A6 peptide-tagged, ultra-small and reduction-sensitive polymersomal vincristine sulfate as a smart and specific treatment for CD44+ acute myeloid leukemia

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ABSTRACT

Acute myeloid leukemia (AML) is a severe blood malignancy associated with a high relapse rate. The current clinical chemotherapy is typically perplexed with serious side effects. Here, A6 peptide-tagged, small and reduction-sensitive polymersomal vincristine sulfate (A6-cPS-VCR) is reported as a novel, smart and specific treatment for CD44 positive AML. A6-cPS-VCR stably loaded with 3.3 wt% VCR displays a size of ≈ 31 nm and pronounced selectivity toward CD44-overexpressed MV4-11 leukemia cells. Intriguingly, A6-cPS-VCR effectively represses the outgrowth of orthotopic MV4-11 AML in vivo, as revealed by significant reduction of leukemia burdens in the circulation, bone marrow, liver and spleen, and significantly extends the median survival time of MV4-11 AML-bearing mice. In addition to active targetability and therapeutic benefits, A6-cPS-VCR has the advantage of easy fabrication, rendering it potentially interesting for clinical translation.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous blood cancer characterized by abnormal clonal expansion and differentiation of myeloid lineage, predominantly occurred in elderly people [1,2]. Without treatment, AML will be fatal within weeks or months in view of the fact that AML typically presents with a rapid onset of symptoms attributable to bone marrow failure [3]. In the past decade, leukemia incidence and mortality increased 45% and 12%, respectively, in the United States of America [4]. Under gold-standard (7 + 3) regimen, patients have typically a 5-year overall survival rate of 27% and high relapse rate of over 60% [5,6]. Notably, several new treatments including midostaurin, enasidenib, ivosidenib, gemtuzumab ozogamicin and CAR-T therapy have recently been approved by the US Food and Drug Administration (FDA) for treating AML [7,8]. These new treatments are typically used in combination with traditional chemical drugs, in relapsed/refractory incidents, and/or in specific mutated cases [9,10]. Chemotherapy remains an indispensable choice of treatment for AML patients.

Low specificity and high toxicity are major problems for chemotherapy of elderly AML patients in which the treatment often has to be discontinued due to unbearable adverse effects. Various nanovehicles such as liposomes [11], nanoparticles [12,13], and micelles [14] have been investigated to improve the anti-AML effect of chemical drugs while reducing their toxicity. Liposome injections, Vyxeos® (5:1 M ratio of cytarabine to daunorubicin) [15,16] and Marqibo® (vincristine sulfate, VCR) [17], have been approved by FDA for leukemia treatment. Both nanoformulations are, however, non-cell-selective. To increase the AML-selectivity and anti-AML efficacy, active-targeting formulations that are preferentially internalized by leukemia cells via their overexpressed receptors e.g. antibody-drug conjugates (ADCs) [18,19], antibody functionalized liposomal formulations [20,21] and specific peptide or hyaluronan decorated nanoparticles [14,22] were developed and explored. The treatment outcomes of these targeted formulations are, however, renounced by their myelotoxicity and inadequate stability [23].

Herein, we designed A6 peptide-tagged, ultra-small and reduction-
sensitive crosslinked polymersomal vincristine sulfate (A6-cPS-VCR) for targeted treatment of CD44+ AML (Scheme 1). CD44 was reported to overexpress on several malignant solid tumor cells and a majority of AML cells as well as leukemic stem cells [24–26]. CD44-targeting with hyaluronic acid and anti-CD44 antibodies appeared to be an interesting approach to enhance the efficacy of chemotherapy or immunotherapy for a variety of solid tumors and leukemic cancers [27–30]. The complicated fabrication, however, sets a constraint for their clinical translation [31]. We have recently showcased that CD44-specific A6 peptide can boost targetability and antitumor efficacy of polymersomal epirubicin to orthotopic multiple myeloma [32]. A6 (KPSSPPEE) is a urokinase-derived short peptide which showed high affinity to CD44 [33]. Notably, A6 peptide has shown excellent safety in the clinical trials.

