Journal of Controlled Release 244 (2016) 63–73

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Co-delivery of polymeric metformin and cisplatin by self-assembled core-membrane nanoparticles to treat non-small cell lung cancer

Yang Xiong [a,b], Yi Zhao [a], Lei Miao [a], C. Michael Lin [a], Leaf Huang [a,*]

[a] Division of Pharmacoengineering and Molecular Pharmaceutics and Center for Nanotechnology in Drug Delivery, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States
[b] Department of Pharmaceutical Science, Zhejiang Chinese Medical University, Hangzhou 310053, Zhejiang, China

ARTICLE INFO

Article history:
Received 9 June 2016
Received in revised form 16 October 2016
Accepted 8 November 2016
Available online 10 November 2016

Keywords:
Cisplatin
Polymeric metformin
Core-membrane nanoparticle
Non-small cell lung cancer

ABSTRACT

Clinically, combined therapy of cisplatin (CDDP) and metformin is an effective treatment for non-small cell lung cancer (NSCLC). The success is attributed to synergistic effects between the two drugs. Therefore, we hypothesize that co-encapsulation of CDDP and metformin will avoid the prominent toxicity of CDDP while maintaining the synergy between the regimens. CDDP was first conjugated to polyglutamic acid (PGA) to form anionic PGA-CDDP which was electrostatically complexed with the cationic polymeric metformin (polymet). The nano-sized complex was then stabilized with cationic liposomes composed of DOTAP (2, 3-Dioleoyloxy-propyl)-trimethylammonium/Cholesterol/DSPE-PEG-anisamide aminoethyl. Both *in vitro* and *in vivo* experiments confirmed the synergy between polymet and CDDP. CDDP delivered with nanoparticles (NPs) exhibited significantly increased tumor accumulation over free CDDP and suppressed tumor growth through apoptosis in NSCLC H460 tumor-bearing mice without nephrotoxicity. The synergistic effect of polymet alongside CDDP demonstrates that polymet-CDDP NPs can activate the AMP-activated protein kinase α (AMPKα) pathway and inhibit mammalian target rapamycin (mTOR) activity to enhance growth suppression. In all, this platform is the first to successfully co-load polymet, a polymeric metformin, and CDDP into the same nanoparticle for successful treatment of NSCLC.

1. Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, and about 85% of all lung cancers are non-small cell lung cancer (NSCLC) [1,2]. The overall 5-year survival rate for patients with NSCLC is 13% in Europe and 16% in the United States [3,4]. Thus, the efficacy and success rate of treatment needs improvement. Cisplatin (CDDP) remains the leading therapy for advanced NSCLC [5]. For patients with advanced (stage III and IV) NSCLC, CDDP was often used in combination with paclitaxel, gemcitabine, docetaxel, vinorelbine or irinotecan, in concurrence with radiotherapy [6].

Recent studies have shown that in addition to the therapies listed above, metformin, a common antidiabetic drug (*N*′,*N*′-dimethylbiguanide), displayed significant growth- inhibition and proapoptotic effects in several cancers, including NSCLC [7,8]. Metformin activates AMP-activated protein kinase (AMPK), inhibits the mammalian target of rapamycin (mTOR) and down-regulates excision repair cross-complementation group 1 (ERCC1) [9,10]. Consequently, metformin may be used in combination with CDDP to treat NSCLC [11,12].

While preclinical studies of free CDDP and metformin in combination exhibited promising outcomes, the clinical application of this combination is severely restricted by a collection of issues. Firstly, CDDP and metformin are administered through different routes, *i.e.* oral for

