Granzyme B-loaded, cell-selective penetrating and reduction-responsive polymersomes effectively inhibit progression of orthotopic human lung tumor in vivo

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**ABSTRACT**

The clinical use of protein therapeutics with intracellular targets is hampered by its in vivo fragility and low cell permeability. Here, we report that cell-selective penetrating and reduction-responsive polymersomes (CPRPs) mediate high-efficiency targeted delivery of granzyme B (GrB) to orthotopic human lung tumor in vivo. Model protein studies using FITC-labeled cytochrome C (FITC-CC) revealed efficient and high protein loading up to 17.2 wt% for CPRPs. FITC-CC-loaded CPRPs exhibited a small size of 82–90 nm, reduction-responsive protein release, as well as greatly enhanced internalization and cytoplasmic protein release in A549 lung cancer cells compared with the non-targeted FITC-CC-loaded RPs control. GrB-loaded CPRPs showed a high potency toward A549 lung cancer cells with a half maximal inhibitory concentration (IC\textsubscript{50}) of 20.7 nM. Under the same condition, free GrB was essentially non-toxic. Importantly, installing cell-selective penetrating peptide did not alter the circulation time but did enhance tumor accumulation of RPs. Orthotopic A549-Luc lung tumor-bearing nude mice administered with GrB-loaded CPRPs at a dosage of 2.88 nmol GrB equiv./kg showed complete tumor growth inhibition with little body weight loss throughout the treatment period, resulting in significantly improved survival rate over the non-targeted and non-treated controls. These cell-selective penetrating and reduction-responsive polymersomes provide a targeted protein therapy for cancers.

1. Introduction

Proteins have emerged as advanced therapeutics for cancer therapy [1–5]. As compared to cytotoxic chemical drugs, proteins display usually better specificity. The clinical use of protein therapeutics with intracellular targets [6,7] is, however, hampered by its in vivo fragility and low cell permeability [8–11]. Cell-penetrating peptides (CPPs) such as transactivator of transcription (TAT) have shown to efficiently chaperone functional proteins into various mammal cells [12–14]. Despite a high in vitro efficacy, protein-CPP conjugates reveal a poor protein delivery in vivo because cationic CPPs potentiate non-specific interactions and have low cell selectivity. Moreover, the conjugation of CPP to proteins demands delicate synthesis and would possibly reduce protein bioactivity.

In the past decade, nanosystems such as polyion complexed micelles [15,16], nanogels [17–21], nanocapsules [22–24] and polymersomes [25–27] have been exploited to deliver proteins intracellularly. In particular, polymersomes with large aqueous interiors are interesting for physical encapsulation and systemic delivery of proteins [28–32]. Notably, the membrane of polymersomes not only serves to protect cargos from leakage and degradation but also can be explicitly engineered with stimuli-sensitivity to tailor the site and rate of drug release [33–36]. The polymersome surfaces can further be installed with cell-selective ligands like peptides [37], lactoferrin [38], galactose [39] or antibodies [40] to enhance specific cell internalization [41–45]. It should be noted, however, that inefficient intracellular trafficking especially endosomal entrapment is a limiting step for protein delivery nanosystems [46,47]. Interestingly, Matsushita et al. developed tumor-
selective CPP denoted as CPP33 (sequence: RLWMRWYSPRTRAYG) that was reported to selectively penetrate A549 lung cancer cells [48]. We and He’s group demonstrated that functionalization of polymersomes and nanoparticles, respectively, with CPP33 peptide significantly improved chemotherapy for A549 lung tumor-bearing mice [49,50]. Lung cancer is a malignancy with high mortality and morbidity [51,52]. Despite significant improvement achieved in the past years, chemotherapy for lung cancer patients remains to be associated with notable adverse effects and drug resistance [53,54].

