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Dendritic hexadecapeptide as a cathepsin B degradable carrier for delivery of HSP90 inhibitor

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ABSTRACT

Biodegradable vehicles that degrade specifically at tumor sites are highly desirable since they can cause selective exposure of highly toxic drugs at tumor sites whereas keep the conjugates stable during blood circulation. Here, we evaluate the utility of a dendritic hexadecapeptide comprised of four arms, each having a tetrapeptide sequence recognized by an enzyme cathepsin B as a carrier system for heat shock protein 90 (HSP90) inhibitor geldanamycin (GDM). We report the synthesis of a carrier having GDM conjugated to the terminal end of each arm (>55% wt/wt drug). We further report the stability of the GDM containing peptidic dendrimer in various buffers and in the presence of serum along with its ability to release free drug in the presence of cathepsin B, the enzyme overexpressed in a variety of tumors. Using androgen-independent prostate cancer cell line (DU-145) we further demonstrate that the geldanamycin containing peptidic dendrimer has antiproliferative property similar to the free drug derivative.

Synthetic polymers offer several advantages for delivery of small molecular weight anticancer therapeutics.[1,2] Dendrimers, recognized as new class of synthetic polymers have been widely accepted as drug carriers because of their unique architecture and low polydispersity.[3] However, one of the limitations of currently used dendrimers is that they are nondegradable.[4] Because degradable systems are more biocompatible than their nondegradable counterparts[5] there is a great interest in developing physiologically degradable dendrimers. A few degradable dendrimers reported so far have relied on the chemical hydrolysis in a biological environment;[6] majority of them having ester bonds that can degrade nonspecifically.[4] In contrast, using dendrimers that can be degraded by enzymes is an attractive choice because of degradation specificity associated with the selected enzyme. Enzymatic trigger has been used successfully for releasing the drug from a polymeric backbone.[7] The use of enzymatically degradable polymeric backbone has also been proven useful compared to traditional *N*-(2-hydroxypropyl methacrylamide) (HPMA) copolymeric conjugates.[8,9] However, the use of enzymes to make biodegradable polymers is relatively unexplored.[10]

We intend to use the enzyme cathepsin B as a trigger for complete degradation of a dendritic carrier. Cathepsin B is a cysteine protease overexpressed in several tumor tissues.[11] We have previously demonstrated that a dendritic hexadecapeptide (peptidic dendrimer) synthesized using the tetrapeptide GFLG, which is a substrate for cathepsin B, is stable in serum but degrades rapidly and completely in the presence of cathepsin B.[12] In this letter we demonstrate the utility of the peptidic dendrimer for the delivery of the heat shock protein 90 (HSP90) inhibitor geldanamycin (GDM).

HSP90 inhibitors are highly toxic; thus, the use of a delivery system to facilitate their tumor specific exposure will be highly beneficial.[13–16] Several preclinical and clinical studies have demonstrated the utility of polymeric carriers in reducing toxicity associated with small molecular weight anticancer drugs such as topoisomerase I and II inhibitors (doxorubicin, camptothecin), microtubule inhibitors (taxanes) and platinates.[17–19] Comparatively very few studies have been reported to evaluate the use of polymeric conjugates for HSP90 inhibitors, despite HSP90 being regarded as a validated target for anticancer therapeutics.[20,21] Thus, in this letter we evaluate the utility of a completely degradable peptidic dendrimer for the delivery of geldanamycin, a potent inhibitor of HSP90.

Figure 1 shows the structure of GDM and aminohexyl geldanamycin (AHGDM) that was used to conjugate to the tetrapeptide GFLG. 1,6 Diaminohexane was chosen as a linker based on the optimization studies reported previously using HPMA copolymers.[22] We synthesized aminohexylgeldanamycin (AHGDM) and *p*-nitrophenyl ester of BocGFLG (BocGFLGONp) as described previously.[12,22] AHGDM was conjugated to BocGFLGONp to obtain BocGFLGAHGDM that was deprotected under acidic conditions to

