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Hyaluronate/lactoferrin layer-by-layer-coated lipid nanocarriers for targeted co-delivery of rapamycin and berberine to lung carcinoma

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ABSTRACT

The self-tumor targeting polymers, lactoferrin (LF) and hyaluronic acid (HA) were utilized to develop layer-by-layer (LbL) lipid nanoparticles (NPs) for dual delivery of berberine (BER) and rapamycin (RAP) to lung cancer. To control its release from the NPs, BER was hydrophobically ion paired with SLS prior to incorporation into NPs. Spherical HA/LF-LbL-RAP-BER/SLS-NPs 250.5 nm in diameter, with a surface charge of −18.5 mV were successfully elaborated. The NPs exhibited sequential release pattern with faster release of BER followed by controlled release of RAP which enables sensitization of lung tumor cells to the anti-cancer action of RAP. LbL coating of the NPs was found to enhance the drug cytotoxicity against A549 lung cancer cells as augmented by remarkable increase in their cellular internalization through CD44 receptors overexpressed by tumor cells. *In vivo* studies in lung cancer bearing mice have revealed the superior therapeutic activity of LbL-RAP-BER/SLS-NPs over the free drugs as demonstrated by 88.09% reduction in the average number of microscopic lung foci and 3.1-fold reduction of the angiogenic factor VEGF level compared to positive control. Overall, the developed HA/LF-LbL-coated lipid NPs could be potential carriers for targeted co-delivery of BER and RAP to lung cancer cells.

1. Introduction

Lung cancer is one of the major causes of cancer-associated mortality with about 23% of total cancer related mortality [1]. The nanoparticles (NPs) have the ability to encapsulate therapeutics and release them in a controlled manner to specifically target cells, facilitate intracellular uptake and improve the solubility of poorly soluble drugs [2,3]. Although lipid NPs are characterized by their ability to carry both hydrophilic and lipophilic drugs with a high drug payload, their use is associated with burst or premature drug release in vivo, mainly because the particles tend to crystallize and expel the drug from their cores. In addition, lipid NPs may be rapidly removed from circulation via the reticulo-endothelial system (RES) [4]. Therefore, to use lipid NPs for efficient tumor-targeted drug delivery, it will be necessary to overcome their limitations by enabling controlled drug release and surface modification.

Among surface modification approaches, layer-by-layer (LbL) self-assembly proves its flexibility and simplicity, as it is usually carried out in aqueous medium and does not entail drastic conditions. LbL assembly technique relies on electrostatic deposition of oppositely charged polyelectrolytes on the nanocarrier surface [5]. The LbL construction, mainly using hydrophilic polymers including proteins and polysaccharides, can delay RES clearance, prolong blood circulation and facilitate enhanced permeation and retention (EPR)-based passive diffusion of nanocarriers in interstitial tumors [6]. Additionally, the fact that the layering material is, itself, a targeting moiety could enhance the targeting ability of

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these structures. Recently, protein–polysaccharide LbL assembled nanocarriers have drawn great interest for tumor-targeted drug delivery by virtue of their biocompatibility, minimal opsonization and easy functionalization [7]. Lactoferrin (LF) is a cationic protein with essential role in iron transportation, in addition to its anti-viral, anti-inflammatory, and anti-cancer activity. It was found that bovine LF tends to inhibit esophagus and lung carcinogenesis at very low doses [8]. Moreover, LF can bind to transferrin receptor (TfR) and LDL receptors overexpressed on various cancer cells including breast, colon, nasopharyngeal and lung [9]. On the other hand, hyaluronic acid (HA) is one of the most commonly used anionic polysaccharides in targeted anti-cancer drug delivery via its inherent ability to bind with CD44 receptors overexpressed on tumor cells including lung cancer cells [10,11].

Rapamycin (RAP), a macrolide lactone with antifungal and immunosuppressant actions, was found to inhibit the mTOR pathway that regulates a number of cellular signals and mitogenic growth factors leading to impaired cancer cell metabolism [12]. However, the extremely lipophilic nature (partition coefficient 3.58) of RAP and hence poor aqueous solubility (2.6 μg/ml) and low oral bioavailability (about 17%) handicaps its parenteral administration and requires use of high amounts of solvents such as DMSO, PEG 400 and ethanol that may cause kidney injury and liver damage [12]. Additionally, RAP is unstable in electrolyte solutions and shown to be sensitive to both acids and bases, resulting in ring fragmentation and degradation. Moreover, its serious nephrotoxicity and hyperglycemic effect limit its clinical application [13].

On the other hand, berberine (BER), a quaternary isoquinoline alkaloid present in many medicinal herbs, displayed antimicrobial, anti-inflammatory and anti-cancer properties [14]. BER could inhibit the growth and invasion ability of H460 and A549 lung cancer cells at a relatively low IC_{50} through a cell cycle arrest at the G0/G1 phase [15]. However, the clinical application of this compound has encountered several challenges, particularly in vivo, largely due to its low gastro-intestinal absorption, and rapid metabolism by CYP 450-dependent processes [16]. Thus, a potential solution to overcome the obstacles hindering the use of both drugs, RAP and BER, is to utilize nanoparticulate formulations for their tumor-targeted delivery.

