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Targeting Cell Surface Alpha(v)beta(3) Integrin Increases Therapeutic Efficacies of a Legumain Protease-Activated Auristatin Prodrug

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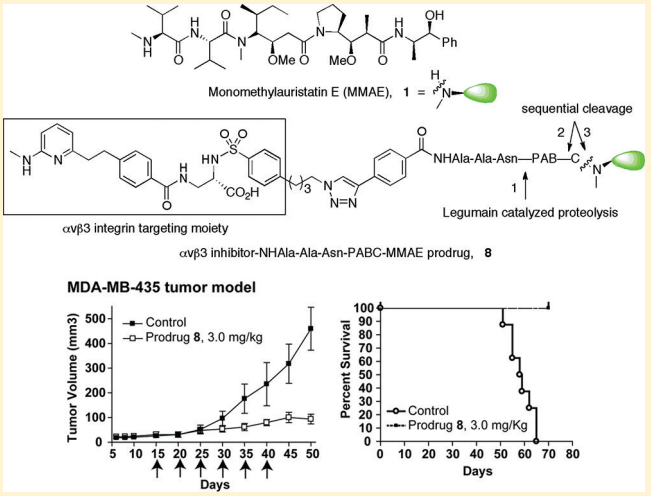
Supporting Information

ABSTRACT: Novel monomethylauristatin E (MMAE) prodrug **8** was designed and prepared that bound cell surface glycoprotein integrin $\alpha v\beta 3$, and was activated using legumain protease as a catalyst. Upon activation, prodrug **8** strongly induced the death of MDA-MB-435 cells that express integrin $\alpha v\beta 3$ on cell surface. Efficacies of prodrug **8** were also determined *in vivo* using animal models of 4T1 murine breast cancer, D121 Lewis lung carcinoma, and MDA-MB-435 human breast cancer. The results demonstrated that prodrug **8** decreased tumor growth and metastasis effectively. In comparison to the parent cytotoxin, MMAE, and prodrug **3**, prodrug **8** was less toxic to mouse white blood cells. The latter caused no loss in weight gain of mice at a dose 3 mg/kg, which is over 30 times in excess to MMAE (0.1 mg/kg). We hypothesize that overexpression and colocalization of integrin $\alpha v\beta 3$ and legumain protease on tumor cells, tumor vasculature, and/or tumor microenvironments can be exploited to enhance the efficacy and selectivity of potent cytotoxins, such as MMAE, which is otherwise too toxic to use for therapy.



KEYWORDS: *monomethylauristatin E (MMAE), prodrug, integrin, legumain, prodrug activation*

INTRODUCTION

Many potent cytotoxins, including enediynes, epothilones, duocarmycins, dolastatins, tubulysins, etc., possess subnanomolar cytotoxicity in *in vitro* assays, yet they have only limited therapeutic efficacies *in vivo* at a dose that is not lethal to animals.[1] Fortunately, such potent cytotoxins can be formulated as prodrugs and delivered to tumor or in tumor microenvironments (TMEs) using various tumor-targeting agents, including monoclonal antibodies (Abs) or small molecule inhibitors.[2] Previously, we have synthesized and examined a series of prodrugs of the doxorubicin,[3,4] enediyne,[5] and duocarmycin analogue prodrugs[6] that are activated using the monoclonal aldolase Ab 38C2[7] or 93F3.[8] We have also prepared and evaluated several monomethylauristatin E (MMAE, **1**) and didesmethylauristatin E (DDAE, **2**) prodrugs, including **3** and **4** (Figure 1),[9] that recruit tumor-associated protease (TAP), legumain, for their activation.[10] Based on our *in vitro* studies with these prodrugs, prodrug **3** was chosen and its efficacy was determined *in vivo* using animal models of murine breast cancer 4T1; prodrug **3** reduced growth of 4T1 tumor by 57% at 0.5 mg/kg, whereas all animals died when parent compound **1** was used at this dose. We argued that the therapeutic efficacies of prodrug **3** could be further enhanced by directing it, in the form of a small molecule−prodrug or an antibody (Ab)−prodrug conjugate, to tumor cells and in TMEs, where legumain protease will catalyze the release of free drugs.

Legumain is an asparaginylendopeptidase with a remarkably restricted specificity for asparagine at P1 site of the substrate sequence.[11] It is an evolutionary offshoot of the C13 family of cysteine proteases,[12] initially identified in plants as a processing enzyme of storage proteins during seed germination, and later also identified in parasites and in mammals. Legumain is present intracellularly in endosome/lysosome systems[13,14] and associated with intracellular protein degradation, but also overexpressed in a majority of tumors, including carcinomas of the breast, colon, and prostate, in central nervous system neoplasms,[10] and secreted to tumor cell surface and in the TMEs. Because legumain is active at low pH, i.e., 4.0−6.5, and gets deactivated at a physiologically neutral pH, it is uniquely

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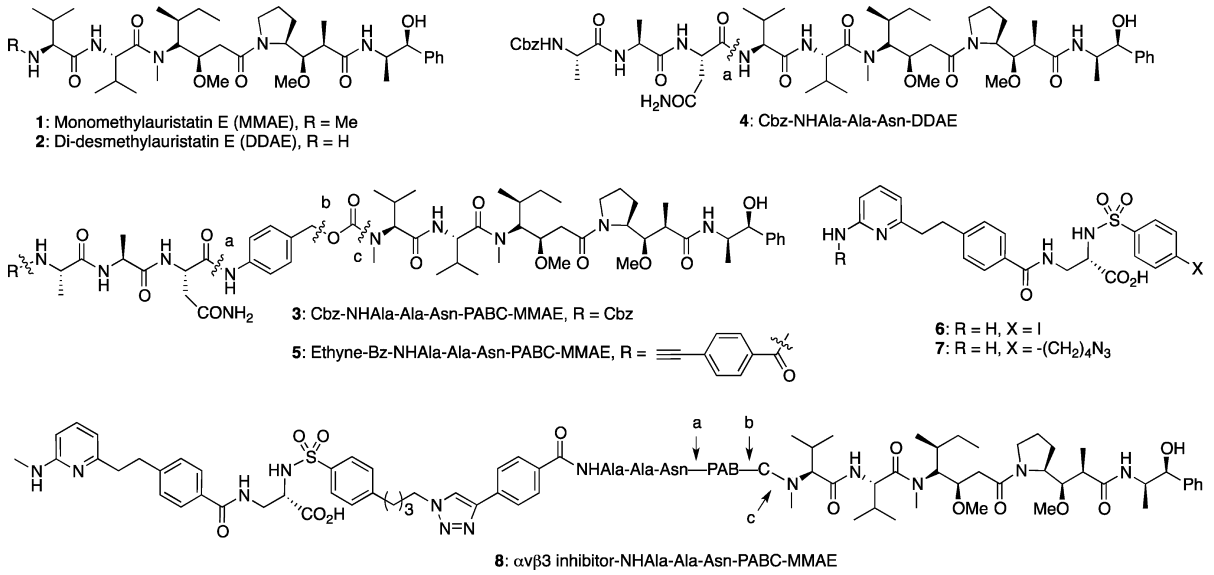

