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Cephalosporin Prodrugs of Paclitaxel for Immunologically Specific Activation by L-49-sFv-β-Lactamase Fusion Protein

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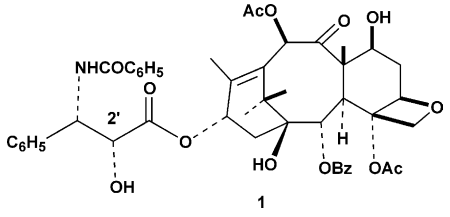
Abstract—Paclitaxel conjugates of 7-phenylacetamidocephalosporanic acid were prepared as prodrugs for site specific activation by targeted β-lactamase. Immunologically specific activation of the prodrug **5** containing 3,3-dimethyl-4-amino-butyric acid as linker in combination with the fusion protein L-49-sFv-β-lactamase was demonstrated in vitro on 3677 melanoma cells.

The aim of Antibody Directed Enzyme Prodrug Therapy (ADEPT) is to achieve selective toxicity to tumor cells while sparing normal cells from chemotherapeutic damage. In this approach a monoclonal antibody (mAb) enzyme conjugate is administered first and localizes at the tumor. After allowing sufficient time for systemic clearance of the mAb conjugate, a prodrug of a cytotoxic agent capable of being activated by the targeted enzyme is administered, leading to site selective generation of the cytotoxic substance at the tumor site. A plethora of prodrugs of potent cytotoxic agents have been employed in combination with a variety of mAb enzyme conjugates, and this methodology has been thoroughly reviewed.[1]

The diterpenoid natural product paclitaxel[2a] (**1**) is an important anticancer drug discovered in 1971. Paclitaxel exerts its antitumor activity by stabilizing tubulin polymers. The *N*-benzoyl-β-phenyl isoserine side chain in **1** is crucial for its activity. Baccatin III, a taxoid devoid of this side chain is 50-fold less effective in tubulin polymerization assay in vitro and 1000-fold less cytotoxic to KB cells, thus illustrating the importance of this side chain for activity.[2b] Esters derived from the 2′-OH group of **1** generally exhibit diminished cytotoxicity.[2b] The 2′-OH therefore serves as a good handle for the design of prodrugs. Because paclitaxel is a bulky molecule, it was anticipated that successful ADEPT prodrugs would require the intermediacy of a less sterically-demanding spacer that would release free drug quickly following enzymatic hydrolysis. During the synthesis of water soluble esters of paclitaxel, Kingston and coworkers found that the 2′-γ-aminobutyrate ester of **1** underwent cyclization slowly in methanol to release paclitaxel in 15 h.[3] Utilizing this observation, a cephalosporin paclitaxel prodrug (**2**) incorporating γ-aminobutyric acid (GABA) as a linker was prepared for ADEPT.[4] When exposed to β-lactamase, **2** released **1** slowly in phosphate buffered saline (PBS). The half-life for this rate limiting cyclization was found to be approximately 6 h at 37°.[4] While this compound was the first published example of a cephalosporin prodrug of paclitaxel, we sought to further improve upon the kinetics of the rate limiting cyclization reaction. A further limitation with sterically unhindered 2′-esters of



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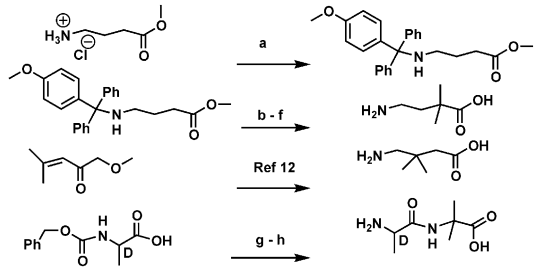
paclitaxel is the potential for direct hydrolysis by endogenous esterases.[5] The important issues to be addressed therefore were: (a) modifications of the linker to ensure more rapid release of **1**, (b) stabilization of the 2′-ester bond to prevent premature hydrolysis by endogenous esterases and finally (c) demonstration of immunologically specific prodrug activation. Efforts in this direction and the results obtained are described here.

The 7-phenylacetamido side chain-containing cephalosporin prodrugs of doxorubicin, phenylenediamine mustard and melphalan displayed favorable properties and pronounced antitumor effects in our earlier studies.[6,7] Hence, it was decided to retain this side chain at position 7 on the cephem nucleus. The sulfoxide oxidation state of the cephem sulfur prevents undesirable Δ^3 to Δ^2 isomerization of the double bond, a common occurrence with many esters of 3-cephem-4-carboxylic acid.[8] In designing the linkers, a geminal dimethyl group in the vicinity of the carbonyl group was expected to increase the rate of cyclization to form both five and six-membered rings, due to the Thorpe–Ingold effect.[9] In addition, such a steric constraint was also expected to improve the stability toward hydrolysis by endogenous esterases. For a different linker strategy, we tried to take advantage of the cyclization of the aminoacid linkers which leads to the formation of diketopiperazine. This is a facile process employed in the synthesis of Ara C prodrugs.[10] Since an L-aminoacid derived peptide may be a substrate for endogenous amidases, the D-alanyl-amide of α-aminoisobutyric acid was chosen as the linker for the diketopiperazine strategy. Compounds **4**–**6** (Scheme 1) thus became the preferred targets. In addition, **3** was prepared as an analogue of **2** in the present series.

