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The Legumain Protease-Activated Auristatin Prodrugs Suppress Tumor Growth and Metastasis without Toxicity

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Keywords

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Naturally occurring antimitotic pentapeptide dolastatin 10 (**1**, Figure 1)^[1] and its synthetic analogue auristatin E (AE, **2**)^[2] possess subnanomolar cytotoxicity against many human cancer cell lines and are over a hundred- to a thousand-times more potent than many pharmaceuticals, including taxol and doxorubicin, respectively, which are currently used in clinic. These pentapeptides have mechanism of actions similar to that of taxol, but through processes that appear opposing to each other; that is, dolastatins and auristatins inhibit tubulin polymerization, whereas taxol promotes it and stabilizes the microtubules.^[3] In the end, both dolastatins or auristatins and taxol inhibit cell proliferation. Additionally, auristatins function as the vascular disrupting agents and damage the established tumor vessels, thereby likely causing a more pronounced effect than taxol in vivo.^[4] Nonetheless, the therapeutic efficacies of the auristatins and dolastatins are poor as they also cause nonselective toxicity to normal cells.^[5] Seattle Genetics has pioneered the selective delivery of auristatins to tumor cells by further modifying them to suitable analogues, including di-desmethyauristatin E (DDAE, **3**) and monomethyauristatin E (MMAE, **4**), and conjugating them to tumor-targeting antibodies (Ab) in the Fc region through a linker.^[6] Upon uptake of the conjugates in tumor cells, free drugs are released by lysosomal protease-catalyzed hydrolysis of the linker. Many such auristatin–Ab conjugates have shown very high efficacy in tumor models, and several conjugates are in clinical trials for the treatment of various cancers.^[7] We are interested in this class of molecules as they could also feed our ongoing program in the development of prodrugs^[8] and their conjugates to the programmed Abs (progAbs).^[9] First, we prepared and evaluated a series of AE (**2**) analogues and compared their cytotoxicity to the previously described analogues, DDAE and MMAE. An examination of the results revealed that new auristatin analogues were highly potent, but they were not as cytotoxic as compounds **1–4**. Therefore, we focused on MMAE and DDAE, and prepared and evaluated their prodrugs, which undergo tumor-associated protease-catalyzed activation. Herein, we report the results of our preliminary study

comparing their in vitro and in vivo efficacy and selectivity to the parent cytotoxins MMAE and DDAE.

Tumor-associated proteases have been implicated in tumor angiogenesis, growth and metastasis formation, and found as both the positive and negative regulators of the disease.^[10] Often, these proteases are overexpressed both intracellularly and extracellularly in cancer cells. Of particular interest to us in this study is legumain,^[11] which is a cysteine protease and the only asparaginyl endopeptidase in mammals. It is highly implicated in tumorigenesis^[12] and expressed in the majority of human solid tumors, including breast, colon and prostate carcinomas.^[12a] Legumain has also been implicated in other diseases, including parasite infection^[13] and atherosclerosis,^[14] as well as antigen processing^[15] and matrix degradation.^[16] It is produced as a pro-enzyme with a molecular weight (MW) of approximately 50–60 kDa and undergoes autocleavage at low pH (3.0–6.0) to give the active protease (MW=30–40 kDa).^[17] Legumain is not present extracellularly, except on tumor cells and in tumor microenvironments (TMEs).^[12] It is only active at a lower pH, and inactive at physiologic pH. Studies have shown that legumain is a highly relevant protease for cancer therapy both through inhibiting the protease activity, as well as utilizing its catalytic property for prodrug activation.^[18] Nonetheless, the legumain-catalyzed prodrug activation is yet to get the full attention of the scientific community. With the limited data available on legumain expression in tumor and TMEs, its catalytic activity, and the legumain-catalyzed prodrug activation, it is safe to suggest that legumain can indeed be recruited to effectively deliver cytotoxins in TMEs^[18] using the prodrug monotherapy concept.^[19]

