



Design and synthesis of a β -lactamase activated 5-fluorouracil prodrug

Ryan M. Phelan^a, Marc Ostermeier^b, Craig A. Townsend^{a,*}

^a Department of Chemistry, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21212, USA

^b Department of Chemical and Biomolecular Engineering, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21212, USA

ARTICLE INFO

Article history:

Received 10 November 2008

Revised 9 December 2008

Accepted 11 December 2008

Available online 24 December 2008

Keywords:

Beta-lactam

Cephalosporin

5-Fluorouracil

Cephalosporin derivatization

ABSTRACT

An efficient synthesis of a 5-fluorouracil-cephalosporin prodrug is described for use against colorectal and other cancers in antibody and gene-directed therapies. The compound shows stability in aqueous media until specifically activated by β -lactamase (β L). The kinetic parameters of the 5-fluorouracil-cephalosporin conjugate were determined in the presence of *Enterobacter cloacae* P99 β L (ECL β L) revealing a $K_m = 95.4 \mu\text{M}$ and $V_{\text{max}} = 3.21 \mu\text{Mol min}^{-1} \text{mg}^{-1}$. The data compare favorably to related systems that have been reported and enable testing of this prodrug against cancer cell lines in vitro and in vivo.

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Colorectal cancer is the third most common cancer in both men and women in the United States today. American Cancer Society projections for 2008 estimate that 150,000 cases of colorectal cancer will occur, claiming the lives of approximately one-third of those diagnosed. Strategies to better combat colorectal cancer would be of great benefit in lowering the mortality rate of this disease. Current methodology using targeted drug delivery through antibody (ADEPT) or gene-directed prodrug therapy (GDEPT) is able to specifically activate prodrugs at precise locations.^{1,2} The benefit of an ADEPT or GDEPT strategy is that heightened concentrations of the desired chemotherapeutic agent can be delivered directly to infected cells, while avoiding their release in benign regions. This strategy is represented in the literature through the use of a β -lactamase (β L) to activate β -lactam based prodrugs, specifically targeting infection or disease.^{3–5} One particular β -lactam class, the cephalosporins, have proved to be very useful reporter, signaling and antibacterial compounds that are able to lie dormant until activation by a β L (Fig. 1).^{6–9} Many chemotherapeutic compounds using the cephem scaffold have been reported including; mitomycin C,⁴ nitrogen mustards,^{3,10–13} doxorubicin,^{14,15} platinum compounds,¹⁶ taxol¹⁵ and a derivative of the *vinca* alkaloid vinblastine.¹⁷ These compounds have been tested in vivo and show promising activity against various cancer lines when used in conjunction with an ADEPT strategy. Although many of the aforementioned prodrug conjugates have been evaluated in vivo, a common FDA approved drug for colorectal cancer, 5-fluorouracil (5-FU), has not been evaluated in an analogous fashion. 5-Fluorouracil is a

main component of the currently used colorectal cancer therapy regimen FOLFOX, along with oxaliplatin and folinic acid.¹⁸ Ability to specifically deliver 5-fluorouracil to cancerous cells provides protection to benign regions while targeting drug concentrations to areas of concern. It would be a benefit to create a simple, straight-forward synthesis of a cephalosporin-5-fluorouracil prodrug that can then be evaluated in vivo. We describe here a facile synthesis of a cephalosporin-5-fluorouracil prodrug that shows stability in aqueous environments until specifically activated by a β -lactamase.

In order to create a 5-FU-cephalosporin conjugate, it was of primary importance to determine the manner in which 5-FU could be attached to the 3' position of the cephalosporin. More often than not the drug of choice is attached to the 3' carbon by the use of a carbonate or carbamate linker that decomposes upon expulsion from the prodrug to yield the desired free compound. In some instances the compound may be attached directly to the 3' position through a displacement reaction between a 3' leaving group and a suitable nucleophile. With both means of linkage being equal, the deciding factor was the ready availability of a commercial starting material that led to the most direct synthetic route. 7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester (**3**) was selected as a suitable starting material (Otsuka, Osaka Japan).

Cephem **3** provided a nucleus that could be modified to the desired prodrug (Scheme 1). This starting material was amenable to direct attachment of 5-FU via displacement of the 3' chloride with 5-FU. Treatment of **3** with 2-thiophene acetyl chloride and 2,6-lutidine afforded the intermediate **4** in excellent yield.^{8,19} With compound **4** in hand it was desired to then attach 5-FU. This

* Corresponding author. Tel.: +1 410 516 7444; fax: +1 410 261 1233.