While the inhibition of mTOR pathway by RAP and its analogs constitutes an outstanding anti-cancer target, RAP-induced immunosuppression represents a challenge facing its clinical application in cancer therapy [17]. On another avenue, BER was found to inhibit the proliferation of hepatocellular carcinoma cells (HCC) mediated by blocking the mTOR pathway via AMPK activation [18]. Accordingly, co-treatment of HepG2 cells with BER and RAP showed synergistic cytotoxicity where BER could maintain the cytotoxic action of RAP at a lower concentration. Thus, BER may reduce the RAP-associated immunosuppression by allowing its use in a lower dose [17]. Therefore, we hypothesize that a nanocarrier for combined delivery of both RAP and BER would benefit from: (a) the powerful anti-cancer action of RAP, (b) the synergistic mTOR inhibiting activity of BER, and (c) the broad anti-cancer activity of BER mediated via multiple mechanisms other than mTOR blocking.

Therefore, in this study, we propose for the first time up to our knowledge, HA/LF-LbL-coated lipid NPs for combined delivery of RAP and BER to lung cancer. **First,** to enable parenteral delivery of BER and RAP, both drugs were co-encapsulated into the lipid core by oil-in-water emulsification to elaborate lipid NPs. **Second,** to enhance its loading and overcome its relatively fast release, BER was pre-formulated as hydrophobic ion pair (HIP) with sodium lauryl sulfate or sodium deoxycholate. **Finally,** to reduce RES clearance of the NPs and enable active tumor-targeting, the RAP-BER/SLS-loaded lipid core NPs were coated with oppositely charged layers of cationic lactoferrin (LF) and anionic hyaluronic acid (HA) via LbL assembly to enhance their internalization into lung cancer cells via binding to LDL or CD44 receptors overexpressed by human lung cancer cells. The developed delivery system was thoroughly investigated *in vitro* and *in vivo* to prove the anti-tumor superiority of the combined drug nano-delivery compared with the free drug combination.

2. Materials and methods

2.1. Materials

Berberine chloride hydrate (BER), sodium deoxycholate (SDC), Fetal bovine serum (FBS), Triton X100, coumarin-6, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), urethane (ethyl carbamate), Dulbecco's modified eagle medium (DMEM), trypsin-EDTA, Canada balsam, Haematoxylin solution, and Eosin solution (H&E) were purchased from Sigma-Aldrich (St. Louis, USA). Rapamycin (RAP), purity 98% was purchased from Jianshi Yuantong Bioengineering Co., Ltd. (China). Hyaluronic acid (HA) was purchased from Baoding Faithful Industry Co., Ltd. (China). Lactoferrin (Lf) was kindly donated by Westland Milk Products (Hokitika, New Zealand). Phosphatidylcholine (Lipoid S75) was kindly provided by Lipoid (Ludwigshafen, Germany). Glyceryl monostearate (GMS) was a gift from Gattefosse (Lyon, France). Poly(ethylene glycol) 400, sodium lauryl sulfate (SLS), ethanol, and methanol were purchased from ADWIC (Cairo, Egypt). Polyoxyethylene sorbitan monooleate (Tween 80) was obtained from (Riedel de H#XPS##x00E4;en, Germany). Acetonitrile and Methanol HPLC grade were purchased from JT Baker (NJ, USA).

2.2. Preparation of BER-hydrophobic ion pair (BER-HIP)

The BER-HIP complex was prepared by the ionic interaction of BER and SLS or SDC under aqueous conditions. Briefly, 25 mg BER was dissolved in distilled water. The aqueous solution of SLS or SDC as ion pairing agents was added dropwise to BER solution under gentle magnetic stirring till the appearance of strong turbidity indicating the complex formation which was then left for 30 min under gentle magnetic stirring [19]. The HIP complex was formed spontaneously upon mixing of both aqueous solutions in 1:1 molar ratio. The solutions were then centrifuged for 45 min at 17000 rpm (3K-30 centrifuge, Sigma laboratory, Germany) and the sediment complex was lyophilized at −80 °C with LyoQuest lyophilizer (Telstar, Spain) to obtain dry powders. The concentration of non-complexed BER was determined in the supernatant by HPLC analysis and detailed in Supplementary Material. The solubility and partition coefficient of BER and BER-HIP complexes were determined as previously reported [20] and detailed in Supplementary Material.

2.3. Preparation of dual RAP/BER-loaded lipid NPs

BER-loaded NPs were prepared by the hot homogenization method [21]. First, oily phase (100 mg GMS) was heated to 70 °C at shaking water bath (Maxturdy 30, DAIHAN Scientific, South Korea). 10 mg of BER or equivalent amount of BER-HIP complex was added to the melted lipid phase. Keeping the temperature at 70 °C, 50 ml aqueous phase containing 100 mg Tween 80 was added into the oily phase under agitation at 12000 rpm for 5 min using Ultra Turrax T-25 homogenizer (Ika Labortechnik, Germany). This emulsion was cooled to the room temperature upon magnetic stirring for 30 min. For preparation of dual drug (RAP/BER)-loaded NPs, 10 mg of RAP along with BER-HIP eq. to 10 mg were added to the melted lipid phase and completed in the same method mentioned above.