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A novel pH-sensitive liposome to trigger delivery of afatinib to cancer cells: Impact on lung cancer therapy

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ABSTRACT

A novel drug delivery systems based on cationic (CL) and pH-sensitive liposomes (PSL) for tyrosine kinase inhibitor afatinib (AFT) were developed to enhance tumor-targetability against NSCLC cells and therapeutic effect. Optimal lipid to drug ratio was selected to prepare AFT-loaded PSL and CL with desirable physiochemical properties based on particle size, drug encapsulation efficiency (EE%), stability and release profiles. Moreover, antitumor activity was performed *in vitro* on human lung cancer cells (H-1975) using a WST-1 assay and Annexin-V apoptosis assay. The mean particle size of the liposomes was <100 nm, and EE% was >50% with lipid to drug ratio of 1:0.5. Stability data showed that PSL and CL were physically stable for 1 months at 4 and 25 °C. *In vitro* drug release study demonstrated the sustained release of AFT at pH 7.5; while PSL exhibited fast drug release in pH 5.5. This effect revealed that PSL showed pH-sensitive release behaviors. In addition, the *in vitro* cytotoxicity study was employed for AFT-loaded PSL due to optimal characterizations. Thus, *in vitro* anticancer activity revealed that AFT loaded-PSL triggered apoptosis in H-1975 cells. In addition, the inhibitory effect toward H-1975 and HCC-827 was observed, indicating, which indicated high antitumor activity of AFT-loaded PSL. Then, PSL might potentially create practical clinical strategies for better targetability and delivery of AFT for treatment of lung cancer.

1. Introduction

Cancer is a foremost problem of disease worldwide to human health in recent years. Moreover, lung cancer becomes a serious danger to human health, it is about 1.38 million cancer-associated mortality in males and females in recent decades [1,2]. The incidence of lung cancer has increased significantly in recent years in Kingdom of Saudi Arabia and United Kingdom due to the increased prevalence of cigarette smoking [3,4]. Approximately 85% of lung cancer diagnoses are classified as non-small cell lung cancer (NSCLC) and the remaining 15% small cell lung cancer (SCLC) [5]. The unsatisfactory effects after treatment of lung cancer patients using conventional approaches such as surgical resection, radiation, and chemotherapy were perceived [6]. The majority of chemotherapy administration is intravenous, causing pronounced side effects due to their systemic drug distribution. Moreover, the bioavailability of orally administrated anticancer agents is usually compromised by the first-pass metabolism [7]. The cytotoxic effects of chemotherapeutic agents against normal cells, according to dose-response effects, have been recorded, leading to the patient's frail and death [8]. Therefore, the targeted delivery of anticancer drugs has become a focus of scientific research. NSCLC treatment can be improved by targeted delivery of chemotherapeutic agent (s) to suppress the major signaling pathways involved in lung cancer. Then the targeted delivery of anticancer drugs directly into the lungs can increase their accumulation in tumor cells and reduce adverse side effects [9]. To date, numerous epidermal growth factor (EGFR) targeting agents have been approved, including gefitinib, erlotinib, and lapatinib; however, both primary and acquired resistance are significant clinical problems [10]. Afatinib (AFT) is a novel, potent, small-molecule tyrosine kinase inhibitor (TKI), which is now marketed (2013), as a film-coated oral tablet as a dimaleate salt. AFT is an especially effective treatment for nonsmall cell lung cancer (NSCLC) [11]. AFT has ability to bind covalently and irreversibly to the intracellular TK domain, preventing intracellular signaling [12]. By targeting ErbB family receptors, AFT blocks a wide spectrum of cancer-associated ErbB-driven pathways, and thus has broader antitumor activity against receptors with acquired mutations that are resistant to the first generation of TKIs [13]. AFT exposed at

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low concentration in the tumor cells, which reduced their clinical uses [14]. Therefore, the targeted delivery of AFT has become a focus of scientific research. Nanomedicine is extensively used due to their intrinsic properties such as improved cancer therapy with reduced toxicity. The nanoparticles may be considered as a promising antitumor delivery system for AFT, which may trigger its delivery to the cancer tissues [15]. The liposomes were the interest type of nanomedicine for clinical application in the field of cancer therapy [16]. In addition, they have ability to target tumor tissues *via* an enhanced permeation and retention (EPR) effect [15]. Liposomes are suitable carriers for pulmonary drug delivery owing to their capacity for targeting to specific cells/tissues [17]. Although drug-loaded liposomes can extend *in vivo* circulation and increase chemotherapeutic activity, they may also be limited targeting and fast cleared [18]. It has been reported that pH-sensitive liposomes have received great attention due to their efficient accumulation in the tumor cells [19]. It has been described that tumor tissues exhibited an acidic condition (pH 5.0–6.5) than normal tissues (pH 7.4–7.5) [20]. Therefore, pH-sensitive liposomes were able to deliver the drugs into the tumor cells when the pH value is lower than the normal tissue. Therefore, it may be expected that pH-sensitive liposomes are more efficient for the delivery of AFT than conventional liposomes due to their fusogenic character [21]. pH-sensitive liposomes could increase the intracellular delivery of their content in cancer cells [20,21]. To the best of our knowledge, only one study investigated AFT nanoparticles in term of polymeric micelles as a pulmonary delivery system that improved the therapeutic efficacy in HER2-overexpressed HCT-15-induced tumors [22].

