\( \alpha_\text{v} \beta_3 \) integrin-targeted micellar mertansine prodrug effectively inhibits triple-negative breast cancer in vivo

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Abstract: Antibody-mertansine (DM1) conjugates (AMCs) are among the very few active targeting therapeutics that are approved or clinically investigated for treating various cancers including metastatic breast cancer. However, none of the AMCs are effective for the treatment of triple-negative breast cancers (TNBCs). Here, we show that cRGD-decorated, redox-activatable micellar mertansine prodrug (cRGD-MMP) can effectively target and deliver DM1 to \( \alpha_\text{v} \beta_3 \) integrin overexpressing MDA-MB-231 TNBC xenografts in nude mice, resulting in potent tumor growth inhibition. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays showed that cRGD-MMP had obvious targetability to MDA-MB-231 cells with a low half-maximal inhibitory concentration (IC\(_{50}\)) of 0.18 \( \mu \text{M} \), which was close to that of free DM1 and 2.2-fold lower than that of micellar mertansine prodrug (MMP; nontargeting control). The confocal microscopy studies demonstrated that cRGD-MMP mediated a clearly more efficient cellular uptake and intracellular release of doxorubicin (used as a fluorescent anticancer drug model) in MDA-MB-231 cells. Notably, cRGD-MMP loaded with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indotricarbocyanine iodide (DiR; a hydrophobic near-infrared dye) was shown to quickly accumulate in the MDA-MB-231 tumor with strong DiR fluorescence from 2 to 24 h post injection. MMP loaded with DiR could also accumulate in the tumor, although significantly less than cRGD-MMP. The biodistribution studies revealed a high DM1 accumulation of 8.1%ID/g in the tumor for cRGD-MMP at 12 h post injection. The therapeutic results demonstrated that cRGD-MMP effectively suppressed MDA-MB-231 tumor growth at 1.6 mg DM1 equiv./kg without causing noticeable side effects, as shown by little body weight loss and histological analysis. This MMP has appeared as a promising platform for potent treatment of TNBCs.

Keywords: breast cancer, reduction-sensitive, drug conjugates, micelles, cRGD

Introduction

Mertansine (DM1) is a powerful tubulin polymerization inhibitor that can effectively treat various malignancies, including breast cancer, melanoma, multiple myeloma and lung cancer.\(^1-3\) The extremely high potency of DM1 renders it an ideal warhead in the development of antibody–drug conjugates (ADCs) for targeted tumor therapy.\(^4,6\) In 2013, Kadcyla (ado-trastuzumab emtansine) that targets DM1 to HER2 was approved by the US Food and Drug Administration (FDA) for the treatment of patients with HER2-positive metastatic breast cancer.\(^7,8\) The success of ado-trastuzumab emtansine has spurred intensive clinical development of novel ADCs based on DM1 but different antibodies for targeting to various tumors.\(^4,5,9\) Undoubtedly, ADCs with DM1 warhead are among the most advanced active targeting cancer nanomedicines that
are expected to greatly improve the therapeutic outcomes of many refractory cancers.

None of the ADCs including ado-trastuzumab emtansine, however, can be used to treat triple-negative breast cancer (TNBC) that accounts for 12%–17% of globally diagnosed breast cancer. TNBC is a highly heterogeneous subtype of breast cancer that does not express, or expresses, low levels of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor.\textsuperscript{10–12} The lack of the above therapeutic receptors makes TNBC insensitive to hormone therapy such as Nolvadex\textsuperscript{8} (tamoxifen) used in ER-positive cancer treatment and to trastuzumab (Herceptin\textsuperscript{9}) commonly used for HER2-positive tumors.\textsuperscript{13–15} TNBC remains a formidable challenge for current clinical practices.\textsuperscript{16–18}