E-mail address: ctownsend@jhu.edu (C.A. Townsend).

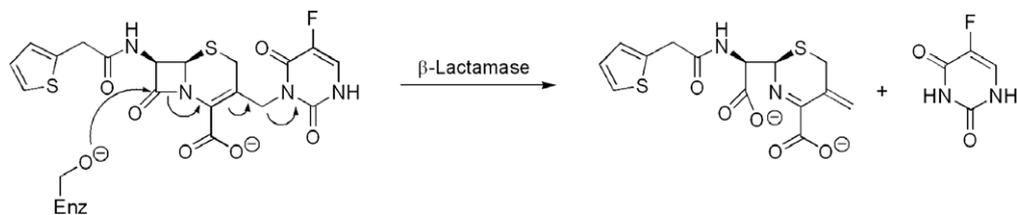
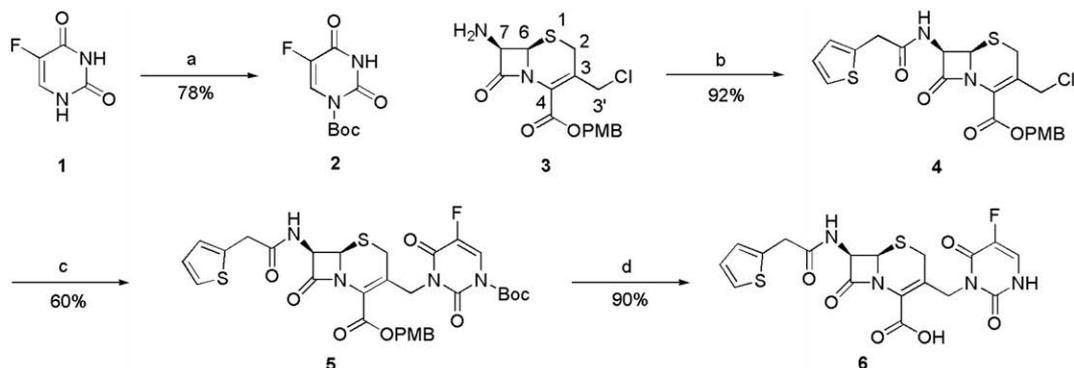


Figure 1. Activation of a cephalosporin prodrug by a β -lactamase. In general, lactam cleavage releases the chemical moiety at the 3'-position, in this case 5-fluorouracil.



Scheme 1. Reagents and conditions: (a) Boc_2O , DMAP, 78%; (b) 2-thiopheneacetyl chloride, TEA, 2,6-lutidine, 92%; (c) NaI, KOSiMe_3 , 3, 60%; (d) CF_3COOH , Et_3SiH , 90%.

proved more difficult than originally thought. A primary difficulty with using 5-FU in organic reactions is its relative insolubility in all common organic solvents. In addition to its poor solubility, it was of concern that both the amide and imide positions could act as the nucleophile, giving rise to regioisomers. To overcome both of these constraints, protection of the amide nitrogen with *t*-butyl carbamate (Boc) was performed.^{20,21} The Boc protection increased 5-FU solubility while also limiting the imide to function as the sole nucleophile. With compounds **2** and **4** in hand, direct coupling to the 3' position was attempted. It was observed that when using carbonate and tertiary amine bases, as well as pyridine, partial isomerization of the cephem nucleus occurred yielding a mixture of the Δ^2 and Δ^3 isomers. Formation of the Δ^2 isomer inactivates the compound by taking the nitrogen of the β -lactam out of conjugation with the 3' position, thus blocking release of the attached compound upon βL activation. A common solution to this problem is oxidation of the cephem sulfur to restore the active Δ^3 isomer, followed by reduction back to the thioether. Oxidation of compound **5** containing a mixture of the Δ^2 and Δ^3 isomers was presumed to restore conjugation.²² It was observed, however, that oxidation of the bridgehead sulfur was not possible as with other related systems, providing no trace of the sulfoxide as a product. One may rationalize this by noting that the cephem contains a highly puckered ring in which the 5-FU moiety could block approach of the oxidant, resulting in no reaction. At this point any attachment of the 5-FU moiety needed to provide a single isomer due to the inability of the oxidation/reduction sequence to restore the required conjugation. A previous report had shown that potassium trimethyl silanoate (KOSiMe_3) could be used to perform the acylation reaction producing **4** without formation of the Δ^2 isomer.²³ Indeed, use of KOSiMe_3 as the stoichiometric base in the formation of compound **5** provided the Δ^3 isomer in good yield.²⁴ It was then possible for acidic deprotection of both the Boc and *p*-methoxybenzyl moieties of **5** providing the final compound in high yield.²⁵ The use of KOSiMe_3 in the direct displacement reaction afforded a facile route to the desired **6** in 3 linear steps. This route to cephem derivatives avoids the oxidation/reduction sequence

that has traditionally dominated in syntheses of similar compounds.

