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Synergistic antitumor activity of a self-assembling camptothecin and capecitabine hybrid prodrug for improved efficacy

Wang Ma [a,1], Hao Su [b,c,1], Andrew G. Cheetham [b,c,1], Weifang Zhang [a], Yuzhu Wang [b,c], QuanCheng Kan [a,*], Honggang Cui [a,b,c,d,e,**]

[a] Department of Oncology, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe Eastern Road, Zhengzhou 450052, Henan, China
[b] Department of Chemical and Biomolecular Engineering, The Johns Hopkins University, Baltimore, MD 21218, USA
[c] Institute for NanoBioTechnology, The Johns Hopkins University, Baltimore, MD 21218, USA
[d] Department of Oncology and Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
[e] Center for Nanomedicine, The Wilmer Eye Institute, Johns Hopkins University School of Medicine, 400 North Broadway, Baltimore, MD 21231, USA

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ABSTRACT

The direct use of anticancer drugs to create their own nanostructures is an emerging concept in the field of drug delivery to obtain nanomedicines of high drug loading and high reproducibility, and the combination use of two or more drugs has been a proven clinical strategy to enhance therapeutic outcomes. We report here the synthesis, assembly and cytotoxicity evaluation of self-assembling hybrid prodrugs containing both camptothecin (CPT) and a capecitabine (Cap) analogue. CPT and Cap molecules were conjugated onto a short β-sheet-forming peptide (Sup35) to yield three different self-assembling prodrugs (dCPT-Sup35, CPT-Cap-Sup35 and dCap-Sup35). We found that the chemical structure of conjugated drugs could strongly influence their assembled morphology as well as their structural stability in aqueous solution. With a decrease in number of CPT units, the resulting nanostructures exhibited a morphological transformation from nanofibers (dCPT-Sup35) to filaments (CPT-Cap-Sup35) then to spherical particles (dCap-Sup35). Notably, the hybrid CPT-Cap prodrug showed a synergistic effect and significantly enhanced potency against three esophageal adenocarcinoma cell lines compared with the two homo-prodrugs (dCPT-Sup35 and dCap-Sup35) as well as free parent drugs (CPT, 5-Fu and CPT/5-FU mixture (1:1)). We believe this work represents a conceptual advancement in integrating two structurally distinct drugs of different action mechanisms into a single self-assembling hybrid prodrug to construct self-deliverable nanomedicines for more effective combination chemotherapy.

1. Introduction

The use of nano-vectors to transport pharmacologically active ingredients has been extensively explored over the past three decades, aiming to improve the treatment efficacy while reducing the potential side effects [1–4]. Encapsulation of hydrophobic small molecule drugs into a nano-carrier can potentially increase their water solubility, prolong their circulation time and enhance their accumulation at target sites. For example, Abraxane®, a carrier-based formulation containing approximately 130 nm albumin-bound paclitaxel particles, was approved by the Food and Drug Administration (FDA) in 2005 for the treatment of metastatic breast cancers [5,6]. Clinical trials in randomized patients clearly revealed that patients administered with Abraxane® had a significantly higher reconciled target lesion response rate of 21.5%, compared to 11.1% response rate for patients receiving a paclitaxel injection (Taxol) [7,8]. Although there are many possible explanations for the improved response rate of Abraxane® (e.g. increased accumulation in the disease sites, and the biological roles of albumin in cellular uptake, transcytosis and intratumoral binding), one indisputable argument is that the replacement of Cremophor-EL with albumin eliminates solvent-related severe hypersensitivity thus allowing for a higher dose of paclitaxel to be administered [6,9]. The clinical success of Abraxane® highlights the importance of choosing a drug carrier for target therapy. However, over the past few years, despite significant progress having been made in the development of carrier-based nanomedicines, their clinical translations are still far below the public expectation [10–14], with only a select few having received FDA approval. One possible hurdle for developing more effective nanomedicines could arise from the complexity of formulating a carrier-based *multicomponent* drug delivery system which contains at least the carrier and one or more therapeutic agents, and often targeting ligands, excipients, and/or co-solvents.