VCR is a highly toxic water soluble drug widely used in the clinics for treatment of acute lymphoblastic leukemia (ALL), lymphoma, neuroblastoma and other cancers [34]. VCR is not a chemotherapy drug commonly used for AML patients, possibly because (i) AML is less sensitive to VCR than ALL, and (ii) VCR is highly toxic and has a particularly narrow therapeutic window. We hypothesized that targeted delivery of VCR would significantly improve its anti-AML potency, and as reported for other therapeutics [35–37], the polymersomal VCR formulation would markedly reduce its adverse effects and improve drug toleration and therapeutic index, offering a novel and effective treatment for AML.

To efficiently load VCR, asymmetric polymersomes with poly(aspartic acid) (PAsp) as inner shell were utilized. In addition to active targetability and therapeutic benefits to human acute myeloid leukemia in vitro and in vivo, A6-cPS-VCR has the advantage of easy fabrication, rendering it potentially interesting for clinical translation.

2. Experimental methods

2.1. Cell culture and animals

Human AML cell lines were obtained from the American Type Culture Collection (ATCC). MV4-11-Luc-GFP stably expressing luciferase (Luc) and green fluorescent protein (GFP) was established.

Venus-Luc-EGFP encoding plasmid and PsPAX2, PMD2.G packaging plasmids were transfected into 293 T cells (ATCC) to produce lentiviruses, followed by the infection of MV4-11 with MOI 20 in culture medium for 16 h. Then, GFP positive cells were sorted by fluorescence-activated cell sorting (FACS, BD Melody) 48 h post infection. Primary leukemia cells in freezing medium composed of 90% FBS (Gibco) and 10% DMSO (Sigma) were thawed, centrifuged and resuspended in 5 mL cell culture medium (RPMI-1640 containing 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin). The cells were cultured in 5% CO₂ in a humidified atmosphere at 37 °C. Female NOD/SCID mice (6 weeks old, 18–22 g) were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). 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.

Scheme 1. Illustration of A6 peptide (KPSSPPEE) -tagged, ultra-small and disulfide-crosslinked polymersomal vincristine sulfate (A6-cPS-VCR) for targeted therapy of CD44+ AML. (a) A6-cPS-VCR is easily prepared via self-assembly of PEG-P(TMC-DTC)-PAsp and A6-PEG-P(TMC-DTC) in the presence of VCR. (b) A6-cPS-VCR selectively targets to CD44+ leukemic blast cells in peripheral blood, liver, spleen and bone marrow. (c) A6-cPS-VCR following efficient internalization by CD44+ leukemic cells quickly releases VCR to the cytosol, resulting in high anti-leukemia effects that inhibition of microtubule formation in mitotic spindle.
and drug loading efficiency (DLE) were calculated according to the following formula:

\[
\text{DLC (wt%)} = \frac{\text{weight of loaded VCR}}{\text{total weight of polymers and loaded VCR}} \times 100
\]

\[
\text{DLE (\%)} = \frac{\text{weight of loaded VCR}}{\text{weight of VCR in feed}} \times 100
\]

cPS-VCR was obtained from PEG-(TMC-DTC)-PAsp under the same protocol. The empty A6-cPS and cPS were fabricated using the same method except in HEPES buffer containing no VCR.

2.3. In vitro GSH triggered drug release from A6-cPS-VCR

The drug release study was conducted using dialysis tubes (MWCO 12000 Da, Spectra/Por) in two different media, i.e. HEPES (10 mM, pH 7.4) with and without 10 mM GSH. 0.5 mL A6-cPS-VCR (1 mg/mL) was added to dialysis tubes, which were immersed into 20 mL corresponding medium in a shaking bath at 37 °C (100 rpm). At prescribed timepoints, 5 mL release medium was withdrawn, and an equal volume corresponding medium in a shaking bath at 37 °C (100 rpm). At prescribed 12000 Da, Spectra/Por) in two different media,

2.4. In vitro targeting and growth inhibition experiment of cell lines

In vitro targeting assays were measured by flow cytometry. A total of 2 × 10⁵ MV4-11 cells/well were cultured in 6 well plates. Cy5-labeled cPS or A6-cPS was added at 10 μg/mL and incubated for 4 h at 37 °C. MV4-11 cells were washed three times with PBS and analyzed via flow cytometry. In vitro growth inhibition experiment was evaluated using CCK8 assays for cell lines. Cells (2 × 10⁴ cells/well) were cultured in 96 well plates. 20 μL VCR, cPS-VCR or A6-cPS-VCR at VCR concentration ranging from 0.5 to 500 ng/mL was added. After 4 h, drug-containing medium was substituted by fresh medium and cultured for 44 h. 10 μL CCK8 solution was added to incubate for 2–4 h, and then the UV absorbance at 450 nm was measured.