Abbreviation: NSCLC, non-small cell lung cancer; CDDP, cisplatin; polymet, polymeric metformin; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; ERCC1, excision repair cross-complementation group 1; IV, intravenous; PK, pharmacokinetic; NPs, nanoparticles; RES, reticuloendothelial system; PGA, polyglutamic acid; PEI, polyethylenimine; EDC, ethylene dichloride; NHS, *N*-hydroxysuccinimide; MTT, 3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide; DAPI, 4′,6-diamidino-2-phenylindole; DIPEA, *N*,*N*-Diisopropylethylamine; DOTAP, 2,3-dioleoyloxy-propyl)-trimethylammonium; RIPA, Radio-Immunoprecipitation Assay; BCA, Bicinchoninine acid; HRP, horseradish peroxidase; DSPE-PEG2000, 1,2-distearoryl-*sn*-glycero-3- phosphoethanolamine-*N*[methoxy (polyethyleneglycol-2000)](ammonium salt); DSPE-PEG-AA, DSPE-PEG-aminoethyl anisamide; TUNEL, TdT-dependent dUTP-biotin nick end labeling; GAPDH, glyceraldehyde-phosphate dehydrogenase; XPA, xeroderma pigmentosum complementation group A; PARP-1, poly ADP-ribose polymerase-1; PBS, phosphate-buffered saline; ^{1}H NMR, nuclear magnetic resonance; DLS, dynamic light scattering; PDI, polydispersity index; TEM, transmission electron microscope; ICP-MS, inductively coupled plasma mass spectrometry; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; RBC, red blood cells; WBC, white blood cells; PLT, platelets; HGB, hemoglobin; HCT, hematocrits; PIC, protease inhibitor cocktail; SDS-PAGE electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; BSA, Bovine Serum Albumin; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MTD, maximum tolerable dose; PI3K, phosphatidyl inositol 3 kinase; ERK, extracellular signal regulated kinase.

* Corresponding author.
E-mail address: leafh@email.unc.edu (L. Huang).

http://dx.doi.org/10.1016/j.jconrel.2016.11.005
0168-3659/

metformin and intravenous (IV) injection for CDDP, which challenges patient compliance. The difference in administrative routes leads to a subsequent discrepancy in the pharmacokinetic (PK) profiles. Second, proper evaluation of the synergy between these two drugs is further complicated by the different physicochemical properties of the drugs, which leads to subpar anti-tumor efficacies. Furthermore, well-known toxicities such as nephro and neurotoxicity limit the use of CDDP. Thus, formulating these two drugs into a single nanoparticle will be a promising strategy to treat NSCLC and overcome the aforementioned limitations.

Formulating CDDP into nanoparticles (NPs), such as liposomal or polymeric formulations, significantly reduces the adverse side effects of CDDP while maintaining its anti-tumor efficacy [13,14]. This advantage can be partially attributed to the fact that NP_S can be modified to avoid undesired uptake by the reticuloendothelial system (RES), leading to prolonged blood circulation and increased tumor accumulation. Due to promising preclinical studies, several NPs incorporating CDDP have already been approved for clinical trials, such as Lipoplatin [15] and Nanoplatin [16]. However, unlike the co-delivery of CDDP with other hydrophobic drugs, which works by modifying CDDP into hydrophobic platinum IV prodrugs, co-delivery of CDDP with a hydrophilic drug (*e.g.* metformin) has rarely been reported [17]. Herein, we report a novel strategy for the co-encapsulation of CDDP and metformin into a single self-assembled core-membrane NPs. We took advantage of recently discovered polymeric metformin (polymet) which exhibits similar anticancer efficacy as metformin and acts through the same mechanistic pathway [18]. Namely, polymet both activates the AMPK pathway, and reduces mTOR activation. It is appropriate to consider polymet as a substitute for metformin. CDDP was chemically conjugated to polyglutamic acid (PGA) through the displacement of chlorine atoms by hydrogen of carboxyl groups on PGA side-chains to form anionic PGA-CDDP as reported before [14,19]. From here, cationic polymet was then subject to an electrostatic interaction with an anionic PGA-CDDP conjugates to produce a negatively charged core which was then coated with PEGylated cationic liposomes to form the final core-membrane structure. The dissociation of polymeric ion pairs then controls the release of both therapeutic moieties.

Herein we report the synergistic anti-cancer activity of NPs containing both polymet and PGA-CDDP. The experiments were carried out in H460 human lung cancer xenograft as a model for human NSCLC.