Figure 1. Auristatin E analogues and their prodrugs; MMAE, monomethylauristatin E; DDAE, di-desmethylauristatin E.

set to catalyze prodrug activation in acidic TMEs and in tumor cells. Interestingly, legumain protease colocalizes with various integrins, including integrin $\alpha v\beta 3$ on cell surface, and they interact with each other, and both are overexpressed in many tumor cells and/or in tumor vasculature and are highly implicated in tumor growth and metastases. Integrin $\alpha v\beta 3$, a transmembrane glycoprotein composed of α and β subunits, is a major target implicated in tumor angiogenesis.[15] In response to a cellular stimulation, the extracellular domain of integrin $\alpha v\beta 3$ is activated, thereby changing its conformation from a low-affinity ligand-binding state to a high-affinity state.[16,17] Integrin $\alpha v\beta 3$ expression level is high, and its activation has been detected in different types of tumors, including prostate,[18] breast,[19] melanomas,[20] gliomas,[21] and ovary.[22] Indeed, $\alpha v\beta 3$ integrin has become one of the most valued targets for imaging and drug targeting.[23]

In this article, we describe *in vitro* and *in vivo* evaluation of an integrin inhibitor−MMAE prodrug conjugate **8**, which combines properties of prodrug **3**[9] with a previously described low molecular weight inhibitor **6** of integrin $\alpha v\beta 3$.[24,25] Presumably, prodrug **8** that is prepared using an MMAE derivative **5** and $\alpha v\beta 3$ integrin inhibitor **7** (see Supporting Information) can target to integrin $\alpha v\beta 3$, and recruit legumain protease to catalyze the prodrug activation selectively in tumor cells or in TMEs. Here, we first examined colocalization of legumain protease with integrins $\alpha v\beta 3$ on tumor cell surface, and catalytic activity of the former in various tissues. Inhibitory effects of the inhibitor−prodrug conjugate **8** on cell proliferation, tumor growth, and metastasis were determined *in vitro* and *in vivo* using 4T1 murine breast cancer and D121 Lewis lung cancer, and MDA-MB-435 human breast cancer cell lines. Notably, MDA-MB-435 has high expression of integrin $\alpha v\beta 3$ on cell surface.[26] With these results, we emphasize that targeting cell surface $\alpha v\beta 3$ integrin and recruiting tumor-associated protease for prodrug activation would greatly increase efficacies of a cytotoxin, such as MMAE, which may otherwise be too toxic to have any therapeutic value.

MATERIALS AND METHODS

Antibody and Tumor Cell Lines. The mouse 4T1, D121, and RAW264.7, and human MDA-MB-435 cell lines were obtained from ATCC. Human embryonic kidney 293 cells stably expressing human legumain (HEK-293L) were kindly provided by Dr. G. David Roodman (Department of Medicine and Hematology, University of Texas Health Science Center, San Antonio, TX). RAW264.7 cells were maintained in Dulbecco's modified Eagle's/Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics. 4T1, D121, and MDA-MB-435 cells were grown in DMEM medium with 10% fetal bovine serum. Mouse anti-human legumain (H00005641-MO2) monoclonal Ab was purchased from (Abnova). Rabbit anti-human legumain polyclonal Ab was affinity purified from rabbit serum, which was prepared by immunization with CGMKRASSPVPLPP peptide conjugated to keyhole limpet hemocyanin (KLH).

Integrin $\alpha v\beta 3$-Targeted Legumain Activated MMAE Prodrug 8. To a mixture of compounds **5** (108 mg, 0.09 mmol) and **7** (50 mg, 0.09 mmol) in DMF (2 mL) were added Cu powder (4 mg, 0.06 mmol) and aqueous $CuSO_4$ (1M, 20 μL, 0.02 mmol), and the mixture was stirred at 40 °C for 24 h. After the reaction was complete, as determined using LC−MS, solvents were removed under reduced pressure, and the residue was purified using HPLC affording compound **8** (90 mg, 57%). HRMS (ESI): Calcd for $C_{94}H_{128}N_{17}O_9S$ $(M + H)^+$ 1830.9217; found 1830.9117.

Confocal Immunoanalysis. Immunofluorescent double staining was performed on hypoxia culture human MDA-MB-435 and HEK-293L cells, which were treated with 10 nM EFG in fibronectin coating flask for 3 days. We dissected MDA-MB-435 tumor to make a frozen section (5 μm) for double staining. For staining of integrin, mouse monoclonal Ab to integrin $\alpha v\beta 3$ (0.1 μg/mL) was used as first Ab, and Texas-red conjugated anti-mouse IgG (Vector Laboratories) was used as the secondary reporting reagent. For legumain identification, legumain polyclonal Ab was used at 0.5 μg/mL, followed by FITC-conjugated anti-rabbit IgG (Vector Laboratories) as the second antibody. Nuclei were stained with DAPI. The slides were analyzed by using Bio-Rad Radiance 2100 Rainbow laser scanning confocal microscope (LSCM). Equal concentrations of mouse IgG, rabbit IgG, and secondary antibodies were used as the negative controls.

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Proteolytic Activities of Cells and Tissues. To determine the proteolytic activity in the cell culture media, cells were incubated under hypoxic conditions for 3 days, and the cultured media (200 μL) were diluted using an activity assay buffer (750 μL) (50 mM citric acid, 121 mM Na_2HPO_4, pH 6.0, containing 1 mM DTT, 1 mM EDTA, and 0.1% CHAPS). Subsequently, a solution of Z-Ala-Ala-Asn-NHMec (50 μL, final concentration 10 μM) was added to the diluted media (950 μL) and incubated at 37 °C for 30 min, and activation was determined by the fluorescence measurement using a Perkin-Elmer LS-50-B spectrofluorometer (excitation, 360 nm; emission, 460 nm). Similarly, to examine the distribution of legumain activity in tumor and various organs, equal weights of 4T1 murine breast cancer tumor and normal tissues were homogenized in OG (octyl glucoside) buffer (50 mM OG, 50 mM Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA pH 6.0) to make a single cell suspension. After the suspensions were incubated with substrate at room temperature for 1 h, fluorescence of NH_2Mec was determined and compared to provide the legumain activity in tumor and in organs.