Legumain specifically cleaves peptides on the C-terminal side of asparagines. Previous examples of the prodrugs that are activated using legumain protease include those derived from doxorubicin and captothecin. In these prodrugs, the tripeptide sequence Ala-Ala-Asn (or A-A-N) was attached to the free amine of the cytotoxin either directly or through an additional amino acid or an amine linker. Upon treatment with a catalytic amount of legumain, these prodrugs undergo rapid activation to give the free toxins.^[18] Therefore, we also designed and prepared DDAE and MMAE prodrugs (pro-DDAEs, **5a,b**; pro-MMAEs, **6a,b**; Figure 1) that possess the A-A-N tripeptide linker tethered directly to the free primary amine in DDAE prodrugs anticipating that legumain would act on the prodrugs at cleavage site 'a' (see Figure 1) giving free DDAE and the tripeptide A-A-N, but through a 4-aminobenzyl carbamate linker in the MMAE prodrugs between the secondary amine and the tripeptide linker, as legumain protease was less likely to cleave a secondary amide. Upon legumain treatment, prodrugs **6a,b** should form intermediate **I** that would further degrade sequentially at cleavage sites 'b' and 'c' under physiologic conditions giving free **4** together with the 4-aminobenzyl alcohol and CO₂ byproducts.^[20] Here, the design of prodrugs **5a,b** and **6a,b** through functionalization at the N-terminal of DDAE and MMAE appeared reasonable as several analogues, prepared by an amide formation with additional amino acid with the secondary amine in MMAE, were found less toxic than the parent compound.

Prodrugs **5a,b** and **6a,b** were synthesized using the parent cytotoxins DDAE and MMAE as described in Scheme 1. Cytotoxins DDAE (**3**) and MMAE (**4**) were prepared^[21] by modifying the dolastatin 10 synthesis.^[20] Thus, compound **4** reacted with asparagine derivative **7** using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt), giving compound **8**, which underwent Fmoc (9H-fluoren-9-ylmethoxycarbonyl) group deprotection followed by peptide coupling with dipeptides **9a** or **9b** using EDC and HOBt. The resulting products were deprotected using trifluoroacetic acid (TFA) to give the pro-DDAE analogues **5a** or **5b**. The pro-MMAE analogues **6a** and **6b** were prepared using MMAE and the fully developed and activated linkers, the acetyl or carbobenzyloxy (Cbz)-protected A-A-N-(4-amidobenzyl)-nitrophenyl

carbonate derivatives **14a** and **14b**. Synthesis of the linkers started with an amide coupling of 4-aminobenzylalcohol (**10**) and the N-protected asparagine derivative **7**. The resulting product **11** underwent Fmoc deprotection followed by peptide coupling with the dipeptides **9a** and **9b** using EDC to give compounds **13a** and **13b**. The latter products were then reacted with chloro-4-nitro-phenylformate giving **14a** and **14b**. Finally, these activated linkers were reacted with **3** in the presence of base, and the products were trityl-deprotected using TFA yielding prodrugs **6a** and **6b**.

With the parent cytotoxins and their prodrugs in hand, we determined their cytotoxicities using the MDA-MB-435 human^[22] and 4T1 murine breast cancer cell lines. MDA-MB-435 cell line was separately transfected with legumain cDNA overexpressing the protease giving second set of cells, *that is*, MDA-MB-435-leg. In comparison to both MDA-MB-435 and MDA-MB-435-leg, 4T1 cells exhibit very weak expression of the legumain protease in vitro. We already confirmed the differential expression of legumain protease in these cell lines using Western blotting experiments (Figure 2 f). Therefore, we anticipated that the prodrugs would be rapidly activated in MDA-MB-435 and MDA-MB-435-leg cells, but not in 4T1 cells, and the toxicity to these cell lines should correlate with the released free drug.

Initially, we carried out the in vitro cytotoxicity assay using MDA-MB-435 and 4T1 cells and analyzed their effect using a general protocol.^[18] The results are shown in Figure 2. Obviously, the parent cytotoxin **4** possessed subnanomolar activity ($IC_{50} < 0.1$ nM in MDA-MB-435 and 3 nM in 4T1 cells), and it was over 100–500-times more potent than **3** (IC_{50} =50 nM in MDA-MB-435 and 0.5 μ M in 4T1 cells). As expected, prodrugs **5a,b** and **6a,b** were more cytotoxic to MDA-MB-435 cells than to 4T1 cells. Prodrugs **6a** and **6b** showed comparable cytotoxicity ($IC_{50} > 5$ nM in MDA-MB-435 and > 10 μ M for both prodrugs in 4T1 cells), and on average they were over 100-times more potent than **5a** and **5b**. The results made it clear that cytotoxin **4** and the related prodrugs **6a,b** were superior therapeutic candidates than compound **3** and its prodrugs **5a,b**. Prodrug **6b** was an appropriate candidate selected for further evaluation, and the MDA-MB-435 cells could be a suitable medium to evaluate the therapeutic efficacy of these cytotoxins and their prodrugs.

