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Cell recognition enhanced enzyme hydrolysis of a model peptide–drug conjugate

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ABSTRACT

A model peptide–drug conjugate designed upon a β-hairpin peptide with the α4β1 integrin recognition sequence LDV appended to the N-terminus and a fluorescent model drug appended to the C-terminus. This model recognizes and binds to α4β1 expressing cells and displays an enhanced rate of enzyme catalyzed hydrolytic model drug release in the presence of the cells compared to the rate in the absence of cells. The present work suggests that peptide–drug conjugate conformation change due to receptor binding may be a viable approach to targeted drug release.

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One of the challenges of developing peptide based drugs and peptide–drug conjugates, is that these chemical entities have variable stability toward enzyme catalyzed hydrolysis. This limits their utility due to issues of poor bioavailability, short serum half-life, and in the case of peptide–drug conjugate based tumor targeted prodrugs, premature prodrug activation and ineffective cell specific targeting ability. Herein we demonstrate that drug conjugates of a β-hairpin peptide bearing the Lue-Asp-Val (LDV) sequence recognize and bind to α4β1 integrin over-expressing tumor cells, are resistant to enzyme catalyzed drug release in the absence of tumor cells, and in the presence of tumor cells exhibit an enzyme catalyzed drug release rate similar to a non-β-hairpin control.

Current clinical trials are investigating a class of therapeutic antibodies that are fused to enzymes that can catalyze the transformation of prodrugs to their corresponding drugs while localized to the tumor.[1–3] A potentially beneficial variation of this method would be to target a prodrug to the tumor site that can resist premature transformation before reaching its target. The use of peptide–drug conjugates for the purpose of cell specific drug targeting is a well established research paradigm in the discipline of drug delivery. The molecular recognition between peptides and cell surface structures parallels that of specific antigen/antibody interactions. For example, very specific cell-type targeting can be discovered from combinatorial peptide libraries.[4–6] Recognition of the tripeptide LDV by the α4β1 integrin is also well known.[7,8] Our hypothesis is that a peptide–drug conjugate may be designed that would be resistant to enzyme catalyzed drug release until it interacts with the target cell, and that this resistance or vulnerability could be controlled by the peptide conformation.

To test our hypothesis a set of model peptide–drug conjugates that target α4β1 integrin was designed and synthesized. The LDV sequence was chosen as the targeting moiety, and 7-amino-4-carbamoylmethylcoumarin (ACC) was used as the model drug, which has been shown to be an excellent fluorescent probe for enzymatic hydrolysis.[9,10] A peptide with controlled β-hairpin structure which incorporates the D-Pro-Gly (dPG) β-turn template and previously shown to adopt a folded β-hairpin conformation in water was used as the core structure.[11,12] Structure **1** represents the model targeted peptide–drug conjugate (Fig. 1). Three additional control peptides were also designed for study. The non-β-hairpin control where the D-proline is replaced with L-proline is represented by structure **2**. Structure **3** bears the Dabcyl fluorescence quenching group, and structure **4** omits the LDV targeting group.

The model peptide–drug conjugates **1**–**4** were synthesized using solid phase peptide synthesis techniques. Briefly, Fmoc-7-aminocoumarin-4-acetic acid was synthesized according to the previously reported procedure and loaded on Rink amide resin and subsequent amino acids were incorporated, following deprotection with piperidine and amino acid coupling with DIC/HOBt cycles. Using a standard cocktail of 5% thioanisole, 2.5% ethandithiol in trifluoroacetic acid the peptide–drug conjugates were cleaved from the resin and purified.

Lyophilized peptide quantities were gravimetrically determined and dissolved in buffer and fluorescence spectra were recorded at various concentrations to determine the linear range of fluorescence increase versus concentration. The fluorescence increase

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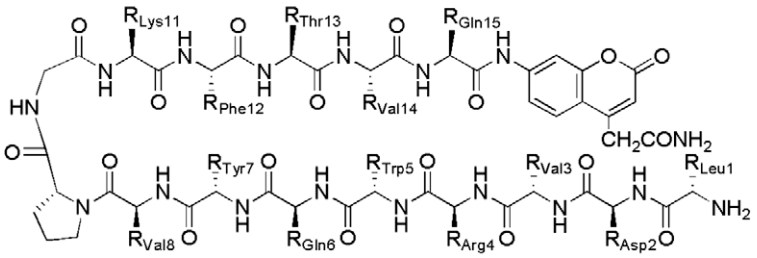

