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Lung cancer specific and reduction-responsive chimaeric polymersomes for highly efficient loading of pemetrexed and targeted suppression of lung tumor *in vivo*

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ABSTRACT

Lung cancer is one of the worldwide leading and fast-growing malignancies. Pemetrexed disodium (PEM, Alimta®), a small hydrophilic drug, is currently used for treating lung cancer patients. However, PEM suffers from issues like fast elimination, low bioavailability, poor tumor cell selectivity and penetration. Here, we report on lung cancer specific CSNIDARAC (CC_9) peptide-functionalized reduction-responsive chimaeric polymersomes (CC_9-RCPs) for efficient encapsulation and targeted delivery of PEM to H460 human lung cancer cells *in vitro* and *in vivo*. PEM-loaded CC_9-RCPs (PEM-CC_9-RCPs) was obtained from co-self-assembly of poly(ethylene glycol)-*b*-poly(trimethylene carbonate-*co*-dithiolane trimethylene carbonate)-*b*-polyethylenimine (PEG-P(TMC-DTC)-PEI) and CC_9-functionalized PEG-P(TMC-DTC) in the presence of PEM followed by self-crosslinking. PEM-CC_9-RCPs displayed an optimal CC_9 density of 9.0% in targeting H460 cells, a high PEM loading content of 14.2 wt%, a small hydrodynamic size of ca. 60 nm and glutathione-triggered PEM release. MTT assays showed that PEM-CC_9-RCPs was 2.6- and 10- fold more potent to H460 cells than the non-targeting PEM-RCPs and free PEM controls, respectively. Interestingly, PEM-CC_9-RCPs exhibited 22-fold longer circulation time and 9.1-fold higher accumulation in H460 tumor than clinical formulation Alimta®. Moreover, CC_9-RCPs showed obviously better tumor penetration than RCPs. Remarkably, PEM-CC_9-RCPs at 12.5 mg PEM equiv./kg effectively suppressed growth of H460 xenografts and significantly prolonged mouse survival time as compared to PEM-RCPs and Alimta® controls. These lung cancer specific and reduction-responsive chimaeric polymersomes provide a unique pemetrexed nanoformulation for targeted lung cancer therapy.

Statement of Significance

Multitargeted antifolate agent pemetrexed (PEM, Alimta®) is currently used for treating lung cancer patients and has low side-effects. However, PEM suffers from issues like fast elimination, low bioavailability, poor tumor cell selectivity and penetration. Scarce work on targeted delivery of PEM has been reported, partly because most conventional nanocarriers show a low and instable loading for hydrophilic, negatively charged drugs like PEM. Herewith, we report on lung cancer specific CSNIDARAC (CC_9) peptide-functionalized reduction-responsive chimaeric polymersomes (CC_9-RCPs) which showed efficient PEM encapsulation (14.2 wt%, 60 nm) and targeted delivery of PEM to H460 human lung cancer cells, leading to effective suppression of H460 tumor xenografts and significantly prolonged survival rates of mice than Alimta®. To the best of our knowledge, this represents a first report on targeted nanosystems that are capable of efficient loading and targeted delivery of PEM to lung tumors.

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Introduction

Lung cancer, among which over 80% is non-small cell lung cancer (NSCLC), becomes one of the worldwide leading and fast growing malignancies [1]. Targeted chemotherapy using Iressa, Tarceva and Camry, though could prolong the survival 2–3 times for patients with EGFR-mutant lung cancer, is not effective to majority of NSCLC patients that are EGFR mutation-negative [2,3]. Chemotherapeutics including *cis*-platinum, carboplatin, paclitaxel (PTX), docetaxel (DTX) and pemetrexed disodium (PEM) are routinely used for treating NSCLC in the clinical settings [4–6]. Notably, PEM, a folate antimetabolite inhibiting DNA and RNA synthesis in cancer cells [4], has received particular attention because it is a multitargeted antifolate agent and has relatively less side-effects [7–9]. PEM for injection (Alimta®) is a second-line drug for NSCLC and first-line drug for malignant pleural mesothelioma [10,11]. However, clinical use of PEM suffers from several problems like fast elimination, low bioavailability, poor tumor cell selectivity and penetration, and potential spleen and kidney toxicity [12,13].

It is interesting to note that despite its clinical significance, scarce work on targeted delivery of PEM has been reported. This is partly due to the fact that most conventional nanocarriers including liposomes, nanoparticles, and micelles show a low and instable loading for hydrophilic, negatively charged, and small drugs like methotrexate disodium (MTX) and PEM [14–16]. For example, a low PEM loading efficiency of <14% was reported for liposomes [17]. We recently reported that chimaeric polymersomes with short polycations such as PDMA and PEI in the watery interior achieved highly efficient and stable encapsulation of MTX and siRNA [18,19].