In this study, we have prepared three types of liposomes, pH-sensitive liposomes compared with conventional and cationic liposomes containing AFT to study their cancer targeting. An HPLC method was developed and validated for AFT for *in vitro* analysis. The liposomes were characterized in term of particle size distribution, zeta potential and encapsulation efficiency. The surface morphology of the desired liposome was observed by TEM. Moreover, the drug release of AFT was investigated for pH sensitivity (pH 5.5) and prolonged circulation (pH 7.4). The stability studies of these liposomes were performed at a different temperature. Afterward, the NSCLC cells (H-1975 cells) were selected for the assessment of antitumor activity of the optimum liposomes using a colorimetric WST-1 assay and flow cytometry. Moreover, the apoptosis in different cells like H-1975, HCC-827 and H-1650 was established after incubation with liposomes.

2. Materials and methods

2.1. Materials

AFT (99.8% purity) was purchased from Green Stone Swiss Co., Limited. 1,2-distearoyl-*sn*-glycero-3-phosphocholine [18:0] (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine [18:1] (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine [18:1] (DOPC), and 1,2-dioleoy-3-trimethylammonium-propane Chloride salt (DOTAP) were kindly gifted by Avanti Polar Lipid. Cholesteryl hemisuccinate (CHEMS) was purchased from Avanti Polar Lipid. H1975, H-1650, and HCC827 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained at 37 °C and 5% CO2 in RPMI 1640 medium (GIBCO®, SIGMA-ALDRICH, Saint Louis, USA) containing 10% fetal bovine serum (FBS) and1% antibiotic/antimycotic which were purchased from (GIBCO®, InvitrogenTM, Carlsbad, USA). All other reagents and chemicals were of analytical grade.

2.2. HPLC assay of AFT

2.2.1. HPLC instrumentation

A Water Breeze2™ HPLC system (Waters Corporation, Milford, U.S.A) was used for method development. The HPLC system equipped with an automated sampling system (Waters™ 2695 Plus Autosampler, USA) at 4 °C and a photodiode array detector (Waters™ 2998, USA). The HPLC system was examined by "Breeze2 (Water™)" software. AFT was analyzed using mobile phase that consisted of A: 0.1% triethanolamine and 1% acetonitrile in HPLC water (pH = 6), and B: acetonitrile and 10% methanol at a flow rate of 1 ml/min. The mobile phase flowed over a reversed-phase C18 column (Water™, 3 × 150 mm, 3.5 μm particle size) coupled with a C18 guard cartridge (4 × 2.0 mm) and maintained at 50 °C. The injection volume of each AFT sample was 10 μl and detected by the UV detector at 253 nm. All the operations were carried out at room temperature.

2.2.2. HPLC assay

A stock solution of AFT was prepared in methanol at a concentration of 1 mg/ml and stored in 4.0 ml amber glass vials at −20 °C. Serial dilutions in mobile phase were performed in the range of 0.01 to 25 μg/ml to produce a standard calibration curve and stored at −20 °C. A daily standard calibration curve ($n = 3$) ranging from 0.01 to 25 μg/ml was prepared to determine the unknown AFT concentrations for entrapment efficiency and drug release.

2.2.3. Method validation

The validation of HPLC method was conducted according to the International Conference on Harmonization (ICH) guidelines. The following items were considered for validation: linearity, accuracy, precision, specificity, limit of detection (LOD), limit of quantification (LOQ) and robustness. Three standard calibration lines were prepared at different times (3 months) to evaluate the linearity, precision, accuracy, and stability of the method.

Linearity was assessed by calculating a regression line by plotting the peak area of AFT *vs.* the AFT concentration ranging from 0.01 to 25 μg/ml.

The accuracy was determined *via* the analysis of multiple replicates ($n = 6$) of AFT concentration. The accuracy of the method was expressed in term of bias.

The precision of a quantitative method was determined by repeatability as intra-day precision by an analysis of three replicates of AFT concentrations over the same day. Inter-day precision was determined by the analysis of three replicates of various AFT concentrations over three different days. The results were expressed as the relative standard deviation (RSD%).

Low, medium, and high concentration quality control (QC) samples at concentrations of (100, 1000 and 10,000 ng/ml AFT, respectively) were analyzed, on three distinct occasions within at least 3 months, as before described.

The LOD and LOQ were determined from the calibration curve obtained using six replicates that were closest to the LOQ. The following equations were used:

$$\mathrm{LOD} = 3.3\,\sigma/S \quad (1)$$

$$\mathrm{LOQ} = 10\,\sigma/S \quad (2)$$

LOD and LOQ were determined based upon the slope (S) of the calibration curve and least standard deviation obtained from the response (σ). It has a low limit of quantitation (5 ng/ml) with satisfactory specificity, no matrix interference was observed. These findings demonstrated that the assay has good selectivity.

2.3. Preparation of liposomes

Different types of AFT-loaded liposomes were fabricated by thin-film hydration method followed by extrusion as described previously [23]. Non-targeting liposomes (NL) were prepared using DSPC, DOPC, and DOPE at molar ratios of 3: 3: 10. Moreover, three parts of the 10 parts of DOPC of the total liposomal contents were replaced by DOTAP or CHEMS to form cationic and pH-sensitive liposomes, respectively. The