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3-Aminoxypropionate-based linker system for cyclization activation in prodrug design

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ABSTRACT

A novel linker system based on 3-aminoxypropionate was designed and evaluated for drug release using proteolysis as an activation trigger followed by intramolecular cyclization. The hydroxylamine moiety present in the linker system enabled faster release of the parent drug from the linker–drug conjugate at lower pH as compared to an aliphatic amine moiety. Introduction of two methyl groups strategically at the α position to the carboxylate in the linker further improved the rate of cyclization by nearly 2-fold. The 3-aminoxypropionate linker was successfully applied to a model prodrug for protease activation using α-chymotrypsin as the activating enzyme; the activation of the model prodrug bearing the 3-aminoxypropionate linker was 136 times faster than the corresponding model prodrug bearing an amine linker.

Non-selective cytotoxicity to normal cells has been the major problem of cancer chemotherapy. The prodrug strategy using a tumor- or tissue-specific enzyme as the activating enzyme is one of the potential solutions that have been investigated.[1–4] Ideally, the prodrug is only activated at the site of tumor tissues through a specific metabolic pathway, such as bioreduction[5] or proteolysis.[6] In the case of proteolysis-activated prodrugs, insertion of a linker between the peptide substrate and the parent drug is often necessary to avoid steric hindrance caused by the bulky parent drug molecule and to enable efficient cleavage by the target protease.[7,8] After proteolytic cleavage, the linker–drug conjugate goes through self-immolation to release the parent drug resulting in activation. Elimination[9] and intramolecular cyclization[10,11] are among the most widely used self-immolation mechanisms in the design of linker systems for proteolysis-activated prodrugs. In this letter, we report a new type of linker system for proteolysis-activated prodrugs, which would go through cyclization, an intramolecular nucleophilic addition elimination sequence, to liberate the parent drug. The nucleophilicity of the trigger group is one critical factor affecting the rate of cyclization in addition to the size of the ring formed. An aliphatic or aromatic amino group is often used as the nucleophilic trigger in the linker and can be deactivated through formation of an amide bond with the C-terminal carboxylic acid of a peptide substrate. Upon proteolysis, a facile cyclization will occur as the free amino group undergoes a nucleophilic attack on the carbonyl group directly attached to the parent drug leading to the release of the free parent drug as shown in Scheme 1. The rate of cyclization is affected by the nucleophilicity of the trigger group, the leaving ability of the parent drug, and the size of the ring formed. Aliphatic amine has been widely used as a trigger group in such linker designs.[12,13] However, the optimum pH for cyclization in this case is much higher than physiological pH; with pK_a between 9 and 11, an aliphatic amine is mostly present in the protonated form with much reduced nucleophilicity under physiological pH.[14]

Herein, we report the development of a linker system that would cyclize readily under physiological conditions and in the interstitial tissues of solid tumors. The solid tumor environment is known to be slightly acidic with a pH of around 6. Hydroxylamine is of great interest to us because the pK_a of its conjugate acid is only 5.9, much lower than that of aliphatic amines. At pH ~6, about 50% of hydroxylamines will be protonated while 99.9% of aliphatic amines will be protonated. This difference prompted us to introduce the more nucleophilic and less basic hydroxylamine in our linkers as shown in model compound **1** to facilitate cyclization. In order to further improve the rate of cyclization of hydroxylamine-containing linkers, two methyl groups were strategically introduced in the linker as in compound **2** to restrict the conformation of the linker, placing the trigger hydroxylamino group

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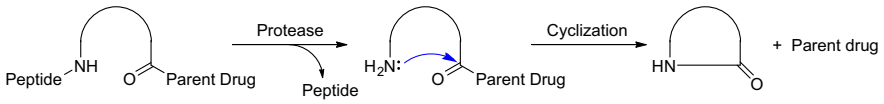
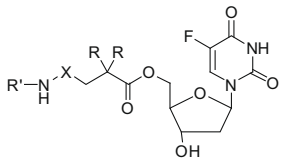