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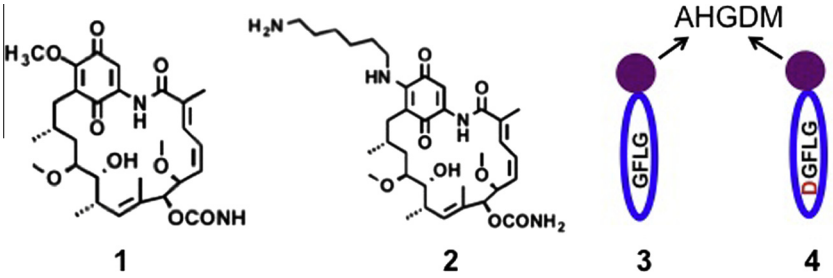


Figure 1. Structure of geldanamycin (**1**) (GDM), aminohexane geldanamycin (**2**) (AHGDM) and monomeric peptides GFLGAHGDM (**3**) and DGFLGAHGDM (**4**).

yield GFLGAHGDM having free terminal amine group. To obtain drug containing peptidic dendrimers we reacted GFLGAHGDM with tetrakis-(*p*-nitrophenyl ester) of EDTA (**5**) (Scheme 1). Briefly, GFLGAHGDM (85 mg, 0.083 mmol) was dissolved in anhydrous DMSO. EDTA(ONp)$_4$ (10.7 mg, 0.014 mmol) and DIPEA (0.23 mL, 1.2 mmol) were added to it. The reaction mixture was stirred for 72 h at room temperature. DMSO was then evaporated under vacuum and the reaction mixture was precipitated in ether. LCMS analysis of the precipitate revealed that it was a mixture of three products having two, three, or four arms of GFLGAHGDM attached to the central core (EDTA). The mixture was separated by preparative HPLC to isolate compound **6** (three peptidic arms conjugated to the core; yield 7%) and **7** (four peptidic arms conjugated to the core; yield 25%). Formation of heterogeneous mixture (two, three, and fours arms conjugated to EDTA core) might be responsible for the lower yields that can be improved in future by using higher equivalents of GFLGAHGDM. The molecular weight of HPLC purified peptidic dendrimers was confirmed using MS and MALDI and the purity of the compounds was determined using HPLC. Table 1 describes characteristics of the AHGDM containing peptides. Both the compounds (**6** and **7**) had very high drug content (>55% wt/wt).

Previously we have reported the stability of a peptidic dendrimer EDTA(GFLGOH)$_4$ lacking drug.[12] Here, we report the stability of drug containing polymer (Fig. 2) as well as drug release profile (Fig. 2B). Thus, compound **7** was incubated in PBS (pH 7.4), and PBS with serum (10%) for 24 and 48 h and intact polymer was quantified by HPLC. The stability of **7** was also checked in acetate buffer to mimic acidic conditions used during studies performed to determine drug release in the presence of cathepsin B. More than 97% of the intact polymer was detected after 24 h incubation with PBS and serum (10% serum in PBS). Further increase in incubation time from 24 to 48 h did not change the percentage of intact polymer detected suggesting the stability of the polymer. Intact peptidic dendrimer (**7**) detected in acetate buffer was slightly lower (94% and 93% at 24 and 48 h, respectively). Overall,

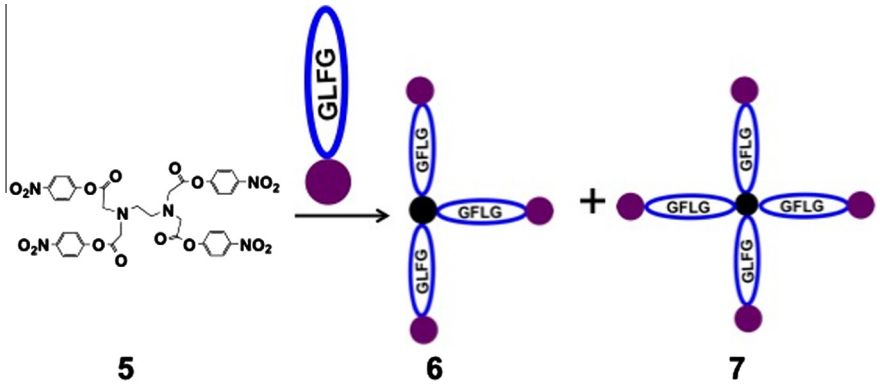
Table 1
Characteristics of monomers and dendrimers