Cytotoxicity Assays. Cytotoxicity assays were performed as described previously. Typically, HEK-293L and MDA-MB-435 cells were incubated with serial dilutions of cytotoxic agent and prodrugs (compounds) in a 96-well tissue culture plate for 2 days. Cells were processed using the CellTiter 96 non-radioactive cell proliferation kit (Promega), as described in the instructions. OD (450−630 nM) of the processed cells was measured by using an ELISA micro plate reader (BIO-RAD450). Dose−response curves were plotted as percent of nonexposed control cells.

Maximum Tolerated Dose. Six-week-old BALB/c mice were used in each experimental group. The mice were weighed individually, and the average weight of the group was used to define the doses. Mice were given ip injections of the indicated amounts of MMAE or prodrugs daily for 5 days. The maximum tolerable dose (MTD) was defined as the maximum drug dose administered to non-tumor-bearing mice once daily for 5 consecutive days without mortality.

Blood Cell Toxicity. 250 μL of fresh blood (with EDTA) was collected from each mouse eye. Cells were diluted to 1 to 1000 in PBS. Ten microliter cells were applied to a hemocytometer and number of RBC per large square were counted. RBC cell number was as follows: counts/large square × 1,000 dilution × 10 large squares/μL = RBC/μL blood. To perform white blood cell count, 10 μL of whole blood was mixed and incubated with 190 μL of RBC lysing reagent for 1 min. Then the number of WBC per large square in 10 μL of lysed blood were counted . WBC cell number was as follows: counts/large square × 20 dilution × 10 large squares/μL = WBC/μL blood.

Mouse Tumor Models. The 4T1 murine breast carcinoma model, D121 Lewis lung cancer, and MDA-MB-435 human breast cancer models were generated and maintained at The Scripps Research Institute. 4T1, D121, and MDA-MB-435 cells (1×10^6) were injected separately sc on the back of 4 to 8 weeks old BALB/c, C57BL/6J, and Hsd:athymic nude mice, repectively. Starting on day 5, when the tumors were approximately 100 mm^3 (for 4T1 and D121 model), the tumor bearing mice were ip injected with saline alone (control group) and prodrug **8** with the indicated dosage and treatment schedule (n = 6 per group). Tumor growth and signs of physical discomfort were monitored daily including for any gross evidence of tumor necrosis or local tumor ulceration as well as evidence of toxicity including the mobility of animals, response to stimulus, piloerection, eating, and weight. Tumor volumes of treated animals were measured every three days in 4T1 and D121 models or five days in MDA-MB-435 model by microcaliper (volume = length × width × width/2). As soon as the tumor volume reached 1500 mm^3 in the control groups (500 mm^3 in the control groups for MDA-MB-435 model), the tumors were removed. All mice were euthanized, and the lungs were removed to Bouin's solution two weeks after removal of the tumor. Spontaneous lung metastases were counted under an anatomy microscope. All experiments were conducted at The Scripps Research Institute facilities using the protocols reviewed and approved by the Institutional Animal Care and Use Committee.

Statistical Analysis. Results are expressed as means ± SEM. Student paired t test is used to analyze the difference between two groups (values significant at $P < 0.05$).

RESULTS

Integrin αvβ3 and Legumain Protease Are Colocalized on MDA-MB-435 Human Breast Cancer Cells. To confirm that integrin αvβ3 and the cysteine protease legumain are indeed colocalized on tumor cells, we used MDA-MB-435 human breast cancer cells, and determined the legumain and integrin expressions using the confocal immunoanalysis after the cells were cultured under the hypoxia condition. Integrin expression was determined using the anti-αvβ3 integrin mouse monoclonal antibody and the anti-legumain polyclonal rabbit antibody together with the appropriate secondary antibodies. As shown in Figure 2A, legumain protease was most abundantly visualized in lysosome and endosome. This is consistent with the fact that legumain is involved in the processing of various intracellular proteins and proteases[27] and the delivery of membranous vesicles containing proteases, actin-binding proteins, and adhesion molecules toward the leading edge of migratory cells has been implicated in cell locomotion.[28] However, legumain is also present on the cell surface of MDA-MB-435 in lamellipodia where it associates and colocalizes with the adhesion protein integrin αvβ3. Presumably, association of the legumain with integrin αvβ3 takes place through the RGD domain present in former to the RGD-dependent integrin.[13] On the other hand, studies with HEK 293L cells show that they do not express integrin αvβ3, nor is there any colocalization of integrin αvβ3 with legumain, as expected (Figure 2B). Colocalization of legumain with integrin αvβ3 is also accompanied by the appearance of legumain activity in MDA-MB-435 culture medium (Figure 2C), but not in HEK 293L culture medium.

Distribution of Active Legumain Protease Is High in Tumor Cells and Tissues. Legumain protease is an essential enzyme found in all cells, however the catalytic activity of tumor tissues is invariably higher in most tumors and TMEs than it is in normal tissues. This is primarily because legumain protease is overexpressed in/on tumor cells and in TMEs, and because the intracellular compartments of the tumor cells and the TMEs are more acidic than the normal cells. It should be noted that the legumain protease is active at low pH and virtually inactive at the physiologic pH.[29] To determine and compare the active legumain protease distribution, tumor tissues and tissues of various organs from a tumor-bearing mouse were used and the protease activity was determined using Z-Ala-Ala-Asn-NHMec as a substrate. The latter

