European Journal of Pharmaceutics and Biopharmaceutics 87 (2014) 187–196

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Combination cytotoxicity of backbone degradable HPMA copolymer gemcitabine and platinum conjugates toward human ovarian carcinoma cells

Acharaporn Duangjai [a,b,1], Kui Luo [a,1], Yan Zhou [a,1], Jiyuan Yang [a], Jindřich Kopeček [a,c,*]

[a] Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT, USA
[b] School of Medical Sciences, University of Phayao, Phayao, Thailand
[c] Department of Bioengineering, University of Utah, Salt Lake City, UT, USA

ARTICLE INFO

Article history:
Received 21 August 2013
Accepted in revised form 28 November 2013
Available online 4 December 2013

Keywords:
HPMA copolymers
Biodegradable polymers
Combination therapy
Ovarian cancer
Gemcitabine
DACH Pt

ABSTRACT

Multiblock, backbone degradable HPMA copolymer–drug conjugates containing gemcitabine and DACH platinum (mP-GEM and mP-DACH Pt), respectively were synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization and subsequent chain extension by click chemistry. Using combination index analysis, the cytotoxicities of the two multiblock conjugates, as single agent and in combination, were evaluated *in vitro* in A2780 human ovarian cancer cells, with free drugs as controls. The greatest synergistic cytotoxic effect was observed when A2780 cells were sequentially exposed to mP-GEM for 24 h and mP-DACH Pt for 48 h. In addition, mechanistic studies support the rationale of the synergy between mP-GEM and mP-DACH Pt: mP-GEM pretreatment was able to enhance the platinum–DNA adduct accumulation and inhibit cell proliferation to a higher extent than single mP-DACH Pt treatment. These observations are useful for the development of combination macromolecular therapeutics for ovarian cancer based on the second-generation backbone degradable HPMA copolymers.

1. Introduction

Ovarian cancer is the fifth most common cause of death in women and most lethal cancer of the female reproductive system [1,2]. It caused approximately 16,000 deaths and about 22,000 new cases were diagnosed in the United States in 2012 [1]. The traditional anti-ovarian cancer treatment includes surgical debulking followed by chemotherapy [2]. However, the prognosis of ovarian cancer following treatment is very poor in the majority of ovarian cancer patients, especially the patients at stages III or IV when diagnosed.

To improve the efficacy of chemotherapy, combination therapies of platinum and nonplatinum agents were commonly employed, including the combination of gemcitabine and platinum agent such as oxaliplatin, cisplatin, or carboplatin [2,3]. Gemcitabine, a synthetic nucleoside analog of cytidine, is activated inside the cells into its triphosphate analog (dFdCTP). dFdCTP is an inhibitor of DNA polymerase and can inhibit DNA synthesis by incorporation into DNA [4,5]. Platinum agents, including cisplatin, carboplatin, or oxaliplatin, have been used clinically for the treatment of many types of cancers [6]. Platinum–DNA adducts can cause cell cycle arrest, cell replication arrest and apoptosis [7]. In particular, platinum agents containing DACH ligand (such as carboplatin or oxaliplatin) possess low toxicity and induce DNA lesions which are more resistant to DNA repair pathways than that caused by cisplatin [8]. Because of the synergistic anti-cancer effects as well as the non-overlapping side effects of the two drugs, the combination of gemcitabine and platinum agent such as carboplatin has been approved to treat patients with advanced ovarian cancer [9–11]. Nonetheless, this combination often shows suboptimal anti-cancer response or unfavorable toxicity profile clinically [3,12]. Therefore, strategies to further improve the anti-cancer efficacy and reduce the toxicity of the combination chemotherapies are still of great need.