Here, we investigated cell-selective penetrating and reduction-responsive polymersomes (CPRPs) for targeted delivery of granzyme B (GrB), a natural and potent apoptotic protein secreted by cytotoxic T cells and NK cells, to orthotopic human lung tumor xenografts (Scheme 1). Unlike chemotherapeutics, free GrB is practically non-cytotoxic to cells [39,55]. However, upon releasing into cancer cells, it induces superior inhibitory effects with a low IC_{50} in the range of 1.6 nM to 500 nM depending on the intracellular delivery tools [29,30,39,56,57]. We reported previously that cNGQ peptide (sequence: cNGQGEQc, specific to α3β1 integrin) functionalized, reversibly-crosslinked chimeraic polymersomes based on poly(ethylene glycol)-b-poly(trimethylene carbonate-co-dithiolane trimethylene carbonate)-b-polyethylenimine (PEG-P(TMC-DTC)-PEI) mediated efficient systemic delivery of siRNA [58]. Remarkably, our results show that GrB-loaded CPRPs (2.88 nmol GrB equiv./kg) induce complete growth inhibition of orthotopic A549-Luc human lung tumor xenografts in nude mice with little side effects throughout the treatment period, significantly improving mice survival rate. CPP33 peptide-guided and reversibly crosslinked polymersomes provide a targeted protein therapy for lung cancers. CPRPs have many unique advantages over previously reported protein delivery nanosystems including easy fabrication, high protein encapsulation, small size, high specificity toward lung cancer cells, and fast cytoplasmic protein release.

2. Experimental section

2.1. Preparation of protein-loaded CPRPs

Cytochrome C was labeled with FITC (FITC-CC) to visualize protein inside the cells, as reported previously [30]. Protein (FITC-CC or GrB)-loaded CPRPs were prepared via self-assembly process by adding 50 μL of CPP33-PEG-P(TMC-DTC) and PEG-P(TMC-DTC)-PEI at predetermined molar ratios in DMSO (10 mg/mL) into 950 μL HEPES buffer (pH 6.8, 5 mM) containing different proteins under stirring. After standing still for 20 min and incubating for 6 h in a shaking bath (200 rpm, 37 °C), the polymersomes were extensively dialyzed (MWCO 300 KDa) 24 h against phosphate buffer (PB, pH 7.4, 5 mM). Using FITC-CC as a model protein, the protein loading content (PLC) and protein loading efficiency (PLE) were determined with UV–vis (492 nm). The in vitro release of proteins was described in the supporting information.

Scheme 1. Schematic of cell-selective penetrating and reduction-responsive polymersomes (CPRPs) for targeted delivery of GrB to nude mice bearing orthotopic A549-Luc lung tumor xenograft. GrB is efficiently loaded into the lumen of CPRPs which are made from CPP33-PEG-P(TMC-DTC) and PEG-P(TMC-DTC)-PEI. GrB-loaded CPRPs are stable in circulation and selectively home to A549 lung tumor. CPP33 peptide mediates efficient penetration of GrB-loaded CPRPs to A549 lung cancer cells. In the cytoplasm, GrB is quickly released as a result of reduction-triggered de-crosslinking of CPRPs.
fetal bovine serum (FBS), 1% L-glutamine, antibiotics penicillin and streptomycin (100 μg/mL). For determination of the cytotoxicity of empty polymersomes, A549 cells were incubated with CPRPs or RPs (final polymersome concentration = 0.1, 0.3 or 0.5 mg/mL) at 37 °C for 48 h.

### 2.3. Cellular uptake and intracellular release of FITC-CC

A549 cells were cultured on microscopic coverslips in 24-Well plates (5.0 × 10^5 cells/well), and incubated with FITC-CC-CPRPs, FITC-CC-RPs, or free FITC-CC (FITC-CC dosage: 3.85 μM, FITC: 7.20 nmol equiv./mL) in 100 μL of PBS at 37 °C for 12 h. The culture medium was removed and the cells on coverslips were washed with PBS (×3), fixed with 4% formaldehyde for 15 min and washed with PBS (×3). The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min followed by PBS washing (×3). Fluorescence images of cells were obtained using confocal laser scanning microscope (CLSM, TCS SP5).