Sr. no.	Compound	M_w calcd	M_w MALDI	HPLC ret. time (min)	Drug content% wt/wt
1	EDTA(GFLGOH)$_4$	1788.87	1791.87	27.08	—
3	EDTA(GFLGAHGDM)$_3$	3293.78	3321.28	18.66	58.74
4	EDTA(GFLGAHGDM)$_4$	4294.34	4320	17.83	60.08
5	AHGDM	644.38	645.38	3.31	—
6	GFLGAHGDM	1018.57	1019.57	4.09	56.92

the stability of the drug containing peptidic dendrimer (**7**) was similar to the one without drug as reported previously. When **7** was incubated with cathepsin B only 73% and 66% of the intact polymer was detected after 24 h and 48 h, respectively. Previously we have reported complete degradation of the peptidic dendrimer (without the drug) within 4 h incubation with cathepsin B.[12] These findings suggest that conjugation of drug hinders accessibility of cathepsin B.

The release of AHGDM was quantified by LCMS after incubation of the compound **7** with cathepsin B (Fig. 2B) following the procedure as described before. 14% and 17% of AHGDM release was determined after 24 h and 48 h incubation, respectively. GDM containing HPMA copolymers have been reported to release 20–25% of AHGDM.[23,24] Comparatively the peptidic dendrimers in this study showed lower drug release. LCMS profile also indicated the presence of a peak corresponding to Gly-AHGDM. Borgman et al. have reported the release of 27–36% Gly-AHGDM from HPMA copolymers depending on the presence or absence of targeting moiety.[23] In this study we found only 8% and 10% release of Gly-AHGDM after 24 and 48 h, respectively. Total release of AHGDM and GlyAHGDM was 22% and 27% after 24 and 48 h, respectively, which corresponds well with 73% and 66% of the intact polymer observed from HPLC analysis.

Next, we evaluated the growth inhibition properties of GDM (Fig. 3, Table 2), AHGDM, GFLGAHGDM, and GDM containing



Scheme 1. Schematic representation of the synthesis of drug containing peptidic dendrimers using EDTA as a core.

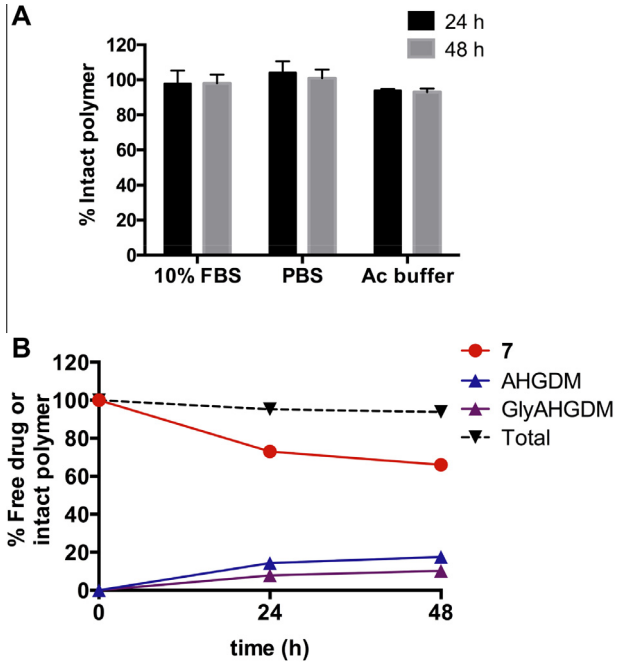


Figure 2. (A) Stability of peptidic dendrimers in PBS, FBS, and acetate buffers and (B) degradation and drug release profile catalyzed by cathepsin (B).

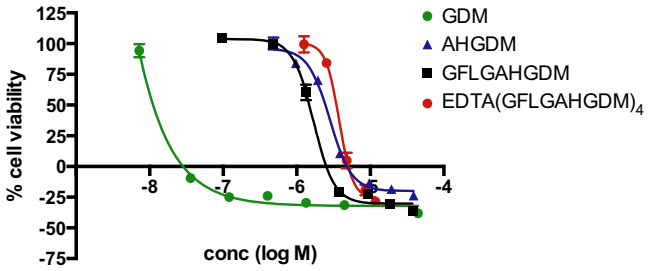


Figure 3. Growth inhibition effect on DU-145 cells after treatment at various concentrations with free drugs (GDM, AHGDM) monomeric peptide (GFLGAHGDM) and peptidic dendrimer for 96 h.