1 Leu-Asp-Val-Arg-Trp-Gln-Tyr-Val-D-Pro-Gly-Lys-Phe-Thr-Val-Gln-ACC
2 Leu-Asp-Val-Arg-Trp-Gln-Tyr-Val-L-Pro-Gly-Lys-Phe-Thr-Val-Gln-ACC
3 Leu-Asp-Val-Lys(Dabcyl)-Arg-Trp-Gln-Tyr-Val-D-Pro-Gly-Lys-Phe-Thr-Val-Gln-ACC
4 Arg-Trp-Gln-Tyr-Val-D-Pro-Gly-Lys-Phe-Thr-Val-Gln-ACC

Figure 1. Sequence of model peptide–drug conjugate **1**, and control peptides **2**–**4**.

was linear for all peptides in the concentration range 0.2–>6.0 μM. Each peptide had a different upper limit of linearity. Deviance of fluorescence from linearity at higher concentration may indicate aggregation as self quenching of coumarin fluorescence would be expected.[13] The fluorescence spectra of compounds **1**–**4** are shown in Figure 2. The ACC fluorophore was excited at 347 nm and the major emission band was observed at 450 nm. The higher intrinsic fluorescence of **1** compared to **2** suggest that **1** adopts a folded β-hairpin conformation where the N-terminus LDV forms a hydrophobic contact with the C-terminus ACC fluorophore. The enhancement of fluorescence by hydrophobic interactions is a recognized phenomenon and is the basis of many ligand–protein binding assays.[14,15] Additional evidence for the β-hairpin conformation of **1** is provided by **3**, which incorporates a Dabcyl bearing lysine near the N-terminus. The Dabcyl group is a fluorescence quencher. If **3** adopts a folded β-hairpin conformation one would expect the Dabcyl group to be held close to and quench some of the fluorescence of the ACC group and therefore have a lower intrinsic fluorescence compared to **1**. Figure 2 demonstrates that **3** has a lower intrinsic fluorescence than **1** and is similar to **2**, which is expected to not adopt a β-hairpin conformation. Peptide **4** exhibits lower intrinsic fluorescence than both **1** and **2**. This may be indicative of that **2** also may exist to a certain extent in a folded conformation and benefit from hydrophobic fluorescent enhancement by ACC and LDV contact. No such contact is possible with **4** because it does not contain the LDV sequence. Furthermore, the fluorescence of **3** was increased (less quenching) in the presence of 2 M guanidine, conditions known to disrupt protein and peptide secondary structure (data not shown).[16]

To determine whether the LDV sequence of **1** retained the ability to recognize cells which express α4β1 integrin. Human melanoma A375 cells were incubated in the presence of **1** or **4**. Analysis of the cells via flowcytometry showed that **1** bound to and fluorescently labeled the cells compared to incubation in the absence of added fluorescent peptide–drug conjugates. Incubation of the cells with **4**, which does not bear the LDV sequence, did not fluorescently label the cells. An LDV–fluorescein conjugate was used as positive control. These data show that **1** can recognize α4β1 expressing cells and suggest that this recognition is not due to non-specific interactions (data not shown).

In order to test the core of our hypothesis, that conformational control of the peptide–drug conjugates could be used to modulate the enzyme catalyzed release of the drug molecule in the vicinity of target cells, we determined the rates of hydrolysis of **1** and **2** by the enzyme papain in the absence and presence of A375 cells. Figure 3 shows the comparative enzyme catalyzed hydrolysis data. Free ACC has stronger fluorescence intensity than when covalently bound to the peptide and make it an excellent hydrolysis probe. Figure 3 is normalized for the difference in intrinsic fluorescence of **1** and **2**. Enzymatic hydrolysis studies show a low ACC release rate from **1** in the absence of A375 cells (11.47 ± 0.94 ng/min). The ACC release rate of **1** increased (15.64 ± 1.26 ng/min) significantly ($p < 0.05$) in the presence of A375 cells, when compared to the absence of A375 cells. The ACC release rate of **2** (15.63 ± 2.34 ng/min) had no statistical difference ($p > 0.4$) from **1** in the presence of A375 cells. The results indicate that the linkage

