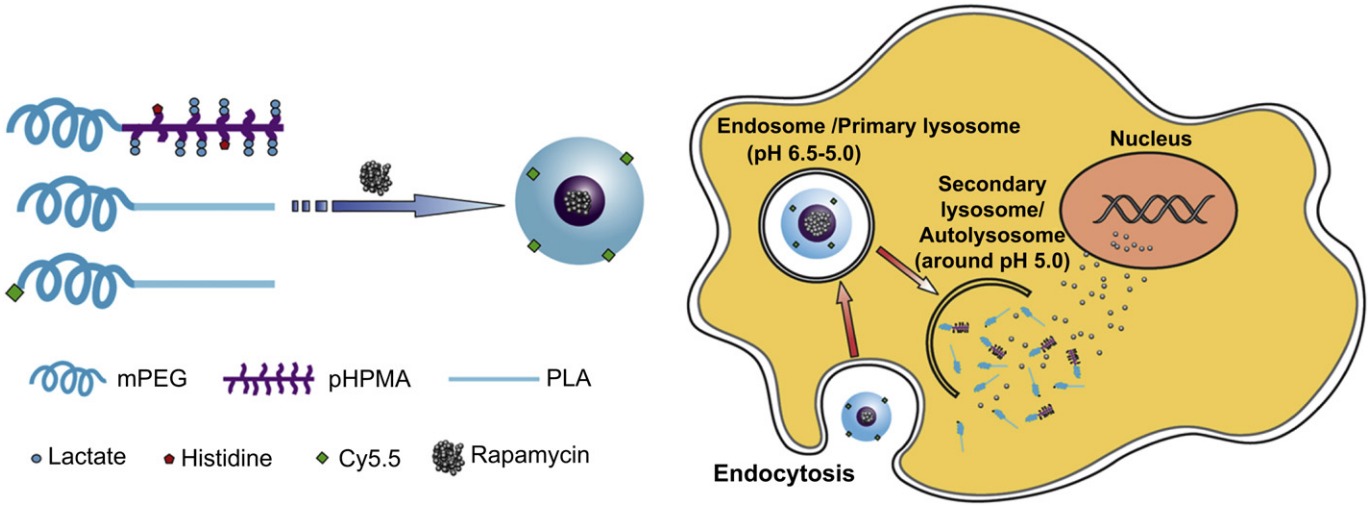
As our previous polymeric micelle systems, dual-responsive micelles from mPEG-*b*-P(HPMA-Lac-*co*-His) (poly(ethylene glycol)-*b*-poly(N-(2-hydroxypropyl) methacrylamide dilactate)-co-(N-(2-hydroxypropyl) methacrylamide-*co*-histidine)) and mPEG-*b*-PLA (poly(ethylene glycol)-*b*-poly(D,L-lactide)) diblock copolymers exhibited a simple, non-toxic property as well as excellent tumor targeting *in vivo* [27]. The current study describes the formulation to improve the solubility of rapamycin for enhanced intracellular delivery and improved antitumor efficacy (Schematic representation in Scheme 1). Hydrophobic interactions of the system are temperature-dependent, thus the efficient use of temperature might provide one method for incorporating drugs with poor water-solubility. This system offers a pH-sensitive structural feature and stability under the neutral serum albumin environment. Rapa-micelles disintegrate because of structural changes in intracellular pH, allowing for the rapid release of the drug from their hydrophobic regions, thus preventing or decreasing leakage of the anticancer drug into systemic circulation, and enhancing the effective concentration of rapamycin. The PEG modified particles are taken up extremely efficiently by a number of different routes [28]. Caveolae represent the main non-clathrin-dependent route from the cell surface to endosomes [29]. This study investigated the encapsulation of rapamycin in dual-responsive micelles by a heat shock protocol, and the stability and pH-dependent release behavior. Intracellular pH topography and cytotoxicity were investigated in human colon cancer HCT116 cells lines and the results were compared with those of free rapamycin. Finally, antitumor therapeutic efficacy and tumor apoptosis of the rapa-micelles and free drug were evaluated in a HCT116 cancer xenograft model.

2. Materials and methods

2.1. Materials

Methoxy poly(ethylene glycol) with Mw 5000 (mPEG) and α-t-butyloxy-carbonylamino-ω-hydroxy-poly(ethylene glycol) (Boc–NH–PEG-OH) with Mw 3000 were purchased from Iris Biotech. D,L-lactide and palladium were purchased from Lancaster. Cy5.5 mono NHS ester was purchased from GE Healthcare, and N-(2-hydroxypropyl) methacrylamide (HPMA) was purchased from Polysciences. 4,4′-Azobis(4-cyanovaleric acid) (ABCPA) and stannous octoate ($Sn(Oct)_2$) were purchased from Aldrich. Sodium hydroxide (NaOH), bovine serum albumin (BSA), potassium chloride (KCl), 2-(*N*-morpholino)ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (*HEPES*), calcium chloride ($CaCl_2$), and magnesium chloride ($MgCl_2$) were purchased from Sigma. 4-(dimethylamino) pyridine (DMAP), N-(tert-butoxycarbonyl)-L-histidine, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) were purchased from TCI. Acetonitrile (ACN), dichloromethane (DCM), and ethanol (EtOH) were purchased from TEDIA. Rapamycin (99%) was purchased from LC Laboratories. LysoSensor Blue DND-167, anti-LC3B (against isoform B of human microtubule-associated protein 1 light chain 3) antibody, Alexa Fluor 488, and Carboxyl-seminaphthorhodafluor-1 acetoxymethylester (SNARF-1-AM) was purchased from Invitrogen. Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO.

The cell lines of human cervical cancer HeLa, human colon cancer HCT116, and normal human foreskin fibroblast HS68 cells were obtained from the Bioresource



Scheme 1. Schematic representation of mixed micelle composed of the diblock copolymers mPEG-b-P(HPMA-Lac-co-His), mPEG-*b*-PLA and Cy5.5-PEG-PLA and loaded with rapamycin. The dual-responsive drug carrier is designed for the intracellular delivery. The acidic lysosomal compartments could induce the deformation of the rapa-micelles to trigger the release of enclosed drug molecules.