Table 2
IC_{50} values of compounds evaluated using DU-145 cell line

Compound	IC_{50} (μM)		
	48 h	72 h	96 h
Geldanamycin (GDM)	0.03	0.01	0.01
AHGDM	2.5 ± 0.2	2.6 ± 0.1	2.4 ± 0.0
GFLGAHGDM	1.2 ± 0.1	1.2 ± 0.1	1.5 ± 0.1
DGFLGAHGDM	8.2 ± 0.3*	2.2 ± 0.2	1.9 ± 0.1
$EDTA(GFLGAHGDM)_3$	13.1 ± 0.5*	—	—
$EDTA(GFLGAHGDM)_4$	13.8 ± 3.9*	3.4 ± 0.5#	3.5 ± 0.0

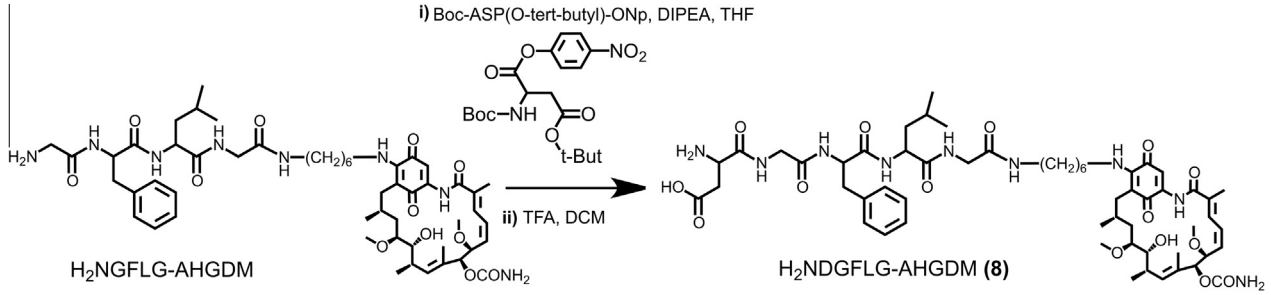
Significant difference (p <0.02) compared to 48 h value for the same compound.
* Significant difference (p <0.005) compared to GFLGAHGDM.

peptidic dendrimers. HSP90 is actively pursued as a therapeutic target for prostate cancer treatment and HSP90 inhibitors have been demonstrated to inhibit the growth of androgen independent prostate cancer cell line DU-145. Thus, we chose DU-145 cell line for our studies to determine their growth inhibition after 48 h incubation in the presence of test compounds as described previously.[25] IC_{50} values (concentrations to inhibit growth by 50%) were calculated from the graphs obtained by cell viabilities at different free drug equivalent doses of test compounds (Fig. 3, Table 2). GDM was highly toxic with an IC_{50} value of 30 nM. AHGDM and GFLGAHGDM were less active than GDM exhibiting IC_{50} values in micromolar range (2.5 μM and 1.2 μM). GFLGAHGDM was about 2-folds more active than AHGDM. Conjugation of GFLGAHGDM to the core yielded compounds (**6** and **7**) with significantly lower activity than GFLGAHGDM (p<0.005 for IC_{50} values). Compounds **6** (three arms) and **7** (four arms) had similar IC_{50} values (13.1 vs 13.6 μM) and both the compounds were 5-folds less active than AHGDM. This could be because of lower drug release from the peptidic dendrimers. We have previously demonstrated that a control peptidic dendrimer having no drug (synthesized from core and GFLGOH) did not exhibit any cytotoxicity up to 66 μM (highest concentration tested).

Because AHGDM release was low at earlier time points (up to 48 h) we evaluated the antiproliferative properties of compound **7** and small molecules after 72 h and 96 h treatment (Table 2). No change in IC_{50} was observed for AHGDM and GFLGAHGDM. However, IC_{50} value for **7** decreased significantly (p<0.02) to 3.5 μM after 72 h incubation (4-folds decrease compared to 48 h period) but did not decrease any further after 96 h treatment. For all the compounds IC_{50} values were similar after 72 and 96 h treatment period.