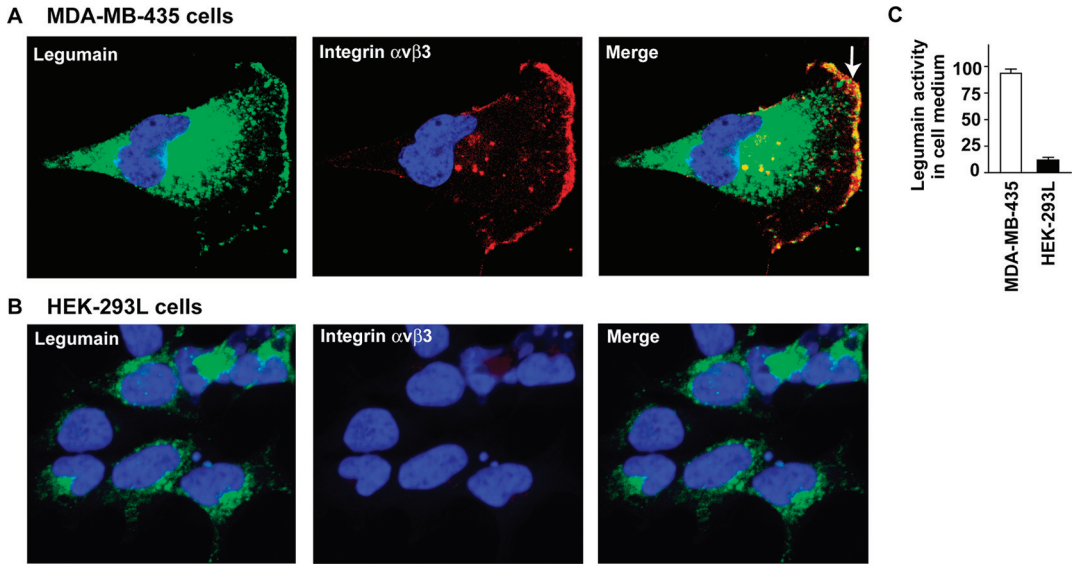

Figure 2. Legumain is exclusively colocalized with integrin $\alpha v\beta 3$ on the MDA-MB-435 cancer cell surface. (A) Legumain (green) is detected in intracellular vesicles and prominently colocalized with integrin $\alpha v\beta 3$ (red) under hypoxia. Cell nuclei are stained with DAPI (blue). Double staining is performed with anti-integrin antibodies and anti-legumain antibody. Stained cells are imaged by confocal microscopy, and the slice closest to the coverslip is presented for each cell. Note that legumain is localized on the MDA-MB-435 cell surface (green, white arrow). (B) High expression legumain is only detected in intracellular vesicles of human HEK-293L cells. HEK-293L cells do not express legumain and integrin $\alpha v\beta 3$ on the cell surface. (C) MDA-MB-435 cells secrete more active legumain into the culture medium than human HEK-293L does. The experiments were repeated three times ($p < 0.001$).

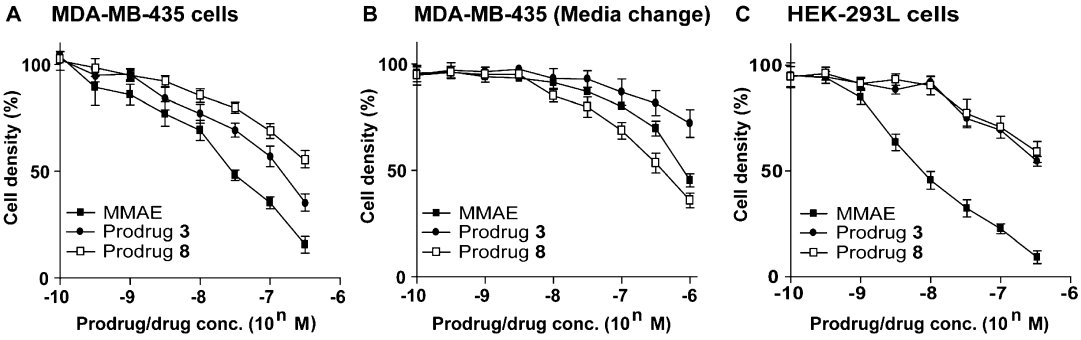

Figure 3. Prodrug **8** enhances cytotoxicity to MDA-MB-435 cancer cell. Cells were incubated with MMAE, prodrug **3**, and prodrug **8** for (A) 48 h and (B) 1 h, and then additional 47 h after unbound compound was removed by change of medium. (C) Similarly, HEK-293L cells were incubated with MMAE, prodrug **3**, and prodrug **8** for 48 h.

undergoes legumain-catalyzed hydrolysis affording the fluorescent 7-aminomethylcoumarin (Mec-NH_2). As shown later in Figure 4D, the legumain activity was indeed high in tumor tissues as compared to that in normal tissues of various organs, indicating that the legumain-catalyzed activation of the MMAE prodrugs would take place primarily in the tumor tissue.

Targeting Integrin $\alpha v\beta 3$ Enhances Tumor-Selectivity of the MMAE Prodrug 8. We have earlier shown that both cytotoxin **1** and prodrug **3** possess subnanomolar to low nanomolar activities to MDA-MB-435 cells (using 72 h incubation period).[9] We anticipated that the integrin-targeted MMAE prodrug **8** should mediate higher and more selective antitumor effects than both the parent cytotoxin **1** and prodrug **3** to MDA-MB-435 cells, which overexpress both legumain protease and $\alpha v\beta 3$ integrin, through localizing in tumor cells and TMEs, *in vivo*. In addition, there should not be a minimum difference in the activity of prodrugs **3** and **8** to HEK-293L cells that do not express cell surface $\alpha v\beta 3$ integrin, but have a high level of legumain protease stably expressed, and the activity of the prodrugs should approach the cytotoxicity of the parent cytotoxin **1**. To assess our hypothesis, first we determined the *in vitro* cytotoxicity of **1**, **3** and **8** to both MDA-MB-435 and HEK-293L cells (using 48 h incubation period). The results are shown in Figures 3A and 3C. Evidently, cytotoxin **1** was more potent than the prodrugs **3** and **8** to both cell lines, and prodrug **8** was also less cytotoxic than **3** to MDA-MB-435 cells, whereas they were equipotent to HEK-293L cells. Next, to mimic the *in vivo* condition and determine the integrin-targeting effects of the prodrug **8**, we examined its activity to MDA-MB-435 cells under a modified cytotoxicity assay condition. Here, cells were incubated with **1**, **3** and **8** at different concentrations at 37 °C for 1 h, and media were replaced with the fresh one with no additional cytotoxin or prodrug and incubation was continued for an additional 47 h. The results are shown in Figure 3B. Under this condition, prodrug **8** was found more effective and superior than both the parent cytotoxin **1** and its prodrug **3**.

Prodrug 8 Has Low Toxicity and Is Stable in Serum. Before carrying out the *in vitro* and *in vivo* assays, we assessed the stability of prodrug **8** by mixing the compound with mouse

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blood, and analyzing the reaction mixture after it was incubated at 37 °C for 16 h using LC−MS. There were no degradation products observed, showing that the prodrug was stable in serum. Prodrug **8** is significantly less toxic than prodrug **3** when evaluated *in vivo*. Prodrug **8** had a much higher cumulative MTD and reduced LD50 compared with prodrug **3** (Table 1A),

Table 1. *In Vitro* and *in Vivo* Toxicity of Prodrug 8 Compared with MMAE and Prodrug 3[a]

(A) Estimated MTD and LD50 of prodrugs **3** and **8** (mg/kg) in BALB/c mice				
	3		**8**	
	MTD	LD50	MTD	LD50
ip comm	15	12	>60	>60
ip 5×	3	1.5	>6	>6
(B) Comparison of gross toxicity to mice; i.p. 5 x				
ip 5× dose (mg/kg)	0.1	0.5	1.5	3
MMAE, **1**				
wt loss (%)	33.4			
death (%)	20	100	100	
prodrug **3**				
wt loss (%)	10.8	12.7	38.7	
death (%)	0	0	40	100
prodrug **8**				
wt loss (%)	0	3.4	8.2	14.9
death (%)	0	0	0	0
(C) Comparison of blood cell density of mice; i.p. 1 x Dose, 9 mg/kg				
		day 0		day 4
PBS				
WBC (10^9/L)		8.1 ± 1.7		7.8 ± 1.5
RBC (10^{12}/L)		9.1 ± 1.5		9.3 ± 1.4
prodrug **3**				
WBC (10^9/L)		7.8 ± 1.7		4.4 ± 2.3
RBC (10^{12}/L)		9.0 ± 1.4		8.1 ± 1.9
prodrug **8**				
WBC (10^9/L)		8.2 ± 1.8		7.6 ± 1.1
RBC (10^{12}/L)		9.4 ± 1.4		9.2 ± 2.0