N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers have been widely used as drug carriers to improve the efficacy and reduce the toxicity of chemotherapeutic agents [13–16]. Nanosized HPMA copolymer–drug conjugates improve the water solubility of chemotherapeutics, increase the circulation time of the drug and lead to enhanced drug accumulation in tumor tissue via the enhanced permeability and retention (EPR) effect [17–19]. Previous studies have shown that HPMA copolymer–DACH platinum (dichloro(1,2-diaminocyclohexane)platinum(II)) conjugate with molecular weight lower than 45 kDa was equally or likely more

* Corresponding author. University of Utah, Center for Controlled Drug Delivery, 2030 E 20 S BPRB, Room 205B, Salt Lake City, UT 84112-9452, USA. Tel.: +1 801 581 7211; fax: +1 801 581 7848.
E-mail address: jindrich.kopecek@utah.edu (J. Kopeček).
[1] These authors contributed equally to this work.

0939-6411/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejpb.2013.11.008

effective than free Oxaliplatin in treating ovarian cancer patients with excellent tolerability in clinical trials [20]. However, the low molecular weight of the first-generation HPMA copolymer–drug conjugates limits their clinical translation.

Molecular weight and molecular weight distribution are important factors in the design of effective macromolecular therapeutics. The higher the molecular weight of the polymer carrier, the higher the accumulation of polymer–drug conjugates in the tumor tissue with concomitant increase in therapeutic efficacy [17,18,21–23]. However, the renal threshold limits the molecular weight of the first-generation (non-degradable) polymeric carriers to below 50 kDa [24]; this lowers the retention time of the conjugate in the circulation with simultaneous decrease in pharmaceutical efficiency. Higher molecular weight drug carriers with a non-degradable backbone deposit and accumulate in various organs, impairing biocompatibility. Clearly, the design of long-circulating backbone degradable HPMA copolymer carriers offers a solution to this problem [21–23,25–27]; it provides a long-circulating polymeric carrier without impairing biocompatibility – the degradation fragments are below the renal threshold.

Recently, backbone degradable multiblock HPMA copolymer drug carriers have been successfully developed through reversible addition-fragmentation chain transfer (RAFT) polymerization and subsequent chain extension by click chemistry [25–27]. The resulting conjugates are composed of alternating low molecular weight HPMA copolymer–drug segments and enzymatically degradable GFLG (glycylphenylalanylleucylglycyl) tetrapeptide segments. The enhanced efficacy of the new, multiblock backbone degradable HPMA copolymer conjugates (when compared to low molecular weight conjugates) has been demonstrated on ovarian cancer xenografts in mice [21,22] and in a rat osteoporosis model [23]. In addition, incorporating free gemcitabine into the multiblock HPMA copolymer carrier helps to stabilize the free drug in circulation. Free gemcitabine can be deactivated in circulation after converting to a uracil metabolite (2′-deoxy-2′-difluorouridine (dFdU)), which limits its efficacy [28].

Previously, we have developed a novel concept of using combination therapy with water-soluble polymer-bound drugs. *In vivo* combination chemotherapy and photodynamic therapy (PDT) studies on two cancer models, Neuro 2A neuroblastoma induced in A/J mice [29] and human ovarian carcinoma heterotransplanted in nude mice [30–32], demonstrated that macromolecular combination therapy produced tumor cures which could not be obtained with either chemotherapy or PDT alone. Other combination systems were quantitatively evaluated by combination index (CI) analysis in A498 renal carcinoma cells [33] and in OVCAR-3 ovarian carcinoma cells [34]. The results demonstrated synergistic effects of HPMA copolymer–drug (SOS thiophene, doxorubicin, and chlorin e_6) conjugate combinations in a wide range of concentrations. Consequently, this manuscript aims to demonstrate that second-generation, backbone degradable conjugates have a potential in combination therapy.

To this end, we synthesized high molecular weight HPMA copolymer conjugates with gemcitabine (mP-GEM) and DACH Pt (mP-DACH Pt), respectively using RAFT copolymerization followed by alkyne-azide click chain extension. The design [25–26,35] provides an innovative therapeutic paradigm; moreover, this design has a competitive advantage with simplicity of structure, proven safety of the polymer carrier, and utilization of current effective drugs. Last but not least, the synthesis procedures proposed are versatile; they provide a platform for the preparation of a large variation of polymer–drug conjugates with tailor-made properties, such as predetermined circulation time and composition. The cytotoxicities of the two multiblock conjugates, as single agent and in combination, were evaluated *in vitro* in A2780 human ovarian cancer cells, with free drugs as controls. The combination effects and possible mechanism of synergy of mP-GEM and mP-DACH Pt were investigated.