2.5. In vitro cell apoptosis and cell cycle assays

A total of 2 × 10⁵ leukemia cells/well were cultured in 24 well plates. VCR, cPS-VCR or A6-cPS-VCR was added to incubate for 4 h. Then, the medium was replaced with fresh medium, and the cells were further incubated for 20 h. Apoptotic cells and cell cycles were detected by staining with an Annexin V-F647/propidium iodide or cell cycle kit (Fmcacs Biotech Co., Ltd.), analyzed using software FlowJo or ModFit LT, respectively.

2.6. Cell growth inhibition and apoptosis of leukemia patients’ primary cells

Bone marrow (BM) samples were collected from 45 AML patients for the determination of CD44 expression, growth inhibition and apoptosis studies, which were approved by the ethics committee of the First Affiliated Hospital of Soochow University in Suzhou, P.R. China in accordance with the Declaration of Helsinki protocol. To determine the CD44 expression, the primary AML cells from patients and leukemia cell lines were washed twice with PBS, and then stained with anti-CD44-PE antibody for 30 min at 4 °C, and finally subjected to flow cytometry analysis. The growth inhibition of the primary cells was evaluated using trypan blue rejection method. The primary CD44 + AML cells (20190121# and 20200707# patients) and CD44- AML cells (20200608# and 20191120# patients) were cultured in 96 well plates at 1 × 10⁵ cells/well. 20 μL A6-cPS-VCR, cPS-VCR or VCR was added at a VCR concentration of 1 μg/mL and cultured for 4 h followed by further incubation in drug-free medium for 44 h. After trypan blue staining, the cells were counted under an inverted microscope. The cell apoptosis analyses were the same as described above.

2.7. In vivo antitumor performance

Human AML mouse model was established by intravenous transplantation of MV4-11-Luc-GFP cells into NOD/SCID mice (female, six weeks, 18–22 g). Briefly, NOD/SCID mice were first X-ray irradiated with a sub-lethal dose of 2.0 Gy and intraperitoneally injected with CD122 antibody (10 μg CD122 per gram mouse) before intravenous transplantation of MV4-11-Luc-GFP cells (1 × 10⁶ cells per mouse). For in vivo monitoring the development of leukemia, peripheral blood from the eye socket was collected in heparinized centrifugation tubes. Following centrifugation, the plasma was removed, erythrocytes were lysed, and the cells were stained with anti-CD45-APC antibody for FACS analysis. Six days after cell injection, tumor load in different organs was evaluated based on in vivo bioluminescence imaging using the PerkinElmer IVIS Lumina III.

For antitumor purpose, MV4-11-Luc-GFP leukemia mice were divided into four groups according to the bioluminescent intensity (n = 10) taken 6 days post inoculation of leukemic cells: PBS, VCR, cPS-VCR, and A6-cPS-VCR. The day on which the drugs were given was designated as day 0. Drugs were intravenously injected on day 0 and 2 (0.2 mg VCR equiv./kg). Animals were weighted daily, and bioluminescence images and peripheral blood were measured at different intervals. The survival rates of the mice were monitored within 30 days (n = 7). The mice were considered dead either when the mice were dead, or over 20% body weight loss.

2.8. Flow cytometry, histological and μCT analyses

To evaluate the influence of antitumor treatment on the leukemia cell engraftment and damages on tissues and bones, the MV4-11-Luc-GFP leukemia mice (n = 3) which had received two injections of PBS, VCR, cPS-VCR, or A6-cPS-VCR on day 0 and 2 (0.2 mg VCR equiv./kg) were sacrificed on day 4. The MV4-11-Luc-GFP cell engraftment in the bone marrow (BM: femur, tibia and ilium), liver, spleen and peripheral blood (PB, collected from eye socket) was harvested in PBS (containing 1% w/w BSA) and quantified by gating APC-positive cells using FACS with anti-CD45-APC antibody. The major organs, hind femurs and tibia were excised for histological analyses and μCT analysis. The extent of leukemic infiltration of different organs was assessed by hematoxylin and eosin (H&E) staining. Tartrate resistant acid phosphatase (TRAP) staining of osteoclasts in tibias and femurs was acquired using TRAP staining kit (Servicebio, Hubei, China). H&E and TRAP staining slices were observed using an Olympus BX41 microscope. The hind femurs and tibia were subject to μCT measurement and analyzed using NRecon software.