In contrast to ADCs, polymeric micellar nanomedicines that can be decorated with different targeting ligands are a more versatile treatment modality for cancer chemotherapy.\textsuperscript{19–21} In the past few years, various micellar nanomedicines have been designed for targeted treatment of TNBC.\textsuperscript{22–27} For instance, cetuximab-conjugated vitamin E d-\textalpha-tocopheryl polyethylene glycol succinate micelles were shown to increase efficacy of docetaxel (DTX) against TNBC.\textsuperscript{22,23} Folate-targeted micelles loaded with orlistat exhibited better treatment of TNBC than free orlistat.\textsuperscript{24} Aminoflavone-loaded EGFR-targeted unimolecular micelles based on poly(amideamine)-poly lactide-poly(ethylene glycol) were reported to significantly enhance treatment of orthotopic TNBC tumor as compared to the nontargeted counterpart and free aminoflavone.\textsuperscript{25} The micellar nanoformulation of lipophilized bortezomib (BTZ) induced significantly better TNBC tumor accumulation and inhibition while lower adverse effects than free BTZ, leading to greatly improved mice survival rate.\textsuperscript{28} It is interesting to note that in spite of clinical use of DM1 in the treatment of HER2-positive metastatic breast cancers, no systems based on DM1 have been developed for targeted treatment of TNBC.

We recently reported that cRGD-functionalized micellar mertansine prodrug based on poly(ethylene glycol)-b-(poly(trimethylene carbonate)-graft-SSDM1) (PEG-P(TMC-g-SSDM1)) mediated targeted treatment of α\textbeta\textsubscript{3} integrin-overexpressed B16F10 melanoma.\textsuperscript{29} Notably, cRGD-MMP has a significantly higher DM1 conjugation, better stability and improved tolerability as compared to ado-trastuzumab emtansine. The aim of the present work was to investigate therapeutic efficacy of cRGD-MMP toward MDA-MB-231 triple-negative breast tumor-bearing nude mice. cRGD peptide has shown a strong binding toward α\textbeta\textsubscript{3} integrin-overexpressed MDA-MB-231 TNBC cells.\textsuperscript{30–32}

Interestingly, our results showed that cRGD-MMP can effectively target to and treat MDA-MB-231 cells in vivo. This micellar mertansine prodrug (MMP) has appeared as a promising platform for potent treatment of TNBC.

### Materials and methods

#### Materials and characterizations

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Co., St Louis, MO, USA), 4′-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich Co.), 1,1′-dioctadecyltetramethyl indotricarbocyanine iodide (DiR; AAT Bioquest Inc., Sunnyvale, CA, USA) and doxorubicin (DOX) hydrochloride (Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd., Beijing, China) were used as received. PEG-P(TMC-g-SSDM1) and cRGD-PEG-P(TMC-g-SSDM1) were prepared by thiol–disulfide exchange reaction between DM1 with PEG-P(TMC-co-poly(pyridyldisulfide cyclic carbonate) (PDSC) and Mal-PEG-P(TMC-co-PDSC) copolymers (Table 1), respectively, as in our previous report.\textsuperscript{29} The characteristics of MMP and cRGD-MMP are summarized in Table 2. The amount of DM1 was determined by high-performance liquid chromatography (HPLC; Agilent 1260; Agilent Technologies, Santa Clara, CA, USA) using a mixture of acetoniitrile and water (v/v = 6/4) as a mobile phase with ultraviolet (UV) detection at 245 nm.

#### Cell culture and animals

MDA-MB-231 TNBCs that express a high level of α\textbeta\textsubscript{3} integrins were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. MDA-MB-231 cancer cells were cultured in the Dulbecco’s Modified Eagle’s Medium (DMEM; Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum and antibiotics penicillin (100 IU/mL) and streptomycin (100 μg/mL; Jinuo Biomedical Technology, Hangzhou, Zhejiang, China) under 5% CO\textsubscript{2} at 37°C.