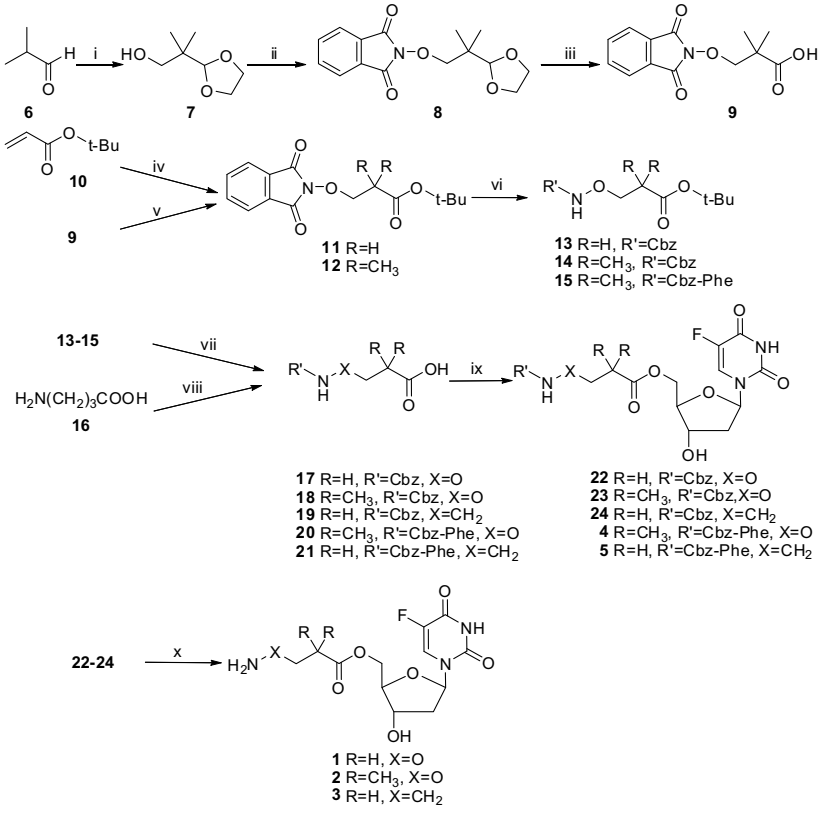
Scheme 1. Two-step activation of peptide-based prodrugs through the cyclization of an amine-containing linker.

spatially close to the carboxylate for nucleophilic attack. Fluorodeoxyuridine (FUDR) was used as the parent drug to evaluate our linker system as in model compounds **1**–**2** and a Cbz–Phe–linker–FUDR prodrug **4** for detailed kinetic analysis of cyclization, model enzyme cleavage, and drug release. The corresponding amine analog **3** and its Cbz–Phe conjugate **5** were used as controls to show the effect of introducing hydroxylamine functionality on the rate of cyclization and proteolysis.



1 R=H, R'=H, X=O
2 R=CH_3, R'=H, X=O
3 R=H, R'=H, X=CH_2
4 R=CH_3, R'=Cbz-Phe, X=O
5 R=H, R'=Cbz-Phe, X=CH_2

The target compounds were synthesized from the commercially available isobutyraldehyde (**6**) as shown in Scheme 2. Aldol condensation with 30% formaldehyde in a sodium hydroxide solution followed by protection of the aldehyde with ethylene glycol gave compound **7**. *N*-Hydroxyl phthalimide was attached to the hydroxyl group in compound **7** through Mitsunobu reaction[15] affording intermediate **8**. After hydrolysis of the acetal with concentrated sulfuric acid, Jones oxidation of the resulting aldehyde gave the corresponding carboxylic acid **9**. The carboxylic acid **9** was converted to the corresponding tertiary butyl ester **12** with *tert*-butyl trichloroacetimidate in the presence of catalytic amount of $BF_3{\cdot}Et_2O$. The intermediate **11** without the two methyl substitutions were obtained by the Michael addition of *N*-hydroxylphthalimide to *tert*-butyl acrylate (**10**).[16] After removal of the phthalimide protecting group with anhydrous hydrazine, the free amines were acylated with benzyl chloroformate in the presence of 2,6-lutidine to give compounds **13** and **14**. Coupling of the free amine to N^{α}-Cbz-phenylalanine through the mixed anhydride protocol gave compound **15**. The *tert*-butyl in compounds **13**–**15** was removed with 50% TFA in CH_2Cl_2 affording the corresponding carboxylic acids **17**, **18**, and **20**. Compounds **19** and **21** were synthesized directly from γ-aminobutyric acid (**16**). Following Mitsunobu reaction,[17,18] the carboxylic acids **17**–**21** were selectively coupled to FUDR at the primary hydroxyl to afford compounds **22**–**24**, and the model prodrugs **4** and **5**. Model compounds **1**–**3** were obtained as 10 mM solutions in methanol upon hydrogenolysis of compounds **22**–**24** in methanol