2.9. In vivo and ex vivo fluorescence imaging of MV4-11-Luc-GFP leukemia mice

For in vivo imaging of the location of the polymersomes in the leukemia mice, a single dose of Cy5-A6-cPS or Cy5-cPS (1 μg Cy5 per mouse) in HEPES (200 μL) was intravenously injected via the tail vein into the mice. After 8 h, the mice were anesthetized and imaged in mode of Cy5-fluorescence. After that, the mice were sacrificed, and the hind limb bones and ilium were isolated and ex vivo imaged on an IVIS imaging system using Living Image 2.6 software.

2.10. Statistical analyses

The data were presented as mean ± SD and analyzed for statistical significance by one-way ANOVA with Tukey multiple comparisons tests.
using Prism 7 or two-tailed paired Student t-test. Kaplan-Meier survival curves were analyzed by log-rank test for comparisons using Prism 7. *p < 0.05 was considered significant, and **p < 0.01 and ***p < 0.001 were highly significant.

3. Results and discussion

3.1. Preparation and characterization of A6-cPS-VCR

VCR, a water-soluble drug, is often chosen as part of poly-chemotherapy because of its clinical anticancer efficacy related to the inhibition of microtubule formation in mitotic spindle and of no significant bone marrow suppression at recommended doses [34]. To accomplish its efficient loading, targeted delivery and overcoming multidrug resistance [39], we designed A6 functionalized chimaeric polymersomes from co-self-assembly of poly(ethylene glycol)-b-poly (trimethylene carbonate-co-dithiolane trimethylene carbonate)-b-poly (aspartic acid) (PEG-P(TMC-DTC)-PAsp) and A6 peptide modified PEG-P(TMC-DTC) (A6-PEG-P(TMC-DTC)) with negatively charged PAsp as inner shell for active loading of VCR and A6 peptide at the outer shell for selective binding to CD44 (A6-cPS-VCR). Such chimaeric structure was thermodynamically favorable and reported earlier for ABC type triblock copolymers by Eisenberg and our group experimentally [40–42] and theoretically [43].

In contrast to complicated emulsion or nanoprecipitation method of loading VCR in previous reports [39,44], A6-cPS-VCR was facilely fabricated from self-assembly of PEG-P(TMC-DTC)-PAsp/A6-PEG-P (TMC-DTC) at varying molar ratios in HEPES buffer (10 mM, pH 6.8) which contained VCR. The average hydrodynamic diameter of A6-cPS-VCR increased slightly from 26 nm for PS-VCR (non-targeted) to 28, 31 and 35 nm with narrow size distributions (PDI < 0.15) for A6 densities of 10%, 20% and 30%, respectively, as measured by dynamic light scattering (DLS, Table 1). These polymersomes were much smaller than polymersomes made of diblock copolymer PEG-P(TMC-DTC) of 62 nm [32] owing to the higher curvature of the polyelectrolyte membrane of the triblock copolymers. Notably, cPS-VCR and A6-cPS-VCR had almost identical drug loading content (DLC) and drug loading efficiency (DLE) at VCR feed ratio of 5.0 wt% (Table 1). The DLC (62%) of cPS-VCR was much higher than that (14%) of PS-VCR made of PEG-P(TMC-DTC), due to the strong electrostatic interaction between PAsp and VCR at pH 6.8. It is critical, therefore, to introduce PAsp to form chimeric polymersomes, which not only affords increased and stable loading of VCR in the triblock copolymers. Notably, cPS-VCR and A6-cPS-VCR had almost [32] owing to the higher curvature of the polymersomal membrane of both formulations based on liposomes, biodegradable micelles and nanoparticles that typically show poor stability and/or slow VCR release [46–48].