* Corresponding author.
** Correspondence to: H. Cui, Department of Chemical and Biomolecular Engineering, The Johns Hopkins University, Baltimore, MD 21218, USA.
E-mail addresses: quanchengk0371@sina.com (Q. Kan), hcui6@jhu.edu (H. Cui).
[1] These authors contributed equally to the work.

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The use of rationally designed prodrugs to create well-defined nanostructures has become an attractive strategy to construct so called one-component nanomedicines, essentially blurring the line between the carrier and the drug [10,15–24]. This approach offers much greater control over the structural features and physicochemical properties of the resulting nanomedicines. A common strategy to achieve this involves the chemical conjugation of an auxiliary segment to anticancer drugs to furnish the resulting prodrugs with an overall amphiphilicity. These rationally designed amphiphilic prodrugs could potentially serve as molecular building units to construct nano-objects of various size, shape and surface chemistries, with self-deliverable characteristics. For example, the Kataoka group pioneered a micelle-forming block copolymer-drug conjugate system with doxorubicin and poly(ethylene glycol)-poly(α,β-aspartic acid) (PEG-*b*-PAsp) [17,18]. Cheng and co-workers reported the use of drugs as initiators to trigger polymerizations and subsequent formulation of nanoconjugates using nanoprecipitation methods [25,26]. The Chilkoti group covalently bonded multiple hydrophobic drug molecules to artificial recombinant chimeric polypeptides to obtain sub-100-nm-sized nanoparticles [19]. Xu and co-workers made filamentous assemblies from peptidic amphiphilic prodrugs to form supramolecular hydrogels [24,27]. Our lab has been motivated by the intriguing assembly potential and biological roles of small molecule peptides to design and synthesize a great diversity of self-assembling peptide-drug conjugates [28–36]. These self-assembling prodrugs can give rise to interesting self-assembly characteristics through rational adjustment of the mass ratio/number of hydrophobic drugs within the prodrug, while maintaining their high potency against a variety of cancer cell lines. It has been shown in our studies that the chemical structure of the conjugated drug can have a strong impact on the hydrophilic–lipophilic balance (HLB) value and the degree of molecular packing of the resultant conjugates, thus affecting their assembled morphologies and structural stability [28]. We have reported that altering the number of camptothecin (CPT) molecules from one to four in the prodrug design could tune the resulting nanostructures from long filaments to short filaments and then to nanotubes [29]. In addition, reduced potency is often an unavoidable outcome of the prodrug design strategy due to the retarded drug release rate and the decreased cellular uptake efficiency [37]. Although we have demonstrated that combining effective drug release with a balanced HLB value in the molecular design can give prodrugs as potent as the parent drug [37,38], further optimization and improvement of the drug potency is difficult to achieve with a single type of drug.