[a]See Materials and Methods for the experimental details.

when given by five repeat ip administrations. For a comparison, the previously described prodrug **3** and cytotoxin **1** were administered at 0.1, 0.5, and 1.5 mg/kg. Buffer alone was used as a control. All animals were weighed periodically starting on day 1 for 3 weeks. The results shown in Table 1B confirmed that the prodrug **8** (3 mg/kg) had lower toxicity than the previously described legumain activated prodrug **3** (1.5 mg/kg). Again cytotoxin **1** was extremely toxic at (0.1 mg/kg), and comparable to the previously reported results. We further evaluated the toxicity of compound **8** and compared to **3** by examining their effects on white blood cells and red blood cells (Table 1C). Whereas both compounds had no appreciable effect on red blood cells when animals were treated with a single injection of the compounds at 9 mg/kg, WBC counts were significantly reduced for the prodrug **3**, but not for compound **8**, which showed no appreciable reduction in the WBC counts at the same dose.

The Integrin Inhibitor-Targeted Prodrug 8 Suppresses Tumor Growth and Metastasis Effectively. Next, we examined the antitumor effects of the integrin-targeted prodrug **8** using the primary tumor models of murine 4T1 mammary carcinoma and D121 Levis lung carcinoma cell lines. Both 4T1 and D121 cells have high legumain expression.[30,31] 4T1 tumor models were generated by sc injection of 1×10^6 cells in the right flank of six-week-old BALB/c mice. A dose-response assessment shows that administration of compound **8** (0.5, 1.5, or 3.0 mg/kg) on day 5, 8, 11, 14, and 17 suppresses tumor growth (Figure 4A). Prodrug **8** has a better effect in suppression of tumor growth than prodrug **3** in dosage of 0.5 mg/kg. High dosage of prodrug **8** (3 mg/kg) could also be applied, whereas 1.5 mg/kg dosage of prodrug **3** caused mouse death. TUNEL assay, which is based on *in situ* labeling of DNA fragmentation sites in nuclei of intact fixed cells on tumor sections, shows that the treatment with prodrug **8** induced tumor cell death (Figure 4B). Significantly, the treatment with prodrug **8** also blocked spontaneous metastasis of 4T1 tumor to lungs (Figure 4C). Similarly, to determine the effect of compound **8** on growth of the D121 Lewis lung tumor model, tumors were generated by sc injection of 1×10^6 D121 cells in the right flank of six-week-old C57BL/6J mice, and compound **8** (3.0 mg/kg) was ip administered on days 5, 8, 11, 14, and 17. Evidently, compound **8** suppressed tumor growth significantly and blocked the spontaneous metastasis to lungs (Figures 5A and 5B).

Prodrug 8 Eradicates Tumors of Human Breast Carcinoma. The expression and presence of colocalization of legumain and integrin $\alpha v\beta 3$ is supported by confocal analysis of MDA-MB435 tumor double staining sections (Figure 6A). We further examined the efficacy of prodrug **8** using human MDA-MB-435 tumor model in Hsd:athymic nude mice, which showed a significant growth inhibition in animals treated with 3 mg/kg with the prodrug (Figure 6B, left). Animals showed no weight loss or any apparent signs of toxicity, and were live during the whole period of the *in vivo* study. In contrast, animals treated with only vehicle had large tumors, and died within 65 days (Figure 6B, right).

DISCUSSION

Cell-targeted prodrug therapy approach, involving cell surface adhesion molecules to direct a prodrug to tumor cells and tumor-associated proteases for the prodrug activation, is likely to enhance the therapeutic indexes of many anticancer agents that are otherwise toxic and not suitable for therapy. Prior studies with MMAE and analogous cytotoxin prodrugs that are activated by cathepsin B protease and conjugated to monoclonal antibodies or the RGD peptides revealed that tumor-targeting is essential to achieve high efficacies with these prodrugs.[32−34] The concept of such dual targeted prodrugs can further benefit by the readily available technologies, including the positional gene expression profiling of tumor tissues and imaging studies, which can identify suitable tumor-associated cell surface receptors and proteases for the prodrug targeting and activation.[35] Indeed, the MMAE prodrug **8** was designed after we established that integrin $\alpha v\beta 3$ and the legumain protease not only are overexpressed but also colocalized on certain cancer cells, including MDA-MB-435 cells (Figure 2). As expected, prodrug **8** possessed the tripeptide linker (P1-Asn, P2-Ala, P3-Ala) identical to that in prodrug **3** (Figure 1), which is in keeping with other legumain-activatable prodrugs, and both prodrugs **8** and **3** were fully activated upon overnight incubation with a catalytic amount of legumain.[9]

The cytotoxicity studies using MMAE and its prodrugs, **3** and **8**, revealed that the dual targeted prodrug **8** was least toxic and more effective than both MMAE and prodrug **3**. It should be noted that prodrug **8** was indeed more potent than MMAE