To investigate the cell entry and endosomal escape, A549 cells cultured on coverslips in 24-Well plates (5.0 × 10^5 cells/well) were incubated with FITC-CC-CPRPs (FITC-CC dosage: 3.85 μM, FITC: 7.20 nmol equiv./mL) for 0.5, 1 or 2 h. Then the cells on microscope coverslips were treated with lysotracker-red (100 μM, 150 mM) for 50 min to stain the endosomes, followed by fixation (4% formaldehyde, 15 min), DAPI staining and CLSM observation. The cells were washed with PBS (×3) between each step.

To quantify the cellular uptake, A549 cells in a 6-well plate (1 × 10^6 cells/well) after incubation with FITC-CC-CPRPs, FITC-CC-RPs or free FITC-CC in 0.2 mL PBS (FITC-CC dosage: 3.85 μM, FITC: 7.20 nmol equiv./mL) at 37 °C for 2 h. PBS treated cells were taken as a blank. The cells were digested by 0.25% trypsin/0.03 w/v% EDTA, as reported earlier [30], by immediately recording the fluorescence histograms with a BD FACS Calibur flow cytometer and analysis using Cell Quest. 10,000 Gated events were analyzed to generate each histogram and the gate was arbitrarily set for the detection of FITC fluorescence.

### Table 1

Characteristics of FITC-CC-CPRPs and FITC-CC-RPs.

<table>
<thead>
<tr>
<th>Polymersomes</th>
<th>PLC (wt%)</th>
<th>PLE (%)</th>
<th>Size (nm)</th>
<th>PDF</th>
<th>ζ (mV)</th>
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<tr>
<td>FITC-CC-CPRPs</td>
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<td>-</td>
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<td>0.15</td>
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<tr>
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<td>4.8</td>
<td>-</td>
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<td>0.19</td>
<td>+1.9</td>
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<td>87</td>
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<td>83.2</td>
<td>90</td>
<td>0.21</td>
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<tr>
<td>FITC-CC-RPs</td>
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<td>0.16</td>
<td>+1.8</td>
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<tr>
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<td>86.4</td>
<td>78</td>
<td>0.20</td>
<td>+2.3</td>
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</table>

- CPP33 molar content: 18.5%;
- Determined by UV-vis
- Determined by DLS in PB (pH 7.4, 5 mM)
- Determined by electrophoresis in PB (pH 7.4, 5 mM).

A549 cells were plated 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, antibiotics penicillin (100 IU/mL) and streptomycin (100 μg/mL). For determination of the effect of CPP33 contents, GrB-CPRPs with CPP33 molar ratio of 9.1%, 18.5% and 28.0% (GrB concentration = 5 μg/mL) were used as controls. The cells were incubated in an atmosphere containing 5% CO₂ at 37 °C for 4 h. Then the cells were incubated in an atmosphere containing 5% CO₂ at 37 °C for 4 h. Then, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) solution in PBS (5.0 mg/mL) was added. MTT assays were performed as described in our previous report [30].

Similarly, for determination of half-maximal inhibitory concentration (IC₅₀), GrB-CPRPs (CPP33 molar ratio of 18.5%), GrB-RPs and free GrB (GrB concentrations varying from 0.0038 to 45.6 nM) in 20 μL PBS were added to A549 cells.

To determine the cytotoxicity of empty polymersomes, A549 cells were incubated with CPRPs or RPs (final polymersome concentration = 0.1, 0.3 or 0.5 mg/mL) at 37 °C for 48 h.

**Fig. 1.** (A) Size distribution of GrB-CPRPs (CPP33 content: 18.5 mol%) and GrB-RPs determined by DLS. (B) In vitro FITC-CC release in PBS (pH 7.4, 10 mM, 150 mM NaCl) with or without 10 mM DTT at 37 °C (Polymersome concentration = 100 μg/mL, FITC-CC concentration = 17.2 μg/mL, n = 3). (C) CD spectra of CC released from CC-CPRPs and native CC at 100 μg/mL. (D) The fluorescence spectra of the substrate Ac-IETD-AFC following 1 h treatment with GrB released from GrB-CPRPs, native GrB and PBS, respectively, at 37 °C.
2.4. Blood circulation of CPRPs-Cy5 in mice