ADEPT and GDEPT strategies to combat disease have the distinct advantage that the prodrug is specifically activated in areas of infection while benign regions are left relatively untouched. For any ADEPT or GDPET strategy to be successful, the prodrug must first be stable under non-activating conditions, yet quickly activated under the desired conditions. In the case of **6** this would amount to the compound lying dormant until acted upon by a βL . In addition to the specific activation by βL , it is important to verify that **6** is a good substrate for βL by evaluation of its fundamental kinetic parameters. Compound **6** was prepared in a 10 mg/mL stock solution and subsequently diluted for all in vitro experiments. To first verify that the compound can specifically be activated only in the presence of a βL , a HPLC assay was performed.²⁶ The prodrug substrate **6** was incubated in phosphate buffered saline (50 mM, pH 7.0) for 12 h at 28 °C under activating and non-activating conditions. The results show that **6**, in the presence of βL , was completely cleaved to 5-FU and the hydrolyzed

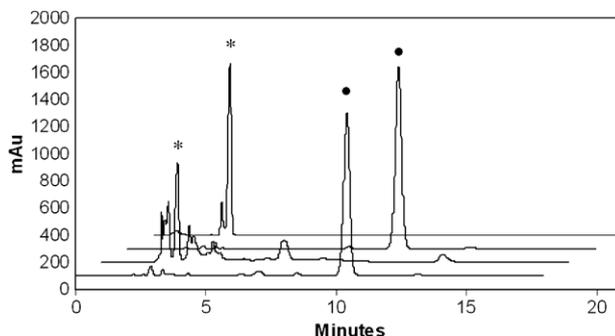


Figure 2. HPLC trace of compound **6** and products released by βL activation. Front to back: **6** incubated 20 h in 50 mM PBS (pH 7.0) without βL , **6** incubated 20 h in 50 mM PBS (pH 7.0) with 1 mg/mL $\text{ECL}\beta\text{L}$, Compound **6** standard, 5-FU standard. (*) 5-FU. (●) Compound **6**.

cephalosporin while **6** was otherwise stable in the absence of β L (Fig. 2). To better evaluate how efficient a substrate **6** is for the aforementioned β L, kinetic parameters were measured using an assay previously described.¹³ It was determined that compound **6** had a molar absorptivity (ϵ) of $9640 \mu\text{mol}^{-1} \text{cm}^{-1}$, which enabled monitoring of β -lactam hydrolysis at 267 nm. Compound **6** was evaluated against the commercially available *Enterobacter cloacae* β -lactamase (EC β L) (Sigma, St. Louis, MO). Initial velocities were measured at varying concentrations of **6** (25–250 μM) and fit to the Michaelis–Menton equation to establish K_m and V_{max} . Compound **6** was found to have a K_m of 95.4 μM and a V_{max} of $3.21 \mu\text{mol min}^{-1} \text{mg}^{-1}$, which compare favorably to previously reported cephalosporin conjugates.^{3,4,13} To determine the 5-FU yield when **6** is activated, a reaction of **6** with the EC β L was run under conditions identical to the previous stability assay. The products of this reaction were compared to a standard curve of known concentrations of 5-FU to determine the percentage of 5-FU released using the previously established HPLC method.²⁶ It was determined that compound **6** is activated to 5-FU in a $78 \pm 2\%$ yield. The combined data show that compound **6** is a good substrate for a β L, activation occurs in an efficient manner and compound **6** is stable in a neutral aqueous environment until specifically activated by the β L (Fig. 2).

We have designed a straight-forward synthesis of a β L-activatable 5-FU-cephalosporin conjugate to release the cytotoxic agent 5-FU. Preparation of this compound was readily achieved through careful attention to two key steps in the synthesis. The protection of the 5-FU amide nitrogen with a Boc group improved solubility in organic solvents and avoided the formation of regioisomers. Second, the use of KOSiMe₃ in the cephem 3'-chloride displacement reaction proceeded without formation of the Δ^2 isomer, circumventing the customary oxidation and reduction sequence at the cephem sulfur to restore the Δ^3 double bond. The use of KOSiMe₃ offers a general approach for attachment of nucleophilic chemical moieties at the 3' carbon while keeping the Δ^3 cephem intact. The stability of compound **6** in the absence of a β L and its favorable kinetic parameters for hydrolysis in its presence demonstrate the potential for compound **6** to be used in ADEPT or GDEPT strategies against a range of human carcinoma cell lines, and open the way for its evaluation in vivo.