#### Table 1 Characterization of PEG-P(TMC-co-PDSC) and Mal-PEG-P(TMC-co-PDSC) copolymers

| Copolymer       | M\textsubscript{w} (g/mol) | M\textsubscript{w}/M\textsubscript{\textalpha}  
<table>
<thead>
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<tbody>
<tr>
<td>PEG-P(TMC-co-PDSC)</td>
<td>5,000–(3,980/2,850)</td>
<td>23,300 1.53</td>
</tr>
<tr>
<td>Mal-PEG-P(TMC-co-PDSC)</td>
<td>5,000–(3,980/2,980)</td>
<td>26,100 1.42</td>
</tr>
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**Notes:** M\textsubscript{w} and M\textsubscript{w}/M\textsubscript{\textalpha} determined by GPC (eluent: DMF, flow rate: 1 mL/min, standards: polystyrene, 30°C). M\textsubscript{\textalpha} calculated from 1H NMR. **Abbreviations:** NMR, nuclear magnetic resonance; GPC, gel permeation chromatography; DMF, dimethylformamide.
In all, 5–6 week-old female nude mice (16–18 g) were provided by Beijing Vital River Laboratory Animal Technology (Beijing, China). The animals were housed at 25°C and 55% humidity under natural 12 h light/dark cycle conditions at the Experimental Animal Center of Soochow University. The mice were all handled under the guideline approved by the Soochow University Laboratory Animal Center. This research was approved by the Animal Care and Use Committee of Soochow University.

In vitro cytotoxicity of cRGD-MMP

The in vitro cytotoxicities of DM1, MMP and cRGD-MMP against MDA-MB-231 TNBCs were investigated by MTT assays. Briefly, MDA-MB-231 cells were grown in 96-well plates at 5×10^4 cells/well overnight. The different drug formulations at concentrations of 0.001355–13.55 μM were added. After 4 h, the medium was replaced by 100 μL fresh medium. The cells were cultured for another 44 h. In all, 20 μL of MTT solution (5 mg/mL) was added. The cells were further cultured for 4 h, the medium was removed and 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve the MTT-formazan crystals. The absorbance of the above solution in each well was recorded using a microplate reader (Multiscan FC USD4600; Thermo Fisher Scientific) at 492 nm. The cell viability (%) was determined by comparing the absorbance at 492 nm with control wells containing only cell culture media. The results are presented as the mean ± standard deviation (n=4).

In vitro cellular targetability of cRGD-MMP

To study its targeting effect in vitro, cRGD-MMP was loaded with fluorescent DOX.33 MDA-MB-231 cells were seeded on microscope slides at 4×10^3 cells/well in 400 μL cell culture medium overnight. DOX-loaded cRGD-MMP or MMP in 100 μL of phosphate-buffered saline (PBS; DOX concentration: 10 μg/mL) was added. After 4 or 8 h incubation, the cells were washed three times and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells following washing three times with PBS, staining with DAPI, blue, and again washing three times with PBS were visualized using a confocal microscope (TCS-SP2; Leica Microsystems, Wetzlar, Germany).

In vivo imaging and biodistribution of cRGD-MMP

To assess the tumor-targeting efficacy of cRGD-MMP in vivo, the fluorescent dye DiR was encapsulated into cRGD-MMP and MMP. Typically, 0.2 mL of mixed dimethylformamide (DMF) solution (5 mg/mL) of PEG-P(TMC-g-SSDM1) and cRGD-PEG-P(TMC-g-SSDM1; w/w, 80/20) with 10 μL DiR solution (1 mg/mL, 1% DiR loading content) was added dropwise to 0.8 mL of PBS (10 mM; pH 7.4) and stirred for another 2 h, followed by extensive dialysis (Spectra/Por; molecular weight cut-off [MWCO] 7000) against PBS for 12 h. MDA-MB-231 triple-negative breast tumor xenograft model was established subcutaneously. In brief, ~2×10^6 MDA-MB-231 cells in 50 μL of serum-free DMEM media were subcutaneously inoculated into the hind flank of nude mice. When the tumor sizes reached 200–300 mm^3, 200 μL of DiR-loaded cRGD-MMP or MMP was intravenously administrated into the tail vein of tumor-bearing mice (dosage: 1 μg DiR equiv./mouse). At 0, 2, 4, 8, 12 or 24 h post injection, the fluorescence images of mice were acquired by the Maestro near-infrared fluorescence imaging system (CRi Inc., USA) with an excitation band filter at 748 nm and an emission at 780 nm.