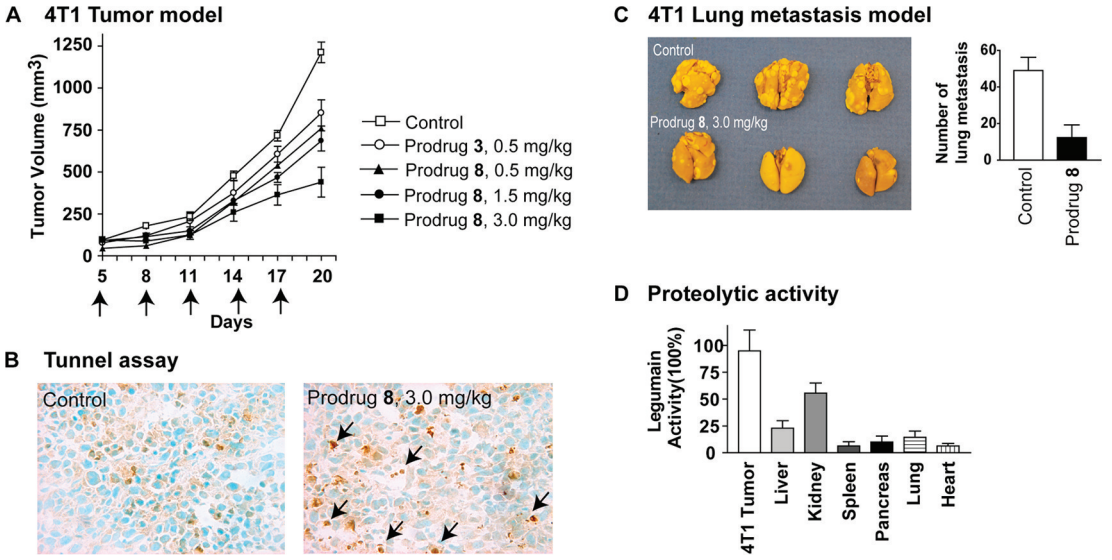

Figure 4. Prodrug **8** suppresses 4T1 tumor growth and lung metastasis. (A) *In vivo* effect of prodrug **8** on 4T1 mammary carcinoma. Starting on day 5, when the tumors averaged ≈4 mm in diameter, the 4T1 tumor mice were treated with 0.5, 1.5, and 3 mg/kg of the prodrug **8** separately on days 5, 8, 11, 14, and 17 without any toxicity, but 3 mg/kg dosage of prodrug **3** caused mouse death. Six mice were treated in each group ($p < 0.001$). (B) Prodrug **8** treatment induces cell death in TUNEL staining of 4T1 tumor sections. (C) Prodrug **8** suppresses spontaneous lung metastasis in 4T1 mouse breast cancer models ($p < 0.001$). (D) Legumain activity of tumor tissue is significantly higher than that of normal tissues. The experiments were repeated in three mice ($p < 0.01$).

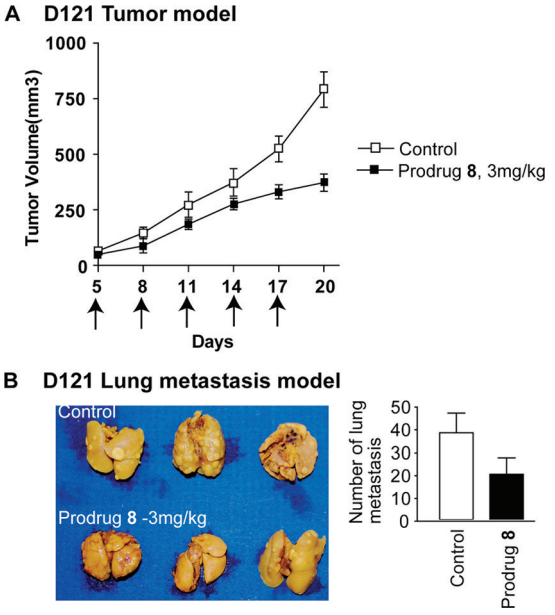

Figure 5. Prodrug **8** suppresses D121 tumor growth and lung metastasis. (A) *In vivo* effect of prodrug **8** on D121 Lewis lung carcinoma. Starting on day 5, when the tumors averaged ≈4 mm in diameter, the D121 tumor mice were treated with 3 mg/kg separately on days 5, 8, 11, 14, and 17. Six mice were used in each group; ($p < 0.001$). (B) Prodrug **8** suppresses spontaneous lung metastasis in D121 mouse models ($p < 0.001$).

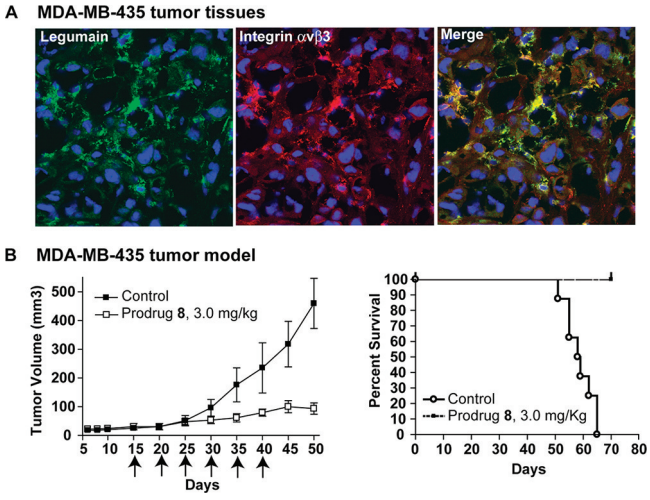

Figure 6. Tumoricidal effect of prodrug **8** in MDA-MB-435 carcinoma in vivo. (A) Confocal analysis of legumain (green) and integrin $\alpha v\beta 3$ (red) in MDA-MB-435 tumor tissue. (B) (Left) *In vivo* effect of prodrug **8** on MDA-MB-435 carcinoma. Starting on day 15, when the tumors averaged ≈5 mm in diameter, the MDA-MB-435 tumor mice were treated with saline alone, and prodrug **8** (3 mg/kg) on days 15, 20, 25, 30, 35, and 40. The experiments were repeated twice, and 6 mice were used in each treated group ($p < 0.01$). (Right) Kaplan–Meier survival curves of mice bearing MDA-MB-435 tumors in control and prodrug **8** groups. The survival was based on the primary tumor diameter (>1.5 cm) and natural death.

and prodrug **3** when the cell culture media were exchanged after a brief incubation (Figure 3B), which supports the hypothesis that targeting integrin $\alpha v\beta 3$ enhances the accumulation of prodrug **8** in tumor, tumor vasculature and/or TMEs in high concentration, where legumain converts the prodrug to the active drug MMAE, **1**. In turn, the latter induces tumor cell death and prevents cancer cell invasion and distant metastasis. The fact that prodrug **3** showed higher cytotoxicity than prodrug **8** under the normal cytotoxicity evaluation conditions to MDA-MB-435 cells is however interesting. This could be explained, if the prodrug **8** is more weakly activated than **3**, which appears less likely as both prodrugs showed comparable cytotoxicity to HEK-293L cells that expresses neither integrin $\alpha v\beta 3$ nor the legumain protease on the cell surface. We argue that prodrug **8** functions both as an integrin inhibitor and as a prodrug, and the selective toxicity due to

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prodrug activation is realized only after the prodrug gets internalized. In contrast, prodrug **3** is also activated extracellularly. It should be noted that the legumain protease is secreted to cell surface in MDA-MB-435 cells, but not in HEK-293L cells.