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 pharmacokinetic studies, Cy5 labeled polymersomes CPRPs-Cy5 or RPs-Cy5 (0.25 mg Cy5 equiv./kg) in 200 μL PB was intravenously injected into Balb/c mice (18–22 g) via the tail vein (n = 3). At prescribed time points post injection, ca. ~30 μL of blood was withdrawn from the orbit of mouse. The blood samples upon withdrawing were immediately dissolved in 0.1 mL of Triton X-100 with brief sonification. Drug was extracted by incubating blood samples in 0.6 mL of extraction solution (DMSO containing 20 mM DTT) in a shaking bath (37°C, 200 rpm) overnight followed by centrifugation (14.8 krpm, 30 min). The Cy5 fluorescence intensity of the supernatant was determined using a fluorometer. Cy5 levels were expressed as injected dose per gram of blood (% ID/g). The blood circulation curves were obtained and the half-lives of two phases (t1/2,α and t1/2,β) were determined by fitting the experimental data using Software Origin 8 exponential decay 2 model: $y = A_1 \times \exp(-x/t_1) + A_2 \times \exp(-x/t_2) + y_0$, taking $t_{1/2,\alpha} = 0.693 \times t_1$ and $t_{1/2,\beta} = 0.693 \times t_2$.

2.5. The in vivo imaging of CPRPs-Cy5

The mice bearing orthotopic A549-Luc lung tumors were established as described in our early report [58]. Briefly, 0.1 mL of A549-Luc...
cell suspension (1 × 10^7 cells) was injected into the left lung parenchyma of nude mice (18–22 g). The bioluminescence of mouse lung and tumor volume was monitored using imaging system in live animals. After 2 weeks when tumors reached 1.0 × 10^7 p/s/cm^2/sr, the tumor-bearing mice were randomly grouped and intravenously injected with Cy5-labeled polymersomes (CPRPs-Cy5 or RPs-Cy5) in 0.2 mL PB (pH 7.4, 5 mM) via the tail veins (0.25 mg Cy5 equiv./kg). The mice were scanned at different time post-injection using IVIS II fluorescence imaging system.

2.6. In vivo antitumor efficacy of GrB-CPRPs

The mice bearing orthotopic A549-Luc lung tumors (1.0 × 10^7 p/s/cm^2/sr) were weighed and randomly divided into four groups (n = 6), and this day was designated as day 0. GrB-CPRPs and GrB-RPs (75 μg GrB equiv./kg) were injected via tail vein every 4 days. Empty CPRPs and PBS were used as controls. The treatment efficacy of mice was evaluated by monitoring tumor bioluminescence intensity. The relative body weight of the mice was normalized to their initial weight. On day 20, one mouse of each group was sacrificed by cervical vertebra dislocation. The heart, liver, spleen, lung and kidney were excised, fixed with 10% formalin and embedded in paraffin. The sliced organ tissues (thickness: 4 μm) mounted on the glass slides were stained by hematoxylin and eosin (H&E) and observed by a Leica digital microscope (Olympus BX41) at magnification (400×).

The Kaplan-Meier survival curve was determined within 60 days (n = 5). Mice in each cohort were considered to be dead either when the mice died during treatment or when the body weight decreased by 15% compared with initial value.

2.7. Statistical analysis

Data were expressed as mean ± SD. Differences between groups were assessed by one-way ANOVA with Tukey multiple comparison tests. Kaplan-Meier survival curves were analyzed by one-way ANOVA with a log-rank test for comparisons using GraphPad Prism 7. *p < .05 was considered significant, and **p < .01, ***p < .001 were considered highly significant.