Here, we report for the first time on lung cancer specific CSNIDARAC peptide-functionalized and reduction-responsive chimaeric polymersomes (CC_9-RCPs) for efficient encapsulation and targeted delivery of PEM to H460 human lung cancer cells *in vitro* and *in vivo* (Scheme 1). PEM-CC_9-RCPs were readily prepared by co-self-assembly of poly(ethylene glycol)-*b*-poly(trimethylene carbonate-*co*-dithiolane trimethylene carbonate)-*b*-polyethylenimine (PEG-P(TMC-DTC)-PEI) and CC_9-functionalized PEG-P(TMC-DTC) copolymers in the presence of PEM. CC_9 peptide screened by phage display technology was shown to be specific to lung cancer cells such as H460 cells [20]. CC_9-RCPs were expected to be self-crosslinkable, robust, and glutathione-responsive, as previously reported for dithiolane trimethylene carbonate (DTC)-containing polymersomes and micelles [21,22]. Strikingly, PEM-loaded CC_9-RCPs show significantly enhanced *in vitro* antitumor activity, circulation time, as well as tumor accumulation and suppression in human H460 lung tumor-bearing nude mice as compared to the clinical formulation Alimta®. These lung cancer specific and reduction-responsive chimaeric polymersomes provide a unique pemetrexed nanoformulation for targeted lung cancer therapy.

Experimental

2.1. Preparation of CC_9-RCPs and PEM-CC_9-RCPs

PEM-CC_9-RCPs and CC_9-RCPs were prepared from co-self-assembly of PEG-P(TMC-DTC)-PEI [19] and CC_9-PEG-P(TMC-DTC) at CC_9 molar ratios of 0, 4.5%, 9.0% or 13.5%. Briefly, 50 μL of mixed polymer solution in DMSO (10 mg/mL) was injected into 0.95 mL of HEPES buffer (pH 5.5, 5 mM) with or without PEM. After standing still for 20 min, the polymersomes were gently mixed and placed into shaking bath (200 rpm, 37 °C) for 12 h and followed by extensive dialysis for 24 h (MWCO 3500). Dynamic light scattering (DLS), static light scattering (SLS) and TEM measurements were measured. The drug loading content (DLC) and drug loading efficiency (DLE) of PEM were determined with UV–Vis spectroscopy at 246 nm. The *in vitro* PEM release was studied at polymersome concentration of 100 μg/mL using HPLC as described in supporting information.

2.2. MTT assays

H460 cells were seeded in a 96-well plate (5×10^3 cells/well) and cultured for 24 h using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (final conc. 2 mM), antibiotics penicillin (100 IU/mL) and streptomycin (100 μg/mL). For assessment of their targeting effect, PEM-CC_9-RCPs with CC_9 molar ratio of 0, 4.5%, 9.0% or 13.5% (PEM concentration: 5 μg/mL) were added. The cells were incubated in an atmosphere containing 5% CO_2 at 37 °C for 4 h. The culture medium was replaced with fresh medium and the cells were incubated for 68 h. MTT assays (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) were performed as described in our previous report [23]. PEM-RCPs and free PEM were used as controls.

For determination of the half-maximal inhibitory concentration (IC_{50}), H460 cells were incubated with PEM-CC_9-RCPs with 9.0 mol.% CC_9, PEM-RCPs or free PEM in 20 μL PB (PEM concentrations: 0.001–20 μg/mL) for 4 h. The rest was performed as above. The IC_{50} values were derived by fitting the cell viability data using Prism 7. For evaluation of the cytotoxicity of empty polymersomes, H460 cells were incubated with CC_9-RCPs and RCPs at varying concentrations of 0.1, 0.3 and 0.5 mg/mL at 37 °C for 48 h before MTT assays.

2.3. Animal models

All animal experiments were approved by the Animal Care and Use Committee of Soochow University (P.R. China) and all protocols of animal studies conformed to the Guide for the Care and Use of Laboratory Animals. For analysis of blood circulation, female Balb/c mice (18–22 g) were used. Mice bearing H460 tumor xenografts were built by subcutaneous injection of 0.05 mL of H460 cells (1×10^7) into the right hind flank of female nude mice. Tumor inhibition experiments started when the tumors reached 100 mm^3 after ca. 2 weeks. At tumor size of 150–200 mm^3, tumor penetration of polymersomes was studied. At tumor size of 200–300 mm^3, the *in vivo* imaging and biodistribution experiments were conducted. Before each experiment, the mice were weighed and randomly grouped.

2.4. *Biodistribution of PEM-CC_9-RCPs in H460 tumor bearing nude mice*

A single dose of PEM-CC_9-RCPs or PEM-RCPs in 0.2 mL PB was administrated into H460 tumor bearing mice via tail veins at 12.5 mg PEM equiv./kg. At 8 h post injection, the mice were sacrificed, the tumors and major organs were collected, and homogenized and treated as described in previous report [18] before PEM determination by HPLC (expressed as percentage of injected dose per gram of tissue, %ID/g).

2.5. *Tumor penetration of PEM/Cy5-CC_9-RCPs*

To investigate PEM accumulation and penetration in the tumors, PEM and Cy5-sulfo co-loaded polymersomes, i.e. PEM/Cy5-CC_9-RCPs and PEM/Cy5-RCPs were prepared and used to track the location of drugs in the tumors. A single dose of PEM/Cy5-CC_9-RCPs or PEM/Cy5-RCPs in 0.2 mL of PB (0.4 μmol Cy5 equiv./kg, 12.5 mg PEM equiv./kg) was injected via tail veins into H460 tumor

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