Overall, **7** exhibited good antiproliferative activity after longer incubation time. One of the limitations noticed during growth inhibitions studies was the limited solubility of **7**, probably because of its higher drug content (>50% wt/wt). Although final solutions contained less than 1% DMSO while testing, stock solutions of drug containing peptidic dendrimers were required to be made in DMSO. To increase the solubility we decided to add a polar amino acid (aspartic acid) at the amine terminus of the tetrapeptide GFLG. Thus, *p*-nitrophenyl ester of aspartic acid that has alpha-acidic group protected as tert-butyl ester was reacted with GFLGAHGDM to form a pentapeptide that was deprotected under basic conditions to yield the expected product DGFLGAHGDM (**8**) as shown in Scheme 2. It should be noted that the use of methyl ester instead of *tert*-butyl ester as an alpha-acid protecting group led to the formation of a pentapeptide but with an unwanted imide bond between the two acidic groups of aspartic acid and an amine from GFLGAHGDM.

The solubility of GFLGAHGDM and DGFLGAHGDM in PBS was determined from the absorbance of saturated solutions and relating them to the calibration curve obtained from known concentrations of the compounds in DMSO (Fig. 4A). DGFLGAHGDM



Scheme 2. Synthesis of AHGDM conjugated pentapeptide DGFLG.

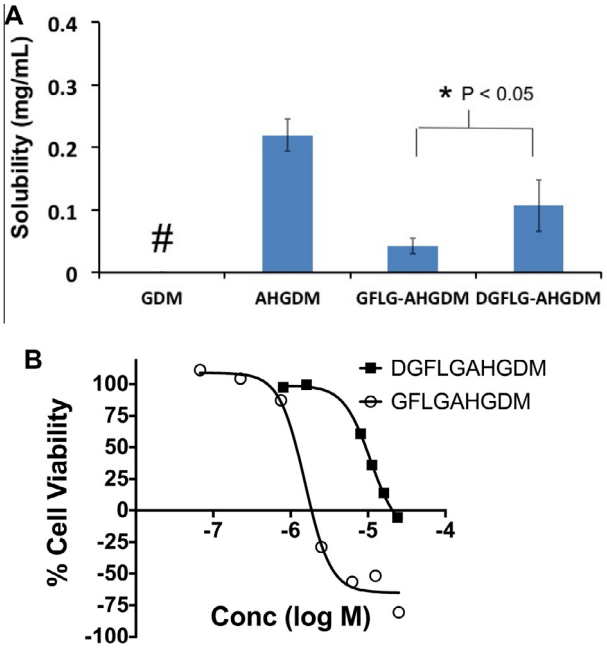


Figure 4. (A) Solubility (mg/mL) of GDM, AHGDM and peptide conjugated AHGDM in PBS. #The solubility of GDM was below detection limit. (B) Growth inhibition effect of tetrapeptide and pentapeptide conjugated AHGDM on DU-145 cells after 48 h treatment.

(94 nmol/mL; 0.1 mg/mL) was two folds more soluble than GFLGAHGDM (41 nmol/mL; 0.04 mg/mL). Both peptide conjugates were several folds more soluble than the free drug GDM; however, they were less soluble than AHGDM (340 nmol/mL; 0.2 mg/mL). Growth inhibition studies using DU-145 suggested a significant loss in activity ($p<0.005$) for DGFLGAHGDM compared to GFLGAHGDM (Fig. 4B) after 48 h treatment but the activities of the two compounds were similar after longer treatment (96 h) (Table 2). Higher hydrophilicity of DGFLGAHGDM might limit its cellular uptake; thus, lowering its activity at earlier time point (48 h). Alternatively, differences in release kinetics of free drug might be responsible for observed difference in the activity of GFLGAHGDM and DGFLGAHGDM. However, further studies are required to support these claims.

In this letter we demonstrate the utility of peptidic dendrimers as GDM carriers. GDM containing peptidic dendrimers were stable in several buffers and serum but released about 27% of the drug within 48 h incubation with cathepsin B. Although the amount of drug release was low compared to GDM containing HPMA copolymers, the activity of peptidic dendrimers was as good as free AHGDM. In contrast, GDM containing HPMA copolymers have been reported to exhibit lower activity than the free drug.[22,23] In addition, peptidic dendrimers had 55% wt/wt of AHGDM. Overall, these studies demonstrate the utility of peptidic dendrimers as drug carrier and provide the rationale for obtaining in vivo profile for completely degradable carrier. Good antiproliferative activity of conjugate having only three arms of EDTA functionalized with drug containing peptide (compound **6**) further suggests that the remaining free arm can be used for conjugation of hydrophilic polymer such as polyethylene glycol to increase water solubility. Further studies are currently under progress to target these constructs to tumor sites to avoid non-specific toxicity associated with HSP90 inhibitors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.06.012.

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