2. Materials and methods

2.1. Materials

Gemcitabine hydrochloride (GEM, ⩾99.0%) was purchased from NetQem LLC (Research Triangle Park, NC). $DACHPtCl_2$ and common reagents were purchased from Sigma–Aldrich (St. Louis, MO) and used as received unless otherwise specified. Materials for peptide synthesis (including *N*-α-Fmoc protected amino acids, resin and coupling reagents) were purchased from AAPPTec Biosciences (Louisville, KY). HPMA [36], *N*-methacryloylglycylglycine p-nitrophenylester (MA-GG-ONp) [37], *N*-methacryloylglycylphenylalanylleucylglycyl gemcitabine (MA-GFLG-GEM) [26], 4-cyanopentanoic acid dithiobenzoate [38], and peptide2CTA (N^{α},N^{ε}-bis(4-cyano-4-(phenylcarbonothioylthio)pentanoylglycylphenylalanylleucylglycyl) lysine) [27] were synthesized as previously described. Initiators: V-65 (2,2′-azobis-(2,4-dimethylvaleronitrile)) was from Wako and V-501 (4,4′-azobis-(4-cyanovaleric acid)) was from Fluka-Sigma–Aldrich. 4,4′-Azobis(*N*,*N*′-propargyl-4-cyanopentanamide) (dialkyne-V-501) [25] and N^{α},N^{δ}-(bis(azidobenzoylglycylphenylalanylleucylglycylalanyl)lysine (diazide-GFLGK) were prepared according to the described procedures [25].

2.2. Synthesis and characterization of multiblock HPMA copolymer–drug conjugates

2.2.1. Synthesis and characterization of HPMA copolymer–gemcitabine conjugate (mP-GEM)

Polymerizable gemcitabine derivative, MA-GFLG-GEM, was copolymerized with HPMA via RAFT polymerization as previously reported [26] with modifications. Briefly, MA-GFLG-GEM (63.5 mg, 0.09 mmol) and HPMA (130 mg, 0.91 mmol) were dissolved in degassed DMSO and DI H_2O under nitrogen atmosphere, respectively. The solutions were transferred into an ampoule via syringe. After addition of peptide2CTA RAFT chain transfer agent, and initiator V-501, the ampoule was sealed, and then kept stirring at 70 °C for 16 h. The polymer was isolated by precipitation into acetone and purified by re-dissolving in methanol and precipitating into acetone two more times. The diblock HPMA copolymer–gemcitabine conjugate (2P-GEM) was obtained as a light pink powder. M_w 62 kDa; M_w/M_n 1.17.

Multiblock backbone degradable HPMA copolymer–gemcitabine conjugate with higher M_w (mP-GEM) were synthesized in two steps: first, 2P-GEM was post-polymerization end-modified with dialkyne-V-501 to produce a telechelic dialkyne conjugate; in the second step the conjugate was chain extended by click reaction with diazide-GFLGK in dimethylformamide (DMF) in the presence of $CuSO_4$ and sodium ascorbate (Fig. 1) [21]. The chain extended conjugate was fractionated on a preparative Superose 6 HR 16/60 column using acetate buffer pH 6.5/30% acetonitrile as the mobile phase. The fraction G2 of M_w 139 kDa (M_w/M_n 1.03) was used for further evaluation.

The molecular weight (weight average, M_w and number average, M_n) and molecular weight distribution of the conjugates were determined by size exclusion chromatography using a Superose 6 HR/16/30 column on an ÄKTA FPLC system (GE Healthcare) equipped with miniDAWN TREOS and OptilabEX detectors (Wyatt Technology, Santa Barbara, CA) with sodium acetate buffer containing 30% acetonitrile (pH 6.5) as mobile phase. HPMA homopolymer fractions were used as molecular weight standards.