Acknowledgments

We gratefully acknowledge a seed grant from the Institute for NanoBioTechnology at The Johns Hopkins University for financial support. Otsuka Chemical Corporation (Osaka, Japan) is thanked for donation of a sample of 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxy-benzyl ester. We thank T. Soka for helpful discussions in preparation for the in vivo applications of the conjugate.

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- Compound **4**: ¹H NMR (300 MHz, CDCl₃) δ 3.53 (2H, q_{ab}, J = 18.6, 30.6 Hz), 3.81 (3H, s), 3.85 (2H, s), 4.45 (2H, q_{ab}, J = 11.7, 17.7 Hz), 4.94 (1H, d, J = 7.8 Hz), 5.12 (2H, s), 5.82 (1H, dd, J = 3.0 Hz), 6.23 (1H, d, J = 9.3 Hz), 6.89 (2H, d, J = 11.7 Hz), 6.96 (2H, m), 7.30 (1H, m), 7.34 (2H, d, J = 11.7 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 27.9, 37.0, 43.2, 55.2, 57.5, 59.1, 68.2, 114.0, 125.5, 126.0, 126.1, 126.5, 127.6, 127.9, 130.7, 134.6, 159.9, 161.0, 164.5, 169.9; HRMS (FAB, MH⁺) calcd for C₂₂H₂₁ClN₂O₅S₂: 493.1. Found: 493.1.
- Compound **2**: ¹H NMR (300 MHz, CDCl₃) δ 1.52 (9H, s), 8.18 (1H, d, J = 7.2 MHz), 11.9 (1H, s); ¹³C NMR (75 MHz, CDCl₃) δ 27.7, 88.1, 123.5, 139.0, 146.1, 147.5; HRMS (FAB, MH⁺) calcd for C₉H₁₁FN₂O₄: 231. Found: 231.
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- Compound **5**: ¹H NMR (400 MHz, CDCl₃) δ 1.60 (9H, s) 3.36 (2H, d, J = 7.6 Hz), 3.81 (3H, s), 3.84 (2H, s), 4.89 (1H, d, J = 4.8 Hz), 4.95 (2H, q_{ab}, J_{ab} = 15.2, 116.0 Hz), 5.24 (2H, s), 5.78 (1H, dd, J = 4.8 Hz), 6.41 (1H, d, J = 9.2 Hz), 6.98 (2H, d, J = 6.8 Hz), 7.02 (3H, m), 7.36 (2H, d, J = 6.4 Hz), 7.93 (1H, d, J = 5.0 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 26.1, 27.7, 29.6, 37.0, 43.1, 55.2, 57.1, 59.1, 68.0, 88.1, 113.9, 122.3, 122.7, 123.3, 126.0, 126.5, 126.8, 127.5, 127.8, 130.5, 134.5, 138.3, 140.7, 161.4, 164.2, 169.9; HRMS (FAB, MH⁺) calcd for C₃₁H₃₁FN₄O₉S₂: 687.1. Found: 687.1.
- Compound **6**: ¹H (300 MHz, DMSO-*d*₆) δ 3.51 (2H, q_{ab}, J = 17.4, 29.4 Hz), 3.75 (2H, s), 4.79 (2H, q_{ab}, J = 14.7, 67.2 Hz), 5.00 (1H, d, J = 4.8 Hz), 5.64 (1H, q, J = 4.2 Hz), 6.95 (2H, m), 7.35 (1H, d, J = 3.6 Hz), 9.08 (1H, d, J = 8.4 Hz); ¹³C (75 MHz, DMSO-*d*₆) δ 24.7, 35.5, 57.0, 58.6, 124.9, 126.0, 126.4, 129.2, 136.6, 137.7, 140.7, 149.9, 157.3, 157.6, 162.8, 164.3, 169.7; HRMS (FAB, MH⁺) calcd for C₁₈H₁₅FN₄O₆S₂: 467. Found: 467.
- The β L cleavage assay was performed on an Agilent (Santa Clara, CA) 1100 HPLC using a Phenomenex (Torrance, CA) Luna C-18 reverse phase column. All compounds were run using an isocratic mobile phase consisting of 30% acetonitrile in 0.1% trifluoroacetic acid. All compounds were monitored at 267 nm.