3.2. Specific antitumor activity of A6-cPS-VCR

The development of targeted anti-AML therapy could utilize the AML tumor-associated antigens [49]. CD44 is a transmembrane glycoprotein that overexpresses on the majority of leukemic blasts and leukemic stem cells [26]. Here, we firstly examined the CD44 expression of 14 AML cell lines with diverse French-American-British (FAB) and 45 leukemic blasts from patients (43 first-visit and 2 relapsed) using anti-CD44-PE antibody. The FACS results showed that 11 cell lines (79.1%), 36 first-visit patients (86.0%) and 2 relapsed patients (100%) were CD44 positive (Fig. 1c, Table S1 and Fig. S3), in accordance with the findings of a phase I clinical study in which 42 out of 44 patients (95%) refractory/relapsed were found CD44 positive on leukemic blasts [47]. These results confirm that CD44 is a promising target for AML therapy including relapsed AML. CD44 + MV4-11 cell line was chosen for in vitro and in vivo antitumor investigations. The highest cytotoxicity to MV4-11 cells was seen for A6-cPS-VCR at 20%A6 (Fig. 1d), which was selected for the following studies if not otherwise specified. Flow cytometry revealed over 2-fold increase in uptake of Cy5-labeled A6-cPS (Cy5-A6-cPS) by MV4-11 cells compared with non-targeting Cy5-cPS control (Fig. 1e). In contrast, A6 modification caused no difference in cell uptake in CD44 negative YNH-1 leukemia cells (Fig. S4a).

We further investigated the cytotoxicity of A6-cPS-VCR in CD44 + cell lines (MV4-11, HL-60 and SHI-1) and CD44- cell lines (YNH-1 and OCI-AML-3). The CCK8 assays demonstrated a clearly lower half-maximal inhibitory concentration (IC50) of A6-cPS-VCR than cPS-VCR or free VCR in all three CD44 + cell lines (Fig. 1f), while little difference in IC50 for two CD44- cell lines (Fig. S4b), confirming that A6 peptide augments the antitumor activity of cPS-VCR via CD44 mediated internalization. To investigate the apoptosis of leukemia cells using flow cytometry, Annexin V-F647/PI was utilized to stain the cells, and the upper and lower right quadrants denoted as total apoptotic cells in the quadrant diagrams. The results showed that A6-cPS-VCR induced much higher apoptosis than cPS-VCR and VCR in CD44 + MV4-11 and HL-60 cells (Fig. 1g, and Fig. S5a). In contrast, little difference in apoptosis was observed for all three formulations in CD44- YNH-1 cells (Fig. S5b).

The cell cycle assays displayed that A6-cPS-VCR gave obviously more cell arrest in G2/M phase compared with cPS-VCR (63.6% versus 41.9%) (Fig. 1h), which is in line with the action mechanism of VCR by inhibiting the microtubule formation in mitotic spindle. We further selected four primary leukemic cells (two CD44+, two CD44-) from patients to study the anti-leukemic activity of A6-cPS-VCR. The results confirmed that A6-cPS-VCR induced more apoptosis than cPS-VCR and VCR in two CD44+ AML cells (Fig. S6). The trypan blue rejection assays showed better inhibition of A6-cPS-VCR to CD44 + AML cells (cell viability < 60%) than the two CD44- cells (viability ca. 70–90%).

These results indicated that A6 peptide active-targeting played a vital role in leukemia cell inhibition. Of note, this short A6 peptide exhibited a similar CD44-targetability to hyaluronic acid and anti-CD44 antibodies [50,51]. Unlike A6 peptide, anti-CD44 antibody and hyaluronic acid targeted nanosystems were mostly prepared by post-modification, which complicates fabrication and characterization.