Scheme 2. Synthesis of the aminoxypropionate–FUDR conjugates. Reagents and conditions: (i) a—formaldehyde (30%), NaOH; b—ethylene glycol, *p*-TsOH·H_2O, toluene, reflux; (ii) PhtOH, Ph_3P, DIAD, THF; (iii) a—concd H_2SO_4, acetone/water (1:1); b—$K_2Cr_2O_7$; (iv) PhtOH, CH_3OH; (v) *tert*-butyl trichloroacetimidate, cat BF3·Et_2O, cyclohexane/dichloromethylene (2:1); (vi) a—H_2NNH_2, dichloromethylene; b—for **13**/**14**: CbzCl, 2,6-lutidine, CH_2Cl_2; for **15**: Cbz-Phe-OH, $ClCOO^iPr$, TEA, 0 °C; (vii) for **17**, **18**, and **20**: 50% TFA/CH_2Cl_2; (viii) for **19:** NaOH, CbzCl; for **21**: Cbz-Phe-OSu, NaOH; (ix) FUDR, Ph_3P, DIAD, THF; (x) H_2, Pd/C, CH_3OH, cat concd HCl.

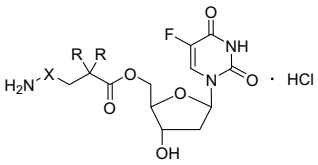
under H_2 atmosphere in the presence of 5% Pd/C and a drop of concentrated HCl.

The kinetics of cyclization for compounds **1**, **2**, and **3** were evaluated in 100 mM phosphate buffer at pH 6.0 and 7.4, 37 °C. Aliquots withdrawn at different time intervals were quenched with 0.5 N HCl and quickly frozen in dry ice/isopropanol for HPLC analysis. The kinetics of enzyme cleavage for model prodrugs **4** and **5** were conducted in 100 mM phosphate buffer at pH 7.4 and 37 °C in the presence of α-chymotrypsin. The stability of the model prodrug **4** was carried out in 100 mM phosphate buffer at pH 7.4 and 37 °C in the absence of the enzyme. Aliquots withdrawn at different time intervals were quenched with acetonitrile and quickly frozen for later HPLC analysis.

The results of kinetic analysis of cyclization are shown in Table 1. When incubated at pH 7.4, 37 °C, compound **2** with two methyl groups at the α position to the carboxylate gave the fastest cyclization rate with a half life of 43.3 min compared to that of compounds **1** and **3** ($t_{1/2}$ = 68.6 min and 24.2 h, respectively). When incubated at a lower pH of 6.0, 37 °C, the rate of cyclization for compounds **1** and **2** decreased by 20- and 32-fold, respectively, while compound **3** was stable and did not cyclize after 48 h of incubation. Compounds **1** and **3** are structurally similar and should assume similar conformation. The difference in the rate of cyclization was presumably due to the difference in nucleophilicity and degree of protonation at the assay pH between hydroxylamine and aliphatic amine. Comparing the rate of cyclization between compounds **1** and **2** at pH 7.4 indicates that the two α-methyl groups are able to restrict the conformation to favor cyclization as shown in Figure 1. This type of conformational restriction employing the Thorpe-Ingold effect is well-known in prodrug design through cyclization activation.[11,19] It should be noted that the rate of cyclization was much slower at pH 6 and the effect of this enhancement was not observed.

α-Chymotrypsin is known to cleave peptides after hydrophobic amino acids like phenylalanine and was used as the model enzyme in our study to evaluate the kinetics of proteolysis of the model prodrug **4** in 100 mM phosphate buffer at pH 7.4 and 37 °C (Scheme 3). The model prodrug **4** was cleaved by α-chymotrypsin with $t_{1/2}$ of 36.5 min (Fig. 2, closed circle) with formation of the intermediate **2** (Fig. 2, closed square). The model prodrug **4** was stable under the assay conditions in the absence of α-chymotrypsin (open circle) indicating the release of the parent drug FUDR (Fig. 2, open square) was dependent on enzyme cleavage rather than chemical hydrolysis. The results also suggest that the peptide–linker–drug conjugate can help avoid steric hindrance that renders direct peptide–drug conjugates resistant to proteolysis. The rate of proteolysis for model prodrug **4** was 136 times faster than the control prodrug **5** (k_1: 1.9×10^{-2} min^{-1} for **4** vs 1.4×10^{-4} min^{-1} for **5**). This indicates that the prodrug activation through proteolysis is facilitated by replacing the amide with hydroxamate as in our linker system.