Integrin $\alpha v\beta 3$, which is overexpressed and activated on invasive tumor cells and on angiogenic blood vessels in tumor tissues, fulfills growth promoting and invasive enhancement of the sprouting endothelial cells.[36] As expected, an inhibition of $\alpha v\beta 3$ integrin using low molecular weight inhibitors and Abs, including cyclic RGD peptide inhibitors and a humanized anti-$\alpha v\beta 3$ Ab MEDI-522, has shown positive effects in patients. Thus, in addition to selective targeting and prodrug activation, prodrug **8** could also have inhibited tumor growth and metastases through inhibiting integrin $\alpha v\beta 3$ owing to the inhibitor component of the molecule. Importantly, the integrin-targeting MMAE prodrug **8** is also likely to mediate a highly selective antitumor effect through localizing in tumor cells and TMEs, but not in normal tissues, especially from kidney, liver, and lung, which also possess considerable protease activity.[30]

Previous examples of the prodrugs that are activated using legumain protease include those derived from doxorubicin[30] and camptothecin.[37] For cardiac tissue, previous legumain activated doxorubicin prodrugs have shown reduced accumulation >15-fold and have a notable advantage of less cardiotoxicity. In this study, prodrug **8** is also a tumor microenvironment activated prodrug that is catalytically converted to end product MMAE in the tumor microenvironment. The activation of prodrug **8** is not found in any significant amounts in normal tissues presumably as a result of no active legumain location on healthy native cell surface. Based on LD50, the toxicity of prodrug **8** in mouse was reduced >30-fold compared with MMAE. Prodrug **8** is stable in plasma, and has little effect on cells of myeloid lineage, as mice showed negligible reduction in peripheral blood or marrow myeloid cells at elevated therapeutic doses (Table 1).

Based on the reduced toxicity, a larger cumulative dosage of prodrug **8** can be administered more rapidly, and significant tumor growth and metastasis inhibition were observed, as shown in Figure 4. In contrast, both MMAE and prodrug **3** were found considerably toxic beyond 0.1 mg/kg and 0.5 mg/kg to mice. Consequently, in the 4T1 mouse breast carcinoma model, a significantly reduced tumor growth was obtained after 4T1 tumor cell inoculation into mice, followed by treatment with the prodrug **8**. Fewer lung metastases were found in treatment group as compared to the control group in spontaneous lung metastasis experiments. Similar results were obtained with two other tumor models, D121 Lewis and human MDA-MB-435 carcinoma. There were greater effects on tumor of MDA-MB-435 carcinoma, which completely stopped and started to shrink upon continued treatment. All animals were alive after 70 days in the treatment group. These data strengthen our contention that integrin $\alpha v\beta 3$ targeted and legumain activated prodrug approach could be a useful antitumor strategy for suppressing tumor cell invasion and metastases of many cancers.

In summary, novel dual-targeted MMAE prodrug **8** designed to bind cell surface glycoprotein integrin $\alpha v\beta 3$ and activate using legumain protease as the catalyst was prepared and evaluated both *in vitro* and *in vivo*. The antitumor efficacy of prodrug **8** was critically evaluated by using mouse models of three different cancers, which demonstrated that prodrug activation took place in tumor tissues that effectively decreased tumor growth and metastasis. This strategy greatly reduces toxicity to healthy body cells by synergism of integrin binding $\alpha v\beta 3$ and legumain activation function, indicating that this antitumor strategy could be widely applicable and relevant for possible cancer therapy. We are also using integrin $\alpha v\beta 3$ and the legumain protease here as the target and catalyst, respectively, of our first small molecule−prodrug conjugate, because their combination has never been utilized to realize the efficacy of a cytotoxin. In addition, the fact that a non-peptidic small molecule integrin inhibitor, such as **7**, is used to direct the MMAE prodrug to tumor cells will broaden the pool of procytoxins, which can be delivered to tumor cells and tumor vasculature through the integrin $\alpha v\beta 3$-targeting.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis and physical data of prodrug **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Druker, B. J. Perspectives on the development of a molecularly targeted agent. *Cancer Cell* **2002**, *1*, 31−6.

(2) Singh, Y.; Palombo, M.; Sinko, P. J. Recent trends in targeted anticancer prodrug and conjugate design. *Curr. Med. Chem.* **2008**, *15*, 1802−26.

(3) Sinha, S. C.; Li., L. S.; Watanabe, S.; Kaltgrad, E.; Tanaka, F.; Rader, C.; Lerner, R. A.; Barbas, C. F. III. Aldolase antibody activation of prodrugs of potent aldehyde-containing cytotoxics for selective chemotherapy. *Chemistry* **2004**, *10*, 5467−72.

(4) Abraham, S.; Guo, F.; Li, L.-S.; Rader, C.; Liu, C.; Barbas, C. F. III; Lerner, R. A.; Sinha, S. C. Synthesis of the next-generation therapeutic antibodies that combine cell targeting and antibody-catalyzed prodrug activation. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 5584−9.

(5) Sinha, S. C.; Li, L. S.; Miller, G. P.; Dutta, S.; Rader, C.; Lerner, R. A. Prodrugs of dynemicin analogs for selective chemotherapy mediated by an aldolase catalytic Ab. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 3095−9.

(6) Li, L. S.; Sinha, S. C. Studies toward the duocarmycin prodrugs for the antibody prodrug therapy approach. *Tetrahedron Lett.* **2009**, *50*, 2933−5.

(7) Wagner, J.; Lerner, R. A.; Barbas, C. F. III. Efficient aldolase catalytic antibodies that use the enamine mechanism of natural enzymes. *Science* **1995**, *270*, 1797−800.

(8) Zhong, G.; Lerner, R. A.; Barbas, C. F. III. Broadening the aldolase catalytic antibody repertoire by combining reactive immunization and transition state theory: new enantio- and diastereoselectivities. *Angew. Chem., Int. Ed.* **1999**, *38*, 3738.