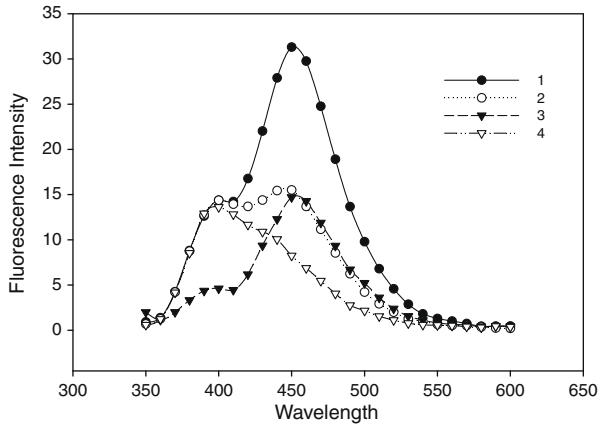

Figure 2. Flourescence spectra of peptides **1**–**4** at 5 μM in HBSS pH 7.4.

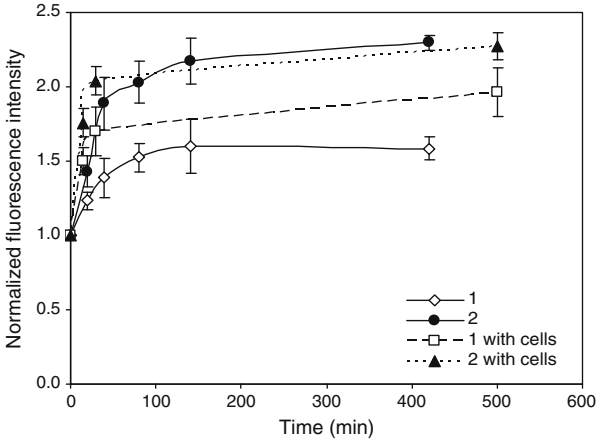

Figure 3. Time course for papain catalyzed release of ACC from peptides **1** and **2** in the absence and presence of A375 melanoma cells. Peptide concentration was 4 μM and papain concentration was 75 nM.

of ACC in **1** is protected from hydrolysis, and hydrolysis can be triggered by binding to A375 cells. It should be noted here that the hydrolysis rates of these peptides by papain are relatively slow even for the non-folded peptide **2**.[17] One possibility of the relatively slow ACC release rate of **2** is the low concentration of **2** in the assay. This is a limitation due to the possibility of aggregation at higher concentrations. A second possibility is that the length of **2** offers some inherent protection or limited folding. These two possibilities may also account for the only modest increase in the rate of hydrolysis of **1** in the presence of cells.

In summary our results suggest that the peptide–drug conjugate model bearing an α4β1 integrin recognition sequence, designed upon a β-hairpin template retain the ability to recognize cells that express the α4β1 integrin. The data also suggest that the β-hairpin based peptide–drug conjugate is resistant to enzyme catalyzed drug release compared to a control peptide, and that this resistance may be ameliorated upon exposure to α4β1 expressing cells. One explanation for the molecular recognition initiated change in enzyme catalyzed hydrolysis susceptibility is that the dynamic equilibrium between folded and non-folded conformations of **1** shifts toward the non-folded conformation, upon binding to the α4β1 integrin, and may be more susceptible to hydrolysis. Why the recognition of folded **1** by α4β1 integrin appears to be more efficient than by papain may be due to the relative shallowness of the integrin LDV recognition site compared to the enzyme active site. Presumably incorporation of other β-hairpin nucleating units such as Asn-Gly[18–20] and $^{\delta}$Orn[21] would provide similar results to those obtained here. Limitations of this drug targeting strategy may be concentration limiting peptide aggregation and the inability to achieve a more pronounced difference in triggered hydrolysis versus non-triggered hydrolysis. Potential solutions to these limitations could be the use of shorter non-aggregating hairpin sequences or a different mechanism to trigger unfolding such as pH.[22] We believe that these preliminary studies lay a foundation that may be built upon in order to deliver an additional degree of specificity to tumor targeting chemotherapeutic strategies.

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