3. Results and discussion

3.1. Preparation and characterization of protein-loaded CPRPs

Cell-selective penetrating and reduction-responsive polymersomes (CPRPs) were fabricated from co-self-assembly of CPP33-PEG-P(TMC-DTC) and PEG-P(TMC-DTC)-PEI copolymers (Table S1). Notably, proteins were conveniently loaded into the lumen of CPRPs by adding copolymer solution in DMSO into a protein-containing HEPES buffer.
(pH 6.8, 5 mM). Model protein studies using FITC-labeled cytochrome C (FITC-CC) and CPRPs with CPP33 peptide surface density of 18.5 mol% revealed that protein loading efficiency (PLE) was 95.1% at theoretical protein loading contents (PLC) of 1–5 wt% (Table 1). Even at a theoretical PLC of 20 wt%, a high PLE of 83.2%, which corresponded to a PLC of 17.2 wt%, was obtained. This high-efficacy protein loading is most probably owing to electrostatic interactions and hydrogen bonding of proteins with PEI in the lumen, as previously reported for different chimaeric polymersomes [49,58]. FITC-CC-loaded CPRPs (FITC-CC-CPRPs) exhibited slightly increased size from 82 to 90 nm with increase in PLC from 1.0 wt% to 17.2 wt% (Table 1). The non-targeted control, reduction-responsive polymersomes (RPs), revealed similar loading of FITC-CC. The size of FITC-CC-loaded RPs (FITC-CC-RPs) was somewhat smaller than that of FITC-CC-CPRPs, ranging from 67 to 78 nm with increase in PLC from 1.0 wt% to 17.8 wt% (Table 1). Notably, all FITC-CC-CPRPs displayed slightly positive surface charges (+1.9 ~ +3.5 mV), which were similar to FITC-CC-RPs, suggesting that decorating RPs with CPP33 peptide has little influence on their surface properties. Minimal positive surface charge is an important prerequisite for long circulation and cell selectivity [59]. In a similar way, GrB-loaded CPRPs (GrB-CPRPs) were fabricated at a low theoretical PLC of 1.8 wt%, given its high potency. Fig. 1A shows that GrB-CPRPs had a good distribution and a hydrodynamic size of 88 nm. GrB-loaded RPs (GrB-RPs, non-targeted control) displayed a smaller size of 75 nm. As for FITC-CC-CPRPs, GrB-CPRPs also had a close to neutral surface charges (Table 2). The similar biophysical properties (size, distribution and surface charge) observed for protein-loaded and blank polymersomes, indicating that loading of proteins has little influence on their structure. TEM micrograph confirmed that FITC-CC-CPRPs retained a vesicular structure (Fig. S1).

Using FITC-CC as a model protein, in vitro release studies demonstrated that protein release from CPRPs was < 13% in PBS within 24 h while ca. 88% FITC-CC was released in 24 h under conditions containing 10 mM dithiothreitol (DTT) (Fig. 1B). FITC-CC-RPs exhibited an identical protein release profile. Circular dichroism (CD) measurements revealed that CC released from CC-CPRPs had the same spectrum to native CC (Fig. 1C), indicating that proteins well preserved their secondary structure. We further evaluated the proteolytic activity of GrB released from GrB-CPRPs using a fluorescent Ac-IETD-AFC as a substrate. Fig. 1D shows that released GrB, similar to native GrB, efficiently cleaved the substrates resulting in fluorescence shift from 440 nm (Ac-IETD-AFC) to 490 nm (AFC). These results support that proteins encapsulated in CPRPs maintain their structure and bioactivity.

### 3.2. Intracellular protein delivery by CPRPs

GrB is a serine protease secreted by cytotoxic T cells and NK cells. As reported previously [21], free GrB caused little toxicity to A549 cells (Fig. 2A), owing to its inferior cellular uptake. Notably, GrB following loading into CPRPs exhibited significantly enhanced inhibitory effect to A549 cells, in which optimal CPP33 peptide molar content appeared to be 18.5 mol%. If not otherwise specified, CPRPs refer to the ones with.