Further analysis of the in vitro cytotoxicity data suggested that these pentapeptide cytotoxins were at least two orders of magnitude less cytotoxic to the murine cell line than to the human cancer cell line. Second, no prodrugs were as active as the parent cytotoxin in either MDA-MB-435 or in 4T1 cells, which we initially attributed to a weak expression of the active legumain protease in these cell lines thereby leading to an incomplete activation of the prodrugs. However, when the cytotoxicity of prodrug **6b** was evaluated using the legumain-over-expressed MDA-MB-435 cells, we found almost identical results, i.e., cytotoxicity of compound **6b** was comparable in both MDA-MB-435 and MDA-MB-435-leg cell lines, which expressed legumain at a normal and higher level, respectively. An exact reason for these observations remains to be established, but we did confirm that the cytotoxicity to MDA-MB-435 cells was indeed due to the legumain protease-catalyzed activation of the prodrugs. For this, the prodrug **6b** was added to a catalytic amount of the legumain protease in assay buffer, and the mixture was incubated at 37°C for 24 hours and periodically analyzed using HPLC. A complete conversion of prodrug **6b** to toxin **4** was observed after hour incubation with the protease (Figure 2 h). Prodrugs **5a** and **6a** were also activated in the presence of legumain though slower than prodrug **6b**.

Next, we used a 4T1 murine mammary carcinoma model to evaluate the efficacy and safety of proMMAE **6b** as compared to the unmodified cytotoxin **4**. Although experiments with 4T1 cells, in vitro, confirmed weak expression of active legumain protease, the latter is abundantly expressed in vivo in TMEs^[18] on the surface of viable endothelial cells and tumor-associated macrophages in 4T1 tumor microenvironment,^[12b] as the legumain

expression is induced under hypoxia and stress condition. Higher expression level of active legumain protease in 4T1 tumor and TMEs, in vivo, was further confirmed in our own study by measuring the legumain activity of the freshly removed tumor tissue and comparing that to the activity of 4T1 cells grown in vitro. As shown in Figure 3, the legumain activity in 4T1 cell was one-third of that observed for the tumor tissue. These results are consistent with that observed previously using the CT26 tumor model.^[12a] Moreover, the 4T1 breast carcinoma model was previously used successfully to assess the efficacy of legumain activated prodrugs.^[12b]

Thus, 4–6 weeks old mice were administered (intraperitoneal; i.p.) with 1×10^6 4T1 cells on day 1, and prodrug (0.1 mg kg^{-1} and 0.5 mg kg^{-1}) and cytotoxin (0.1 mg kg^{-1}) were administered (intravenous; iv) on days 6, 9, 12, 15 and 18, and tumor growth was monitored on every fourth day starting on day 4. Upon 23 days post-treatment, tumor volumes of the prodrug **6b** treated group were significantly smaller than the control group and cytotoxin **4** treated group (Figure 4a; $p < 0.01$, $n=6$). Treatment of 4T1 cells with **6b** also demonstrated growth retardation in this model versus the untreated control. H&E staining of tumor sections demonstrated tumor cells were destroyed in treated mice (Figure 4 c). Treatment with **6b** (0.5 mg kg^{-1}) demonstrated significant inhibitory effects on spontaneous metastasis of 4T1 murine mammary carcinoma (Figure 4 b). These data indicate that prodrug **6b** has a stable efficacy at 0.5 mg kg^{-1} dose and inhibited both tumor growths and tumor metastasis in vivo. Mice treated with **4** show higher weight loss than those treated with **6b** (Figure 4 d). In fact, prodrug **6b** treated mice were nonrecurrence with no weight loss and no other apparent signs of toxicity in in vivo experiments, indicating that **6b** are activated in the tumor microenvironment and have less toxicity to other tissues.

In conclusion, the tumor-associated protease-activated prodrug approach,^[23] first described by Katzenellenbogen,^[24] can be efficiently applied to deliver extremely potent cytotoxins, including the pentapeptide MMAE, which is a synthetic analogue of the subnanomolar potent dolastatin 10. At least one MMAE prodrug, **6b**, was found highly effective in both in vitro and in vivo assays; treatment of mice with **6b** (0.5 mg kg^{-1}) did not give rise to any toxicity, but inhibited the growth of 4T1 murine breast cancer by 57% as compared to those treated with buffer alone. In contrast, mortality rate was 100% in mice treated with MMAE at 0.5 mg kg^{-1} , or lost 16–20% weight without showing any efficacy at a lower dose, 0.1 mg kg^{-1} . Further enhancement in efficacy and selectivity of the protease-activated MMAE prodrugs remains one of our goals, which is likely to increase through targeting the prodrugs to tumor cells using small-molecule inhibitors^[25] or preferably using macromolecules, such as antibodies.^[26] We suggest that the protease-activated prodrug approach can make best use of many subnanomolar potent cytotoxins, including tubulysins, enediynes and duocarmycins, which have been discovered but as yet have no therapeutic values.