3.3. Targeted accumulation in orthotopic MV4-11-Luc-GFP leukemia mice

Unlike solid tumors, leukemia is a blood cancer primarily located at
Fig. 1. Properties and in vitro anticancer performance of A6-cPS-VCR. (a) Hydrodynamic size of 20%A6-cPS-VCR, and Cryo-TEM of blank 20%A6-cPS. (b) Drug release profiles from 20%A6-cPS-VCR with or without GSH (10 mM). \( n = 3 \). (c) CD44 expression of 45 leukemic cells from AML patients (43 first-visit and 2 relapsed) and 14 AML cell lines determined by FACS using anti-CD44-PE antibody. The CD44 expression > 80%, between 50% and 80%, and < 50% were denoted as highly positive, medium positive, or negative, respectively. (d) CCK8 assays of the viability of MV4-11 cells incubated with A6-cPS-VCR with various A6 contents (10 ng VCR equiv./mL). \( n = 6 \). Student t-test: * \( p < 0.05 \). (e) Flow cytometric analyses of MV4-11 cells incubated for 4 h with Cy5-A6-cPS or Cy5-cPS (dose: 50 ng Cy5/mL). (f) Growth inhibition studies of CD44+ cell lines (MV4-11, HL-60 and SHI-1) treated with 20%A6-cPS-VCR, cPS-VCR or free VCR using CCK8 assays. \( n = 3 \). (g) Apoptotic assays and (h) cell cycle assays of MV4-11 cells upon treatment with 20%A6-cPS-VCR, cPS-VCR or free VCR. For d, f, g and h, the cells were treated with VCR formulations for 4 h followed by incubation in drug-free medium for 44 h (d and f) or 20 h (g and h) at a VCR concentration of 6 ng/mL.
Here, we established a systemic leukemic model in NOD/SCID mice through sub-lethal X-ray irradiation and CD122 antibody injection followed by i.v. inoculation of MV4-11-Luc-GFP cells (Fig. 2a). The bulk leukemia population in peripheral blood (PB) was monitored on day 3, 6, 8, and 10 after inoculation. The leukemia percent was low on day 3 while the leukemic clone expanded from day 6 to day 8 (Fig. 2b and Fig. S7), and the engrafted leukemia cells in BM, liver, and spleen was 49.8%, 14.6%, and 0.79%, respectively, on day 10 after inoculation (Fig. S8). These results validated that the established systemic MV4-11-Luc-GFP model was highly malignant with a rapid onset of symptoms.

Selective accumulation of anti-leukemia drugs in the BM is thus crucial for effective management of AML. To investigate their BM homing capability in vivo, Cy5-A6-cPS or Cy5-cPS was intravenously injected into leukemic mice with similar burden (Fig. S9a-b). At 8 h post-injection Cy5-A6-cPS treated mice displayed significantly higher Cy5 fluorescent signal in vivo than Cy5-cPS group with the highest signals in the limbs (Fig. S9c-d). The excised hind limb bones \((p < 0.001)\) and ilium \((p < 0.05)\) of Cy5-A6-cPS group built up significantly more than those of Cy5-cPS group (Fig. 2c-d), suggesting that A6 peptide boosted targeted cPS accumulation in the BM. The peripheral neurotoxicity is the principle clinical toxicity of VCR, which is usually cumulative and dose-dependent [34]. The enrichment of A6-cPS-VCR in the BM would not only improve its anti-AML efficacy but also minimize the side effects of VCR.

### 3.4. Targeted treatment of orthotopic MV4-11-Luc-GFP leukemia mice

The anti-AML activity of A6-cPS-VCR was investigated in orthotopic MV4-11-Luc-GFP leukemia mice. The treatment was started on day 6 post cell implantation, which was designated as day 0. The second injection was given on day 2 (Fig. 3a). The development of leukemia was monitored by the bioluminescence of MV4-11-Luc-GFP cells in vivo. Bioluminescence imaging of PBS group visualized that leukemia cells engrafted primarily in the BM of mice with secondary dissemination to the liver and spleen and progressed rapidly (Fig. 3b). The treatment with A6-cPS-VCR markedly reduced leukemic burden overall. Fig. 3c showed that the bioluminescence intensity of MV4-11-Luc-GFP cells in A6-cPS-VCR group on day 4, 7, and 9 was significantly lower than that in free VCR and non-targeted cPS-VCR groups. The reduction of leukemic burden by A6-cPS-VCR reached 98.8% relative to PBS control on day 4 (Fig. 3c). Furthermore, A6-cPS-VCR and cPS-VCR appeared to be better tolerated than VCR, as revealed by their little drop of mouse body weights over treatments (Fig. 3d).

The mouse of PBS group started to die from day 4 exhibiting a median survival time (MST) of 5 days (Fig. 3e), corroborating the highly aggressive nature of acute leukemia. Kaplan-Meier survival curves showed that A6-cPS-VCR greatly improved the survival of MV4-11-Luc-GFP leukemia-bearing mice, with MST increased to 16 days, which was over 3-fold that of PBS group. Free VCR and cPS-VCR though also improved the survival time of the mice were significantly less effective than A6-cPS-VCR. Notably, superior anti-leukemia efficiency was attained after only two injections rather than constantly repeated administration of drugs in prior reports [22,53,54]. These results supported that A6-cPS-VCR was able to target to CD44-overexpressing systemic leukemia model.