The in vivo biodistribution studies were performed by tail vein injection of cRGD-MMP or MMP (dosage: 5 mg DM1 equiv./kg) to MDA-MB-231 tumor-bearing nude mice, and DM1 amounts in the tumor and major organs were quantified using the HPLC test. At 12 h post injection, the tumor and main organs containing heart, kidney, spleen, lung and liver were collected, washed with PBS, weighed, homogenized in methanol and extracted by DMF solution. The supernatants were treated with excess 1,4-dithio-D,L-threitol (50 mM) which followed centrifugation of the extracted solution at 18 krpm for 20 min. DM1 contents were quantified by the HPLC test. The results are presented as the mean ± standard deviation (n=3).

In vivo antitumor efficacy

The in vivo antitumor efficacy and safety of cRGD-MMP and MMP was investigated in MDA-MB-231 triple-negative breast tumor-bearing mice. In all, 2×10^6 MDA-MB-231 cells in 50 μL of serum-free DMEM media were subcutaneously injected in the hind flank of nude mice. When the tumor sizes
reached ~70 mm³ after inoculation, the mice were randomly divided into five groups (n=5). The mice were intravenously administrated with PBS, free DM1 at 0.8 mg/kg, MMP at 0.8 mg DM1 equiv./kg and cRGD-MMP at 0.8 or 1.6 mg DM1 equiv./kg, respectively, every two days for a total of four injections. Tumor sizes were recorded using calipers and calculated based on the following formula: \( V = 0.5 \times L \times W^2 \), where L and W are the longest and shortest tumor diameters. The relative tumor volumes were normalized to the initial tumor volume when starting the treatment. The body weights of all mice were weighed every two days over the entire treatment period. At day 16, all mice were sacrificed, and tumors were harvested and weighed. Tumor inhibition rate (TIR) was calculated according to the following formula: (1 – mean tumor weight of drug treated group/mean tumor weight of saline treated group) ×100.

Hematoxylin and eosin (H&E) staining
At the end of the treatment, the mice were sacrificed and major organs, including liver, heart, spleen, lung and kidney, were excised. The tissues were fixed with 4% paraformaldehyde solution and embedded in paraffin. The sliced organ tissues (thickness: 4 μm) mounted on the glass slides were stained by H&E and observed by a digital microscope (Leica QWin; Leica Microsystems).

Results and discussion
Targetability and antitumor activity of cRGD-MMP toward MDA-MB-231 cells
We have shown previously that cRGD-MMP with a small size of 45 nm and high DM1 content of ~40 wt% can effectively target to \( \alpha_v\beta_3 \) integrin overexpressing B16F10 melanoma. cRGD-MMP can also be used as a nanocarrier for other hydrophobic drugs like DTX to achieve synergistic melanoma chemotherapy. Given the fact that MDA-MB-231 TNBC cells overexpress \( \alpha_v\beta_3 \) integrins and that ado-trastuzumab emtansine is clinically used for the treatment of HER2-positive metastatic breast cancers, we decided to investigate the targetability and treatment effect of cRGD-MMP toward MDA-MB-231 tumor. MTT assays showed that cRGD-MMP was highly potent against MDA-MB-231 cells in vitro with a low half-maximal inhibitory concentration (IC₅₀) of 0.18 μM, which was comparable to that of free DM1 (0.12 μM) and 2.2-fold lower than that of the nontargeted MMP control (0.39 μM; Figure 1), indicating that cRGD-MMP can target to MDA-MB-231 cancer cells and DM1 can be quickly released inside the cells. cRGD peptide-functionalized nanomedicines were reported to afford good targetability to MDA-MB-231 cells in vitro and in vivo.