Table 1
Kinetics of cyclization of model linker–drug conjugates **1**–**3**[a]



Compound	Structure		k_{obs} (min^{-1})		$t_{1/2}$	
	R	X	pH 6.0	pH 7.4	pH 6.0	pH 7.4
1	H	O	5.05×10^{-4}	1.01×10^{-2}	22.9 h	68.6 min
2	CH_3	O	4.94×10^{-4}	1.60×10^{-2}	23.4 h	43.3 min
3	H	CH_2	—[b]	4.78×10^{-4}	—[b]	24.2 h

[a] The assay was performed at 0.2 mM substrate concentration in 100 mM phosphate buffer at pH 6.0 or 7.4, 37 °C and the cyclization was followed by measuring the peak area change of the starting substrate using HPLC as monitored at 268 nm.

[b] No cyclization was detected after 48 h.

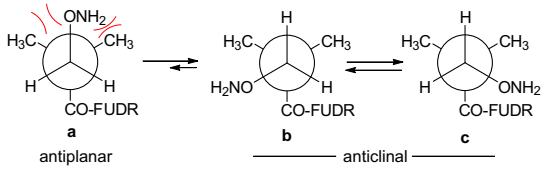


Figure 1. Conformational restriction using the two methyl groups in 3-aminoxypropionate linkers as in **2**. Shown are the three low energy conformations with steric hindrance favoring the anticlinal **b** and **c** conformations, placing the hydroxylamine spatially close to the carboxylate for nucleophilic attack.

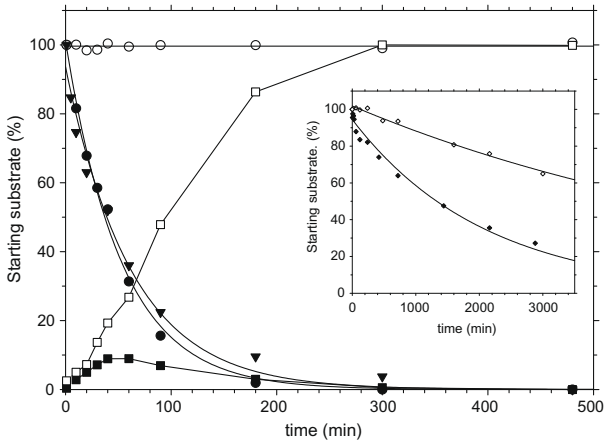
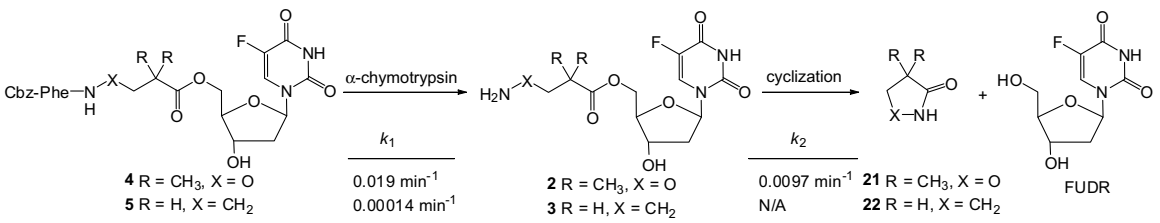


Figure 2. Shown are the kinetic courses of compound **2** at pH 7.4, 37 °C (▼), model prodrug **4** in the presence of α-chymotrypsin at pH 7.4, 37 °C (●), model prodrug **4** in the absence of α-chymotrypsin at pH 7.4, 37 °C (○), intermediate **2** derived from model prodrug **4** in the presence of α-chymotrypsin at pH 7.4, 37 °C (■), and FUDR formed from model prodrug **4** in the presence of α-chymotrypsin at pH 7.4, 37 °C (□). Shown in the inset are those of compound **3** at pH 7.4, 37 °C (♦) and model prodrug **5** in the presence of α-chymotrypsin at pH 7.4, 37 °C (◇).



Scheme 3. Proteolytic cleavage and cyclization activation of model prodrugs **4** and **5** at pH 7.4, 37 °C.

In summary, we developed a novel type of linker system for prodrug design by introducing a hydroxylamine moiety and two methyl groups α to the carbonyl in the linker. The introduction of hydroxylamine moiety enabled faster release of the parent drug than the corresponding aliphatic amine under physiological conditions. The two methyl groups α to the carbonyl in the linker significantly facilitated the release of the parent drug. The 3-aminoxypropionate linker was successfully applied to a model prodrug for protease activation using α-chymotrypsin as the activating enzyme. It was demonstrated that proteolytic cleavage of Cbz–Phe–hydroxamate–linker–drug conjugate by α-chymotrypsin is over 100 times faster than the corresponding conjugate containing the original amide linkage, representing an additional advantage of our new aminoxypropionate linker. The novel linker should be of general applicability in the design of prodrugs for proteolytic activation.

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