Supplementary Material

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Acknowledgments

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Figure 1.

Structure of dolastatin 10, auristatins and auristatin prodrugs. Cleavage sites are indicated and labeled as follows: a) legumain-catalyzed proteolysis; b) autocleavage through 1,6-elimination; c) autocleavage through CO₂ loss.

Figure 2.

Cytotoxicity of cytotoxins and prodrugs to (a–c) MDA-MB-435 human and (d–e) 4T1 murine breast cancer cell lines. In a typical experiment, 5000 cells were plated in 96-well plates and allowed to grow overnight. Different concentration of prodrug and cytotoxin were added to each well, and cells were incubated for 72 h at 37 °C and processed using MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). f) Determination of the legumain expression in the cell lines. Cells were lysed in NP-40 buffer. Using clear lysate, an equal amount of protein was separated on an 8–16% SDS-Tris/glycine gel and transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% milk powder in Tris-buffered saline (TBS) plus Tween 20 before probing with antibody to legumain. g) Table showing EC_{50} values of cytotoxins and their prodrugs. h) Prodrug activation in the presence/absence of the cysteine protease, legumain. Prodrugs (10 mM in DMSO, 2 μ L) were added to the legumain protease (0.1 mgmL⁻¹, 100 μ L in assay buffer containing 50 mM citric acid, 120 mM Na₂HPO₄, 1mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1 propanesulfonate (CHAPS), pH 6.0) and the mixture was incubated at 37°C for 24 h and periodically analyzed using HPLC. Condition A: assay buffer containing 50 mM citric acid, 120 mM disodium hydrogenphosphate, 1 mM DTT, 1 mM EDTA, and 0.1% CHAPS, pH 6.0, and Condition B: legumain protease in assay buffer.

Figure 3.

Comparison of the legumain activity in 4T1 tumor tissues (□) against 4T1 cells (■) grown in vitro. 4T1 tumor tissues and 4T1 cells were homogenized in *n*-octyl glucopyranoside (OG) buffer (50 mM OG, 50 mM Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA pH 6.0) and normalized to equal protein concentrations. To determine the legumain activity, Z-Ala-Ala-Asn-NHMec substrate was added to these samples and incubated at room temperature for 1 h, and fluorescence was measured using Perkin–Elmer LS-50-B spectrofluorometer (λ_{ex} : 360 nm; λ_{em} : 460 nm).

Figure 4.

Tumoricidal effect of prodrug **6b** as compared to cytotoxin **4** in mammary carcinoma in vivo. a) In vivo effect of **4** and **6b** on 4T1 mammary carcinoma. When the tumors averaged ≈ 4 mm in diameter (after around six days), the 4T1 tumor mice were treated with: saline alone (control), cytotoxin **4** (0.1 mg kg^{-1}), or prodrug **6b** (0.1 mg kg^{-1} or 0.5 mg kg^{-1}), every fourth day starting on day 6; total five i.p. injections per mouse. b) Inhibition of 4T1 spontaneous lung metastasis by **6b** (0.5 mg kg^{-1}). c) H&E staining of tumor treated with saline alone, prodrug **4** (0.1 mg kg^{-1}), or prodrug **6b** (0.1 mg kg^{-1} or 0.5 mg kg^{-1}). d) Weight loss of treatment groups with prodrug **4** (0.1 mg kg^{-1}), or prodrug **6b** (0.1 mg kg^{-1} or 0.5 mg kg^{-1}) in 4T1 mammary carcinoma model. All experiments were conducted in compliance with all regulations relating to animal care and welfare.

Scheme 1.

Synthesis of monomethylauristatin E (MMAE) and di-desmethyllauristatin E (DDAE) prodrugs. *Reagents and conditions:* a) **3**, EDC, HOBt, DIPEA, CH₂Cl₂ ; b) 1. Piperidine, DMF; 2. EDC, HOBt, DIPEA, CH₂Cl₂; 3. TFA, CH₂Cl₂; c) **7**, HATU, DIPEA, CH₂Cl₂, DMF; d) 1. Piperidine, DMF; 2. EDC, HOBt, DIPEA, CH₂Cl₂ ; e) Py, CHCl₃; f) 1. **4**, HOBt, DMF, Py, DIPEA; 2. TFA/CH₂Cl₂, 0°C.