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**Fig. 4.** Bioluminescence images of orthotopic A549-Luc lung tumor-bearing mice treated with GrB-CPRPs. GrB-RPs, CPRPs and PBS were used as controls. The mice were via i.v. injected at a dosage of 2.88 nmol GrB /kg on day 0, 4, 8 and 12.
CPP33 peptide molar content of 18.5 mol%. The IC50 of GrB-loaded CPRPs to A549 cells was determined to be 20.7 nM, which was ca. 2-fold lower than that of non-targeting GrB-RPs (Fig. 2B). Both empty polymersomes, CPRPs and RPs, were essentially non-toxic to A549 cells (cell viability > 85%) even at a concentration of 0.5 mg/mL and 48 h incubation (Fig. S2). GrB-CPRPs with 40 nM GrB corresponded to a CPRPs concentration of 0.05 mg/mL. It is clear that the enhanced antitumor activity of GrB-CPRPs over GrB-RPs is not due to direct cytotoxic effects of CPP33 peptide. To further investigate whether the observed killing effects of GrB-CPRPs is due to the specific pro-apoptotic effects of GrB, we conducted apoptosis assays using Annexin V-FITC/PI FACs analyses and caspase 3/7 activity in A549 cells using Apo-ONE homogeneous caspase3/7 assays, respectively. Fig. S3A demonstrates that after treatment with 7.5 nM GrB-CPRPs, 29.6% and 8.0% A549 cells underwent late and early apoptosis, respectively. In comparison, non-targeted GrB-RPs caused 15.2% late apoptosis and 6.7% early apoptosis. Fig. S3B displays that GrB-CPRPs induced pronounced caspase 3/7 activation with 5.5-fold higher caspase 3/7 activity in cleaving Z-DEVD-R110 substrate than empty CPRPs.

The cellular uptake and intercellular trafficking of proteins were investigated using FITC-CC as a model protein. Fig. 2C shows that installation of CPP33 peptide significantly enhanced cellular uptake, in which A549 cells treated with FITC-CC-CPRPs demonstrated 2.1 and 11.4-fold higher FITC-CC levels than cells with non-targeted FITC-CC-RPs and free FITC-CC controls, respectively. CLSM observation revealed that FITC-CC distributed all over the cells treated with FITC-CC-CPRPs (Fig. 2D). In comparison, significantly weaker FITC fluorescence was observed in A549 cells incubated with FITC-CC-RPs or free FITC-CC controls. These results support that CPP33 peptide greatly improves intracellular protein delivery.

We further stained the endo/lyso-somes of A549 cells with lysotracker red. Interestingly, significant amount of FITC-CC was delivered to the cytosol of A549 cells after incubation with FITC-CC-CPRPs for 1 h (Fig. 2E). This fast cytoplasmic delivery of proteins could be due to either direct transduction of FITC-CC-CPRPs or receptor-mediated endocytosis followed by endosomal escape.

Fig. 5. In vivo therapeutic efficacy of GrB-CPRPs toward orthotopic A549-Luc lung tumor-bearing mice. The mice were L.v. injected with 2.88 nmol GrB/kg on day 0, 4, 8 and 12. GrB-RPs, empty CPRPs and PBS were used as controls. (A) Dependence of A549-Luc bioluminescence levels in the lung of mice on time (n = 6). One-way ANOVA with Tukey multiple comparison tests, *p < .05, ***p < .001. (B) Ex vivo images of mice lungs on day 20. (C) Body weight changes of mice (n = 6). (D) Survival rates of mice (n = 5). Kaplan-Meier analysis (log-rank test for comparison): GrB-CPRPs vs. GrB-RPs: *p < .05; GrB-CPRPs vs. CPRPs and PBS: **p < .01; GrB-RPs vs. CPRPs and PBS: *p < .05. (E) Histological analyses of mice lungs (magnification: 400×). Circles and green arrows indicate the tumor region and cells, respectively. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.3. The in vivo pharmacokinetics and biodistribution of Cy5-labeled CPRPs

Pharmacokinetics studies demonstrated that Cy5-labeled polymersomes CPRPs-Cy5 had a long blood circulation time in mice with elimination half-life (t1/2,β) of 6.75 h, similar to that of non-targeted RPs-Cy5 (6.67 h) (Fig. 3A), indicating cell-selective penetrating peptide did not alter the blood circulation. To study in vivo tumor targetability of polymersomes, nude mice inoculated with orthotopic A549-Luc lung tumor were injected with CPRPs-Cy5 or RPs-Cy5 via tail veins of mice which showed similar initial Luc bioluminescence intensity in the lungs. In vivo NIR imaging results displayed that Cy5 fluorescence intensity in the lungs enhanced from 2 to 8 h and reduced from 8 to 24 h (Fig. 3B). It is noteworthy that mice treated by CPRPs-Cy5 had obviously better lung tumor accumulation than RPs-Cy5 treated ones, and strong fluorescence could still be seen at 24 h. The improved tumor accumulation of CPRPs is mainly attributed to the targeting effect of Cyp3 peptide to A549 lung tumors, which leads to not only better cellular uptake but also enhanced tumor retention [48–50].

