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Research paper

Dual drug-loaded biofunctionalized amphiphilic chitosan nanoparticles: Enhanced synergy between cisplatin and demethoxycurcumin against multidrug-resistant stem-like lung cancer cells

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ABSTRACT

Lung cancer kills more humans than any other cancer and multidrug resistance (MDR) in cancer stem-like cells (CSC) is emerging as a reason for failed treatments. One concept that addresses this root cause of treatment failure is the utilization of nanoparticles to simultaneously deliver dual drugs to cancer cells with synergistic performance, easy to envision — hard to achieve. (1) It is challenging to simultaneously load drugs of highly different physicochemical properties into one nanoparticle, (2) release kinetics may differ between drugs and (3) general requirements for biomedical nanoparticles apply. Here self-assembled nanoparticles of amphiphilic carboxymethyl-hexanoyl chitosan (CHC) were shown to present nano-microenvironments enabling simultaneous loading of hydrophilic and hydrophobic drugs. This was expanded into a dual-drug nano-delivery system to treat lung CSC. CHC nanoparticles were loaded/chemically modified with the anticancer drug cisplatin and the MDR-suppressing Chinese herbal extract demethoxycurcumin, followed by biofunctionalization with CD133 antibody for enhanced uptake by lung CSC, all in a feasible one-pot preparation. The nanoparticles were characterized with regard to chemistry, size, zeta potential and drug loading/release. Biofunctionalized and non-functionalized nanoparticles were investigated for uptake by lung CSC. Subsequently the cytotoxicity of single and dual drugs, free in solution or in nanoparticles, was evaluated against lung CSC at different doses. From the dose response at different concentrations the degree of synergy was determined through Chou-Talalay's Plot. The biofunctionalized nanoparticles promoted synergistic effects between the drugs and were highly effective against MDR lung CSC. The efficacy and feasible one-pot preparation suggests preclinical studies using relevant disease models to be justified.

1. Introduction

Amphiphilic carboxymethyl-hexanoyl chitosan (CHC) was modified through a feasible one-pot preparation to achieve antibody-functionalized dual-drug nanoparticles for simultaneous delivery of demethoxycurcumin (DMC) and cisplatin (CDDP). It was hypothesized that the simultaneous intracellular delivery and co-localization of the drugs mediated by the nanoparticles would result in greatly enhanced synergistic effect against lung cancer stem-like cells (CSC) and that this nanomedical technology would thus show potential for combinatorial cancer therapy, an approach already employed in treatment of lung cancer by clinicians using free drugs [1].

Among cancers, lung cancer remains a leading killer [2]. Treatment of non-small cell lung cancer (NSCLC) is particularly important from a healthcare perspective, as it combines high prevalence (about 85% of lung cancer cases) with high mortality [2]. When the cancer has entered the stage at which complete removal by surgery is difficult, chemotherapy, targeted drugs or immunotherapy is commonly employed [2]. Unfortunately the 5-year survivability is quite low, even with treatment [2]. The difficulty in killing of the cancer and the resulting low survival rate may be due to CSC [3,4]. The role of CSC is just beginning to be understood, but they are thought to play critical roles in metastasis

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and cancer relapse after treatment, while also presenting multidrug resistance (MDR) features [3,4]. It is thus critical to develop improved treatments to overcome MDR and effectively kill the CSC.

Dual-drug administration has been discussed for years as a means to improved cancer treatment with potential benefits such as reduced side effects due to lowering of drug concentrations and reduction in MDR. Although nanoparticle-based dual-drug delivery seems highly promising, there are challenges in terms of complex production processes, low encapsulation efficiencies and unfavorable release profiles of the different drugs [5].

In the clinic, CDDP is a first-line drug for NSCLC treatment and combinations with other drugs are commonly employed [1]. However, resistance of cancers toward CDDP is commonly observed through cysteine-rich protein blocking [6,7] and responses from the NF-κB-upregulated MDR CD133 signaling pathway, which is highly active in CSC [3,6,8–10]. Cells with upregulated CD133 present resistance to treatment, self-renewal capability, fast DNA repair and overexpression of multidrug resistance protein 1 (MRP1) and breast cancer resistance protein 1 (BCRP), among others [3,8,10]. Curcuminoids, a traditional Chinese medicine herbal extract, have been reported to downregulate NF-κB, inhibit MDR-related proteins, promote apoptosis through inactivation of Bcl-2 [11,12] and reverse CDDP resistance in lung cancer [13]. Recently, free curcumin has also been shown to act in synergy with CDDP and CDDP/polymer-conjugate nanoparticles to achieve improved efficacy against CDDP-resistant ovarian cancer cells [14]. DMC is a curcuminoid with better stability in blood and under basic condition compared to the commonly used curcumin [15]. Therefore, this work focused on feasible one-pot preparation of CD133-antibody dressed nanoparticles for simultaneous intracellular delivery of the well-proven hydrophilic anticancer drug CDDP and the hydrophobic curcuminoid DMC to overcome MDR in CSC through enhanced synergistic effects. *In vitro* evaluation of cell internalization and effectiveness against MDR lung cancer cells were carried out using A549-ON, a stable cell line presenting stem-like characteristics and overexpression of CD133, prepared by transfection of human A549 lung adenocarcinoma cells using a lentiviral infection system with vectors encoding *Oct4* and *Nanog* cDNA [9].