To visualize its intracellular delivery behavior in MDA-MB-231 cells, confocal studies were performed using cRGD-MMP loaded with 4.94 wt% DOX (a fluorescent anticancer drug). Dynamic light scattering (DLS) showed that DOX-loaded cRGD-MMP had a size of 41.0 nm. The results displayed that cells following 8 h treatment with DOX-loaded cRGD-MMP had much stronger intracellular DOX fluorescence than those with DOX-loaded MMP (nontargeted counterpart; Figure 2), supporting the active role of cRGD in mediating cellular uptake. It should be noted that the fluorescence of DOX could be self-quenched when encapsulated in the micelles resulting from homo-Förster resonance energy transfer (homo-FRET). The observed strong DOX fluorescence in MDA-MB-231 cells confirms efficient internalization of cRGD-MMP by MDA-MB-231 cells and fast intracellular drug release.

In vivo imaging and biodistribution of cRGD-MMP
To assess their tumor targeting effect in vivo, cRGD-MMP and MMP micelles were loaded with DiR. The in vivo near-infrared fluorescence imaging showed strong DiR fluorescence in the tumor at 2 h following intravenous (i.v.) injection of cRGD-MMP (Figure 3). The tumor DiR fluorescence maximized at 12 h and remained strong even at 24 h. In comparison, DiR-loaded MMP (nontargeted control) gave much weaker tumor DiR fluorescence. These results signify that cRGD decoration of MMP can significantly enhance its accumulation in the MDA-MB-231 tumor in vivo.
The in vivo biodistribution of cRGD-MMP and MMP was studied in MDA-MB-231 tumor-bearing nude mice. The mice were sacrificed at 12 h post injection of cRGD-MMP or MMP (dosage: 5 mg DM1 equiv./kg). The quantification of DM1 using HPLC measurements displayed that tumor uptake of DM1 was 8.10% of injected dose per gram of tissue (%ID/g) for cRGD-MMP, which was ~2.5-fold higher than that of nontargeted MMP control (Figure 4). Notably, similar uptake was observed for cRGD-MMP and MMP in the vital organs such as heart, liver, spleen, lung and kidney. The enhanced tumor accumulation of cRGD-MMP likely results from the specific affinity of cRGD peptide to the $\alpha_v\beta_3$ integrins that are overexpressed on tumor neovasculatures and MDA-MB-231 TNBCs.40

**In vivo anticancer effect of cRGD-MMP**

The in vivo therapeutic performance of cRGD-MMP was evaluated using MDA-MB-231 triple-negative breast
tumor-bearing mice. cRGD-MMP, MMP or free DM1 was administered every 3 days for a total of four injections at a dosage of 0.8 or 1.6 mg DM1 equiv./kg. As expected, mice treated with PBS showed rapid tumor growth (Figure 5A). Free DM1 showed modest tumor growth inhibition at 0.8 mg/kg. Considering that free DM1 has an maximum-tolerated dose of 1 mg/kg,\(^41\) no higher dosage was attempted. cRGD-MMP exhibited better tumor suppression at 0.8 mg/kg than free DM1 and MMP controls under otherwise the same conditions. Moreover, cRGD-MMP showed a dose-dependent tumor growth inhibition, in which tumor progression was greatly enhanced at 1.6 mg DM1 equiv./kg. The images of tumors isolated on day 16 showed clearly that mice treated with cRGD-MMP at 1.6 mg DM1 equiv./kg had almost complete growth inhibition (Figure 5B). The tumor weights revealed that cRGD-MMP at 0.8 mg DM1 equiv./kg yielded a TIR of 67.3%, which was significantly higher than that for MMP (TIR: 46.2%) and free DM1 (TIR: 17.5%; Figure 5C). A high TIR of 87.6% was achieved with 1.6 mg DM1 equiv./kg cRGD-MMP. The significantly enhanced tumor growth inhibition of cRGD-MMP as compared to free DM1 is most likely because cRGD-MMP has a longer circulation time, better tumor selectivity and higher drug accumulation in tumor than free DM1. No obvious body weight loss was observed for all treatments over the entire