3.4. Antitumor activity of GrB-CPRPs

The antitumor activity of GrB-CPRPs was assessed using orthotopic A549-Luc-bearing nude mice. The mice were i.v. injected with GrB-CPRPs every four days and for four times at a low GrB dosage of 2.88 nmol/kg. The non-targeted GrB-RPs and blank CPRPs were used as controls. Notably, Fig. 4A shows that mice treated with GrB-CPRPs had little change of A549-Luc bioluminescence within 20 days, indicating effective inhibition of tumor growth. GrB-RPs could also slow down tumor growth though obviously less effective than GrB-CPRPs (Fig. 4B). By contrast, tumor grew rapidly for mice administered with blank CPRPs and PBS (Fig. 4C and D). Semi-quantitative bioluminescence analyses exhibited that tumor growth was nearly completely subdued by GrB-CPRPs (Fig. 5A). GrB-CPRPs brought about significantly more effective inhibition of A549-Luc tumor growth than the non-targeted GrB-RPs, empty CPRPs and PBS. The ex vivo bioluminescence images of lung tumor excised on day 20 revealed aggressive invasion of A549-Luc cancer cells to the lungs of mice treated with blank CPRPs or PBS, and GrB-CPRPs inducing clearly the best inhibition (Fig. 5B).

Fig. 5C displays that mice treated with blank CPRPs or PBS had significant body weight loss (> 20% in 20 days), due to the fact that the invasion of A549-Luc tumor to lung would cause lung malfunction. On the contrary, little body weight loss was shown for the mice treated with GrB-CPRPs or GrB-RPs within 20 days, which on one hand supports their effective inhibition of tumor invasion into the lung, and on the other hand indicates that they have a low systemic toxicity. Remarkably, GrB-RPs (non-targeted control) significantly improved mice survival rate over the PBS control (median survival time: 35 versus 21 days) (Fig. 5D). GrB-CPRPs further improved median survival time to 48 days, which was a significant improvement compared with GrB-RPs. Blank CPRPs caused no change of survival rate relative to PBS, indicating that CPRPs do not induce adverse or therapeutic effects. Rosenblum et al. reported that GrB/scFvFvMEL fusion protein at a dosage of 288 nmol GrB/kg, which was 100-fold higher than GrB-CPRPs used in this study, could efficiently inhibit growth of human melanoma A375 xenograft tumors in nude mice [60]. Histological analyses illustrated that GrB-CPRPs and GrB-RPs treated mice had nearly no tumor tissue in the lung similar as unaffectted lung tissue. However, large tumor tissues were present in mouse lungs treated with PBS or CPRPs (Fig. 5E). No noticeable damage to other major organs like heart, liver, spleen or kidney was observed for all groups (Fig. 5A).

4. Conclusion

We have demonstrated that cell-selective penetrating and reduction-responsive polymersomes (CPRPs) mediate high-efficiency targeted delivery of granzyme B (GrB) to orthotopic human lung tumors in vivo, leading to complete suppression of tumor growth, little body weight loss throughout the treatment period, and significantly improved survival rate over the non-targeted and non-treated controls. GrB-CPRPs are unique for targeted protein therapy for lung tumor in that they show highly efficient protein loading, small size, sufficient in vivo stability, good targetability to A549 cells, strong tumor accumulation and tumor tissue penetration. Considering their biodegradability, biocompatibility and facile preparation, CPRPs are promising for constructing artificial killer cells for targeted protein therapy of non-small cell lung cancer.

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Appendix A. Supporting data

References