2. Methods

2.1. Materials

Cis-diammine platinum(II) dichloride (cisplatin, CDDP), o-phenylenediamine (OPDA), dimethyl sulfoxide (DMSO), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-propanol, sodium hydroxide, chloroacetic acid, deuterium oxide (D_2O), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich. Carboxymethyl-hexanoyl chitosan (CHC) was purchased from Advanced Delivery Technologies, Inc. PSVue®480 was purchased from Molecular Targeting Technologies, Inc. N,N-dimethylformamide (DMF) was purchased from Aencore. Demethoxycurcumin (DMC) was a courteous gift from China Medical University (Taiwan). The CD-133 antibody was purchased from Genetex. All other chemical reagents in the study were of analytical grade and were used as received without further purification. A549-ON, a stable cell line with stem-like characteristic and overexpression of CD133, prepared by transfecting human A549 lung adenocarcinoma cells using a lentiviral infection system with vectors encoding *Oct4* and *Nanog* cDNA [9], were kindly provided by Professor Shih-Hwa Chiou (Yang Ming University, Taiwan). The reader is referred to the given reference for detailed cell characteristics and preparation protocol.

2.2. Preparation of CHC/CDDP and CHC/DMC nanoparticles

CHC/CDDP and CHC/DMC nanoparticles were prepared by mixing 0.4 mL of CDDP or DMC (1 mg/mL) in 1% DMSO in ddH_2O with 1 mg of CHC and 1.6 mL pH 11 ddH_2O. The pH of the resulting solution was about 7 and the particles were mixed on a magnetic stirrer for 12 h to allow self-assembly into drug loaded nanoparticles. Subsequently, the sample was placed into Amicon centrifugal filter and centrifuged at 5000 rpm for 30 min to remove unloaded drug. The gel-like nanoparticle-concentrate was dispersed ddH_2O to required concentrations. Encapsulation efficiency (EE) was measured, as described in Section 2.6, before use of nanoparticles to ensure use of correct drug concentration.

2.3. Analysis of CHC/CDDP interactions

Fourier transformed infrared spectroscopy (FT-IR) was conducted on a Spectrum 100 (Perkin Elmer) as follows: 1 mL nanoparticle samples were concentrated using Amicon centrifugal filters under 5000 rpm for 30 min. Subsequently, 100 μL of ddH_2O was added to disperse the nanoparticle-concentrates. The nanoparticle dispersions were applied on silicon wafers, taking care to avoid bubble formation, and were dried at 50 °C. Spectra were recorded with a resolution of 1 cm^{-1} between 4000 and 400 cm^{-1}.

Samples for X-ray photoelectron spectroscopy (XPS) analysis were prepared as follows: Nanoparticle dispersions were freeze-dried for one day to obtain fiber-like samples that were mounted on silicon wafers. CDDP powder was mounted as delivered onto the silicon wafer. The samples were briefly sputtered with Au to improve the electrical conductivity and were analyzed for elemental composition using a Microlab 350 X-ray photoelectron spectrometer (VG Scientific) equipped with Mg Kα source.

Samples were prepared for platinum nuclear magnetic resonance spectroscopy (^{195}Pt NMR) by freeze-drying of 10 mL of dissolved CDDP or dispersed nanoparticles for one day, followed by dissolution/dispersion in 1 mL of D_2O. ^{195}Pt NMR spectra were recorded using a 600 MHz Mercury NMR spectrometer (Varian).

2.4. Preparation of CHC/DMC-CDDP nanoparticles

CHC/CDDP-DMC nanoparticles were prepared by mixing 0.4 mL of DMC (X μg/mL) in 1% DMSO in ddH_2O with 1 mg of CHC and 1.2 mL pH 9 ddH_2O, followed by addition of 0.4 mL of CDDP (Y μg/mL) in 1% DMSO in ddH_2O. For characterizations and *in vitro* release X and Y = 1000. To investigate the effect of different drug concentrations on *in vitro* cytotoxicity the drug loading was modified to achieve desired concentrations (X, Y = 50, 750; 100, 1500; 150, 2250; 150, 3000; 200, 3000). The pH of the resulting solution was about 7 and the particles were mixed on a magnetic stirrer for 12 h to allow self-assembly into drug loaded nanoparticles. Further preparation was performed as for CHC/CDDP nanoparticles, described above. Before use the EE was measured for each preparation, as described in Section 2.6, to ensure use of correct drug concentrations.

2.5. Modification with CD133 antibodies

After preparing CHC/CDDP-DMC nanoparticles, as described above, but before the centrifugation-filtration step to separate nanoparticles from free drug, 1 μL of anti-CD133 antibodies (1 mg/mL in ddH_2O) was added to the solution. The mixture was stirred at 4 °C for one hour, after which 0.05 mL of EDC solution (0.1%, w/v) was slowly added over 4 h to crosslink between carboxyl groups and amine groups. The CHC/CDDP-DMC/Anti-CD133 nanoparticles were subsequently separated from free drug and