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**Figure 4** In vivo biodistribution of cRGD-MMP and MMP in MDA-MB-231 tumor-bearing mice at 12 h post i.v. injection (DM1 dosage: 5 mg/kg). Notes: The results are presented as mean ± standard deviation (n=3). \(^{**}p<0.01\) (Student’s t-test). Abbreviations: cRGD-MMP, cRGD-functionalized micellar mertansine prodrug; MMP, micellar mertansine prodrug; i.v., intravenous; DM1, mertansine; ID/g, injected dose per gram of tissue.

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**Figure 5** In vivo antitumor performance in subcutaneous MDA-MB-231 human triple-negative breast tumor-bearing nude mice. Notes: (A) Tumor volume changes in MDA-MB-231 breast tumor-bearing mice treated with free DM1, MMP and cRGD-MMP. DM1 drugs were given on days 0, 3, 6 and 9 for a total of four injections. The results are presented as mean ± standard deviation (n=5). (B) Photographs of typical tumor blocks collected from different treatment groups on day 16. (C) Tumor weights of mice following 16 days treatment (n=5). (D) Change in mice body weights over 16 days following different treatments. \(^{*}p<0.05\) and \(^{**}p<0.01\) (ANOVA test). Abbreviations: DM1, mertansine; MMP, micellar mertansine prodrug; cRGD-MMP, cRGD-functionalized micellar mertansine prodrug; ANOVA, analysis of variance; PBS, phosphate-buffered saline.
αβ3 integrin-targeted micellar mertansine prodrug

experimental period (Figure 5D). Notably, formyl peptide receptor-targeting and paclitaxel-encapsulated human serum albumin nanoparticles were reported to cause more effective tumor inhibition though similar toxicity as compared to Taxol in MDA-MB-231 tumor-bearing mice. Johnstone et al. reported that mitaplatin-loaded Poly (lactide-co-glycolide)-PEG nanoparticles with a long-term controlled drug release behavior exhibited a similar TIR to free mitaplatin in MDA-MB-468 tumor-bearing mice. DTX-loaded PEG-poly(epsilon-caprolactone) nanoparticles displayed a comparable in vivo antitumor efficacy and survival rate in MDA-MB-231 TNBC animal model to DTX commercial formulation (Taxotere®).

H&E staining displayed that cRGD-MMP at 0.8 and 1.6 mg DM1 equiv./kg did not cause significant damage to the major organs, while free DM1 induced obvious increased Kupffer cells in liver tissues, fat vacuoles, liver cell cord derangement and dilated intercellular spaces (Figure 6). All the abovementioned results demonstrate that cRGD-MMP has better tumor selectivity and enhanced treatment of MDA-MB-231 TNBC. These cRGD-decorated, polycarbonate-based, reduction-sensitive mertansine prodrug micelles have proven to be an effective platform for TNBC chemotherapy.

**Conclusion**

We have demonstrated that cRGD-MMP mediates highly potent and targeted treatment of αβ3 integrin overexpressing MDA-MB-231 TNBC xenografts in nude mice. Notably, cRGD-MMP presents many great merits, including: 1) high affinity to MDA-MB-231 cells and fast intracellular drug release, leading to a potent antitumor effect comparable to free DM1; 2) high accumulation in the MDA-MB-231 tumor, reaching 8.1%ID/g at 12 h post injection, likely due to its high stability and active targeting ability; 3) significantly more effective inhibition of tumor growth than free DM1 and the nontargeted MMP controls at 0.8 mg DM1 equiv./kg; and 4) reduced systemic toxicity as compared to free DM1, which enables effective treatment of MDA-MB-231 tumor-bearing
mice at a higher dosage without causing obvious side effects. This MMP appears to be a promising platform for potent treatment ofTNBCs.

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Author contributions
All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure
The authors report no conflicts of interest in this work.

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