We report here the synthesis, assembly and toxicity evaluation of self-assembling prodrugs containing both CPT and a capecitabine (Cap) analogue. The use of two or more therapeutic agents of different action mechanisms is a well-known strategy to overcome multidrug resistance [39], and our motivation here is to develop an in-depth understanding of the assembly behavior and the altered potency of self-assembling prodrugs containing two or more structurally and mechanistically distinct drugs. CPT, a DNA topoisomerase I inhibitor, is a natural plant alkaloid isolated from the Chinese tree *Camptotheca acuminate* [40]. Although it exhibits extraordinary in vitro anticancer activity, poor solubility and stability in biological environments have hindered its further clinical usage. Two CPT analogues, topotecan and irinotecan, have been approved by the FDA and are used in cancer chemotherapy [40]. The former is mainly used to treat ovarian cancer (approval in 1996), cervical cancer (approval in 2006) and small cell lung cancer (approval in 2007), while the latter is utilized for colon cancer (full approval in 1998) and usually in combination with other chemotherapeutics [41]. Cap is a FDA-approved prodrug of 5-fluorouracil (5-FU) that is sequentially converted to 5-FU by three enzymes located in the liver and in tumors [42,43]. 5-FU exerts its anticancer effects primarily through the irreversible inhibition of thymidylate synthase, resulting in impaired DNA synthesis and cell death [43,44]. Both 5-FU and Cap are widely used in the treatment of a range of cancers, including colorectal, breast, gastric and esophageal cancers. In particular, esophageal cancer is a highly virulent malignancy, which comprises two different types – squamous cell carcinoma and adenocarcinoma [45,46]. High incidences of squamous cell carcinoma are observed in Asian countries whereas adenocarcinoma is increasingly more common in Western countries. Almost half of the patients are in the advanced stage at diagnosis and the natural course encompasses only 8 to 10 months overall survival time, with a 5-year mortality of 85%–90% [47]. In the treatment of esophageal cancer, systemic chemotherapy is the mainstay of current standard care and the obvious need to improve the therapeutic efficacy leads to the development of combination chemotherapy [48]. A recent phase III study has evaluated the combination usage of 5-FU, irinotecan and leucovorin as the first-line treatment against advanced gastric or esophagogastric junction adenocarcinoma, demonstrating significantly improved therapeutic outcomes [49]. Inspired by the combination regimen of irinotecan and 5-FU, we integrated the hydrophobic drug CPT and water soluble drug Cap with a short β-sheet-forming peptide to yield three self-assembling prodrugs. We found that the molecular structures of the drugs could have a great impact on the resulting assembled morphology as well as the nanostructure stability. More importantly, due to the differing anticancer mechanisms of the two parent drugs, our CPT-Cap hybrid prodrug showed an improved efficacy against esophageal cancer cell lines compared with dual CPT-containing and dual Cap-containing prodrugs as well as free parent drugs. We envision that the merging of two structurally distinct drugs into a single self-assembling prodrug offers a promising strategy to influence the self-assembly behavior and leads to more effective cancer chemotherapies.

2. Experimental sections

2.1. Materials

Fmoc amino acids (except Fmoc-Lys(Fmoc)-OH) were purchased from Advanced Automated Peptide Protein Technologies (AAPPTEC, Louisville, KY, USA). Rink Amide MBHA resin and Fmoc-Lys(Fmoc)-OH were purchased from Novabiochem (San Diego, CA, USA). Camptothecin was obtained from AvaChem Scientific (San Antonio, TX, USA). 5′-Deoxy-5-fluorocytidine (5′-DFCR) was purchased from TCI America (Philadelphia, PA) and all other reagents were sourced from Sigma-Aldrich (St. Louis, MO) or VWR (Radnor, PA, USA), unless indicated otherwise.

2.2. Methods

RP-HPLC was performed on a Varian ProStar Model 325 HPLC (Agilent Technologies Santa Clara, CA). Preparative separations utilized a Varian PLRP-S column (100 Å, 10 μm, 150 × 25 mm), while analytical HPLC used a Varian Pursuit XRs C18 column (5 μm, 150 × 4.6 mm). Acidic solutions (water and acetonitrile containing 0.1% v/v TFA) were used as the mobile phase. Purified materials were lyophilized using a FreeZone − 105 °C 4.5 L freeze dryer (Labconco, Kansas City, MO). ESI-MS mass spectrometric data for characterization was obtained on a Finnigan LDQ Deca ion-trap mass spectrometer (Thermo-Finnigan, Waltham, MA). Bruker Avance 300 or 400 MHz FT-NMR spectrometers were used for the acquisition of ^{1}H and ^{13}C NMR spectra.

2.3. Synthesis and characterization of the self-assembling prodrugs

The peptide $(Ac\text{-}Cys)_2KGN_2Q_2NYK_2\text{–}NH_2$ (dCys-Sup35) sequence was synthesized using standard Fmoc solid-phase synthesis techniques. The synthesis of dCPT-Sup35, CPT-Cap-Sup35 and dCap-Sup35 were carried out in nitrogen-purged DMSO with a 1:1 mixture of CPT-etcSS-Pyr and Cap-etcSS-Pyr prodrugs and dCys-Sup35. After reacting for 2 days, the mixture was diluted with 0.1% TFA in acetonitrile/water and purified by preparative RP-HPLC. Collected fractions were analyzed by ESI-MS, and the appropriate fractions were combined, concentrated