### 3.5. Ex vivo leukemia blasts evaluation

To further evaluate the anti-leukemia effects of A6-cPS-VCR in vivo, representative mice were sacrificed on day 4. MV4-11-Luc-GFP cells engrafted in the BM (femur, tibia and ilium), liver, spleen and peripheral blood (PB) were harvested and assessed by FACS. Fig. 4a showed that free VCR, cPS-VCR and A6-cPS-VCR treatments all led to markedly suppressed expansion of MV4-11-Luc-GFP cells. Strikingly, A6-cPS-VCR resulted in hardly detectable leukemia cells in the BM, liver, spleen and...
PB (0.07%, 0.40%, 0.11%, and 0.91%, respectively), remarkably out-performing free VCR and non-targeted cPS-VCR (Fig. 4b-4e). In particular, leukemia cells alter the BM niche into a leukemia growth-permissive and normal hematopoiesis-suppressive microenvironment thus facilitating their own growth and evading chemotherapy [55]. Here, the reduction of leukemia engraft in the BM of mice treated with A6-cPS-VCR reached 99.92% compared to PBS (\(p < 0.001\)), showcasing stronger antileukemic effects than antibodies and inhibitors [56,57]. Moreover, the engrafted leukemia cells visualized by pathological morphology display that the BM, liver and spleen tissues of A6-cPS-VCR group practically turned to normal (Fig. S10), while massive infiltration of leukemia blasts in BM and liver, and necrosis of splenic red pulp were observed in PBS group (Fig. 4d). In contrast, mice treated with free VCR and cPS-VCR group could only partly suppress leukemia proliferation in BM and liver. These results corroborated that A6-cPS-VCR gives better treatment of MV4-11-Luc-GFP leukemia.

3.6. Bone damage evaluation

Bone formation and resorption is mainly regulated by two molecules, receptor activator of NF-κB ligand (RANKL) and osteoprotegerin. RANKL produced by AML cells in the bone marrow stimulates osteoclast activation causing bone damage [52]. To evaluate the changes in the BM microenvironment induced by AML, here we performed tartrate resistant acid phosphatase (TRAP) staining and micro-compute tomography (μCT) analyses to estimate the activity of osteoclasts and bone
Fig. 4. A6-cPS-VCR reduces leukemia burden in BM, liver, spleen and PB. (a) Representative FACS panel and (b-e) quantitative determination of leukemic blasts in BM, liver, spleen and PB on day 4 after treatment with PBS, VCR, cPS-VCR or A6-cPS-VCR (n = 3). Student t-test: *p < 0.05, **p < 0.01, ***p < 0.001. (f) Representative views of H&E-stained BM, liver and spleen sections on day 4 after treatment. Scale bar: 400 μm.
resorption. Fig. 5a showed numerous osteoclasts (stained red) on patellar bone surfaces in the femur and tibia from leukemic mice of PBS group. The osteoclasts were distinctly reduced by A6-cPS-VCR, supporting that A6-cPS-VCR markedly suppressed expansion of leukemia cells in BM. The μCT analyses displayed loss of trabecular structures, decreased trabecular number (Tb.N) and thickness (Tb.Th), and increased trabecular spacing (Tb.sp) in leukemic mice treated with PBS (Fig. 5b). Notably, A6-cPS-VCR group exhibited nearly intact trabecular tissue and increased bone density compared with PBS and cPS-VCR groups (Fig. 5c, Fig. S11), owing to the negligible leukemia infiltration in BM and thus little production of osteoclasts. All the above results evidenced that A6-cPS-VCR possessed good AML-targetability and anti-AML activity in vitro and in vivo as well as good protection of the bones.

4. Conclusion

We have established that A6 peptide-coupled, small and disulfide-crosslinked polymersomal vincristine sulfate (A6-cPS-VCR) holds a pronounced selectivity and potency toward CD44+ MV4-11 leukemia cells, resulting in higher anti-leukemia activity in vitro and significantly enhanced therapeutic efficacy of orthotopic MV4-11 AML mice in vivo compared with free VCR and non-targeted cPS-VCR. Intriguingly, A6-cPS-VCR led to a remarkable leukemia inhibition rate of 99.92% in the bone marrow, greatly improved survival rate, and decreased bone damage. A6-cPS-VCR with easy fabrication and active targeting ability provides a potentially interesting treatment for CD44+ malignant AML.
Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2020.10.005.

References