(9) Bajjuri, K. M.; Liu, Y.; Liu, C.; Sinha, S. C. The legumain protease-activated auristatin prodrugs suppress tumor growth and metastasis without toxicity. *ChemMedChem* **2011**, *6*, 54−9.
(10) Liu, C.; Sun, C.; Huang, H.; Janda, K.; Edgington, T. Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. *Cancer Res.* **2003**, *63*, 2957−64.
(11) Chen, J. M.; Dando, P. M.; Rawlings, N. D.; Brown, M. A.; Young, N. E.; Stevens, R. A.; Hewitt, E.; Watts, C.; Barrett, A. J. Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. *J. Biol. Chem.* **1997**, *272*, 8090−8.
(12) Ishii, S. Legumain: asparaginyl endopeptidase. *Methods Enzymol.* **1994**, *244*, 604−15.
(13) Chen, J. M.; Dando, P. M.; Stevens, R. A. E.; Fortunato, M.; Barrett, A. J. Cloning and expression of mouse legumain, a lysosomal endopeptidase. *Biochem. J.* **1998**, *335*, 111−7.
(14) Shirahama-Noda, K.; Yamamoto, A.; Sugihara, K.; Hashimoto, N.; Asano, M.; Nishimura, M.; Hara-Nishimura, I. Biosynthetic processing of cathepsins and lysosomal degradation are abolished in asparaginyl endopeptidase-deficient Mice. *J. Biol. Chem.* **2003**, *278*, 33194−9.
(15) Desgrosellier, J. S.; Cheresh, D. A. Integrins in cancer: biological implications and therapeutic opportunities. *Nat. Rev. Cancer* **2010**, *10*, 9−22.
(16) Xiong, J. P.; Stehle, T.; Diefenbach, B.; Zhang, R.; Dunker, R.; Scott, D. L.; Joachimiak, A.; Goodman, S. L.; Arnaout, M. A. Crystal structure of the extracellular segment of integrin alphaVbeta3. *Science* **2001**, *294*, 339−45.
(17) Guo, W.; Giancotti, F. G. Integrin signalling during tumour progression. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 816−26.
(18) Cooper, C. R.; Chay, C. H.; Pienta, K. J. The role of alpha(v)beta(3) in prostate cancer progression. *Neoplasia* **2002**, *4*, 191−4.
(19) Liapis, H.; Flath, A.; Kitazawa, S. Integrin alpha V beta 3 expression by bone-residing breast cancer metastases. *Diagn. Mol. Pathol.* **1996**, *5*, 127−35.
(20) Albelda, S. M.; Mette, S. A.; Elder, D. E.; Stewart, R.; Damjanovich, L.; Herlyn, M.; Buck, C. A. Integrin distribution in malignant melanoma: association of the beta 3 subunit with tumor progression. *Cancer Res.* **1990**, *50*, 6757−64.
(21) Gingras, M. C.; Roussel, E.; Bruner, J. M.; Branch, C. D.; Moser, R. P. Comparison of cell adhesion molecule expression between glioblastoma multiforme and autologous normal brain tissue. *J. Neuroimmunol.* **1995**, *57*, 143−53.
(22) Carreiras, F.; Denoux, Y.; Staedel, C.; Lehmann, M.; Sichel, F.; Gauduchon, P. Expression and localization of alpha v integrins and their ligand vitronectin in normal ovarian epithelium and in ovarian carcinoma. *Gynecol. Oncol.* **1996**, *62*, 260−7.
(23) Chen, K.; Chen, X. Integrin targeted delivery of chemotherapeutics. *Theranostics* **2011**, *1*, 189−200.
(24) Duggan, M. E.; Duong, L. T.; Fisher, J. E.; Hamill, T. G.; Hoffman, W. F.; Huff, J. R.; Ihle, N. C.; Leu, C. T.; Nagy, R. M.; Perkins, J. J.; Rodan, S. B.; Wesolowski, G.; Whitman, D. B.; Zartman, A. E.; Rodan, G. A.; Hartman, G. D. Nonpeptide $\alpha v\beta 3$ Antagonists 1. Transformation of a Potent, Integrin-Selective αIIb$\alpha 3$ Antagonist into a Potent $\alpha v\beta 3$ Antagonist. *J. Med. Chem.* **2000**, *43*, 3736−45.
(25) Li, L. S.; Rader, C.; Matsushita, M.; Das, S.; Barbas, C. F. III; Lerner, R. A.; Sinha, S. C. Chemical-adaptor immunotherapy: design, synthesis and evaluation of novel integrin-targeting devices. *J. Med. Chem.* **2004**, *47*, 5630−40.
(26) Furger, K. A.; Allan, A. L.; Wilson, S. M.; Hota, C.; Vantyghem, S. A.; Postenka, C. O.; Al-Katib, W.; Chambers, A. F.; Tuck, A. B. β_3 Integrin Expression Increases Breast Carcinoma Cell Responsiveness to the Malignancy-Enhancing Effects of Osteopontin. *Mol. Cancer Res.* **2003**, *1*, 810−9.
(27) Chen, J. M.; Fortunato, M.; Stevens, R. A.; Barrett, A. J. Activation of progelatinase A by mammalian legumain, a recently discovered cysteine proteinase. *Biol. Chem.* **2001**, *382*, 777−83.
(28) Wolk, K.; Grutz, G.; Witte, K.; Volk, H. D.; Sabat., R. The expression of legumain, an asparaginyl endopeptidase that controls antigen processing, is reduced in endotoxin-tolerant monocytes. *Genes Immun.* **2005**, *6*, 452−6.
(29) Li, D. N.; Matthews, S. P.; Antoniou, A. N.; Mazzeo, D.; Watts, C. Multistep autoactivation of asparaginyl endopeptidase in vitro and in vivo. *J. Biol. Chem.* **2003**, *278*, 38980−90.
(30) Wu, W.; Luo, Y.; Sun, C.; Liu, Y.; Kuo, P.; Varga, J.; Xiang, R.; Reisfeld, R.; Janda, K. D.; Edgington, T. S.; Liu, C. Targeting cell-impermeable prodrug activation to tumor microenvironment eradicates multiple drug-resistant neoplasms. *Cancer Res.* **2006**, *66*, 970−80.
(31) Luo, Y.; Zhou, H.; Krueger, J.; Kaplan, C.; Lee, S. H.; Dolman, C.; Markowitz, D.; Wu, W.; Liu, C.; Reisfeld, R. A.; Xiang, R. Targeting tumor-associated macrophages as a novel strategy against breast cancer. *J. Clin. Invest.* **2006**, *116*, 2132−41.
(32) Temming, K.; Meyer, D. L.; Zabinski, R.; Dijkers, E. C.; Poelstra, K.; Molema, G.; Kok, R. J. Evaluation of RGD-targeted albumin carriers for specific delivery of auristatin E to tumor blood vessels. *Bioconjugate Chem.* **2006**, *17*, 1385−94.
(33) Temming, K.; Meyer, D. L.; Zabinski, R.; Senter, P. D.; Poelstra, K.; Molema, G.; Kok, R. J. Improved efficacy of alphavbeta3-targeted albumin conjugates by conjugation of a novel auristatin derivative. *Mol. Pharmaceutics* **2007**, *4*, 686−94.
(34) Alley, S. C.; Okeley, N. M.; Senter, P. D. Antibody-drug conjugates: targeted drug delivery for cancer. *Curr. Opin. Chem. Biol.* **2010**, *14*, 529−37.
(35) Rautio, J.; Kumpulainen, H.; Heimbach, T.; Oliyai, R.; Oh, D.; Jarvinen, T.; Savolainen, J. Prodrugs: design and clinical applications. *Nat. Rev. Drug Discovery* **2008**, *7*, 255−70.
(36) Byzova, T. V.; Rabbani, R.; D'Souza, S. E.; Plow, E. F. Role of integrin alpha(v)beta3 in vascular biology. *Thromb. Haemost.* **1998**, *80*, 726−34.
(37) Stern, L.; Perry, R.; Ofek, P.; Many, A.; Shabat, D.; Satchi-Fainaro, R. A novel antitumor prodrug platform designed to be cleaved by the endoprotease legumain. *Bioconjugate Chem.* **2009**, *20*, 500−10.