2. Materials and methods

2.1. Chemicals and materials

CDDP was purchased from Sigma-Aldrich (Dorset, UK). Linear polyethylenimine (PEI) hydrochloride with average molecular weight 8000, Poly-L-glutamic acid sodium salt (molecular weight 3000–15,000), cholesterol, dicyandiamide, *p*-Anisic acid, Ethylene dichloride (EDC), *N*-Hydroxysuccinimide (NHS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), *N*,*N*-Diisopropylethylamine (DIPEA), Radio-Immunoprecipitation Assay (RIPA), dichloromethane and silver nitrate were obtained from Sigma-Aldrich (St Louis, MO) without further purification. (2, 3-Dioleoyloxy-propyl)-trimethylammonium (DOTAP) and 1, 2-distearoryl-*sn*-glycero-3-phosphoethanolamine-*N* [methoxy (polyethyleneglycol-2000)] (ammonium salt) (DSPE-PEG2000) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). DSPE-PEG-aminoethyl anisamide (DSPE-PEG-AA) was synthesized in our lab as described previously [20]. TdT-dependent dUTP-biotin nick end labeling (TUNEL) assay kits was obtained from Promega (Madison, WI). 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Vector laboratories (Burlingame, CA). Bicinchoninine acid (BCA) protein assay reagent kit was purchased from Thermo Fisher Scientific Inc. Rabbit monoclonal antibodies: Phospho-AMPKα (Thr172) (40H9), AMPKα (D5A2), Phospho-mTOR (Ser2448) (D9C2) XP®, mTOR (7C10), Phospho-p70 S6 Kinase (Ser371), Phospho-4E-BP1 (Thr37/46), and horseradish peroxidase (HRP)-linked Antibody were purchased from Cell signaling. Reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) (14C10), mouse monoclonal antibodies: ERCC1 (3H11), anti-rabbit IgG, xeroderma pigmentosum complementation group A (XPA) (12F5), poly ADP-ribose polymerase-1 (PARP-1) (F-2) and goat antimouse-IgG2b, HRP-linked Antibody were purchased from Santa Cruz Biotechnology, Inc.

2.2. Cell lines and experimental animals

H460 human NSCLC cells was obtained from American Type Culture Collection (ATCC) and was cultured in RPMI 1640 Media (Sigma Aldrich, UK) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, California), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Cells were cultivated in a humidified incubator at 37 °C with 5% CO_2 and harvested with 0.05% trypsin-EDTA (ethylene diamine tetraacetic acid) before subculture.

Female nude mice of 6–8 weeks old were purchased from National Cancer Institute (Bethesda, MD) and bred by the Division of Laboratory Animal Medicine (DLAM) at University of North Carolina at Chapel Hill. To establish the xenograft models, 5×10^6 H460 cells in 100 μL of phosphate-buffered saline (PBS) were inoculated subcutaneously into the right flank of each mouse. All procedures involving experimental animals were performed in accordance with the protocols approved by the University of North Carolina Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

2.3. Preparation of polymet

Polymet (*ca.* molecular weight is 4300 Da) was synthesized as previous reported [18]. Briefly, 0.2 g of linear PEI and 2 g of dicyandiamide were mixed in 10 mL deionized water. Two milliliters HCl were added into the solution (Fig. 1A). The compounds reacted in a 100 °C oil bath for 4 h. Polymet was then purified through an ultrafiltration tube with a cutoff of 3000 Da, washed with deionized water for two times and lyophilized.

2.4. Preparation and characterization of polyglutamic acid-CDDP conjugation (PGA-CDDP)

The preparation of PGA-CDDP was synthesized as previous reported [19]. First the *cis*-$[Pt(NH_3)_2(H_2O)_2]$ $(NO_3)_2$ precursor was as previously described [21]. Briefly, $AgNO_3$ (66.2 mg or 0.39 mmol) was added to a suspension of CDDP (60 mg or 0.2 mmol) in 1 mL water. The mixture was heated at 60 °C for 3 h and then stirred overnight in a flask protected from light with aluminum foil. The mixture was centrifuged at 16,000 rpm for 15 min to precipitate AgCl. The supernatant was then filtered using a 0.22 μm syringe filter to afford the desired product (CDDP precursor). PGA was solved in deionized water (25 mM) and then added to the solution of CDDP precursor (6.25 mM) at the same volume. The mixture was then put in dark at room temperature for 72 h with gently stirring to form the PGA-CDDP conjugation (Fig. 1B). The formation of the conjugation was certified by the ^1H NMR nuclear magnetic resonance (^1H NMR) (Fig. S1).

2.5. Preparation and characterization of the NPs

The formulation of polymet-CDDP NPs involves two key steps after the production of PGA-CDDP. Firstly, an anionic core must be formed from a complex of polymet and PGA-CDDP. The anionic core is then combined with cationic liposomes composed of DOTAP and cholesterol to produce the outer membrane of the core-membrane structure (Fig. 1C).