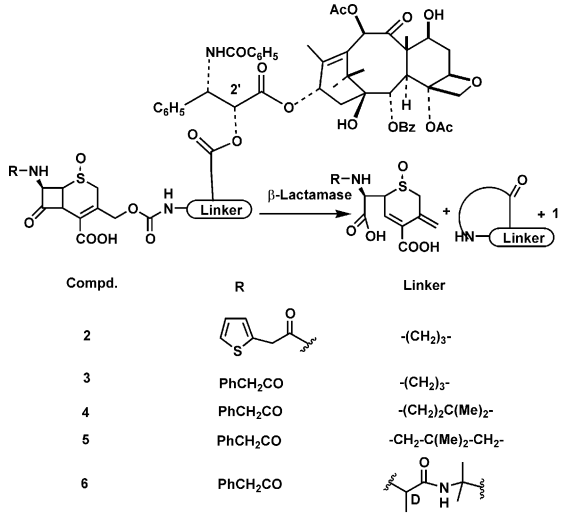
The linkers for attachment to the activated sulfoxide **7**[7] were prepared as shown in Scheme 2: The necessary 2,2-dimethyl-4-aminobutyric acid was prepared by alkylation of the lithium enolate of *N*-*p*-MeO-trityl-GABA methyl ester in THF followed by basic hydrolysis to give the zwitterionic *N*-*p*-MeO-trityl-GABA. Detritylation with TFA provided the necessary linker with the reported properties.[11] Michael addition of nitromethane to ethyl 3,3-dimethylacrylate followed by reduction of the nitro group and hydrolysis provided the necessary 3,3-dimethyl-4-aminobutyric acid.[12] The CBZ protected peptide linker necessary for the diketopiperazine strategy was prepared by coupling α-aminoisobutyric acid with in situ prepared *N*-hydroxysuccinimidate ester of CBZ-D-alanine using disuccinimidyl carbonate (DSC).[13] After reductive removal of the CBZ group the crude product was used to condense with **7**.



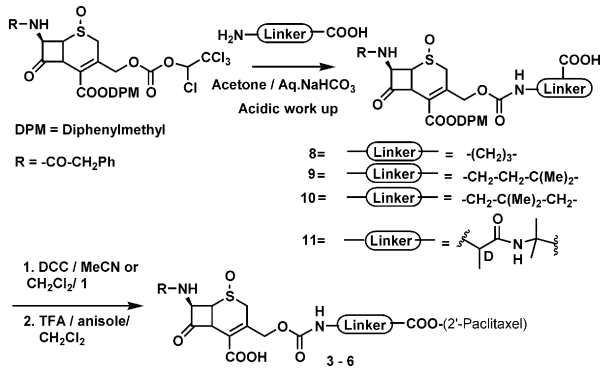
Scheme 2. (a) Methoxy trityl chloride, Et_3N, rt, 14 h, 96%; (b) LDA (2.2 equiv), −78 °C; (c) MeI (−78 °C to rt); (d) repeat (b) and (c); (e) KOH, EtOH reflux 24 h, then acidification (pH 4); (f) TFA, CH_2Cl_2 over all 70% yield; (g) DSC, Et_3N, DMF 0 °C, 1 h/H_2N–$C(CH_3)_2COOH$ 43%; (h) H_2–Pd, MeOH.

Coupling of **7** with these linkers (Scheme 3) in acetone containing aqueous $NaHCO_3$ proceeded in ~60% yield to give **8**–**11**. While **8**, **10** and **11** underwent DCC coupling with paclitaxel smoothly, compound **9** gave only *N*-acyl-dicyclohexyl urea under the same conditions of coupling. Removal of the DPM ester group was carried out using TFA anisole to give **3**, **5** and **6**.[15]

Reversed phase analytical HPLC analyses were carried out to study the stability of **3**, **5**, and **6** in human serum at 37 °C. The rate of cyclization of the intermediate 2′-esters generated by β-lactamase in DMSO:phosphate buffered saline (PBS pH 7.4, 1:9) were also evaluated. A high concentration of *Enterobacter cloacae* P99 β-lactamase (0.07 mg/mL) was used in these studies. Under these conditions the enzymatic hydrolysis is almost instantaneous to form the intermediate 2′-esters. The half-life values for cyclization and the half life values in



Scheme 1.



Scheme 3.

Table 1. Half lives of cephalosporin paclitaxel derivatives in the human serum and in presence of β-lactamase at 37 °C

Compd	$t_{1/2}$ in human serum (min)	$t_{1/2}$ for cyclization after β-lactamase hydrolysis (min)
3	275	230
5	350	50
6	410	60

human serum are listed in Table 1. Geminal dimethylation not only improved serum stability, but also resulted in approximately 4- to 5-fold increase in the rate of cyclization irrespective of whether the cyclization led to the formation of five-membered lactam from compound **5** or the diketopiperazine from compound **6**.

In vitro cytotoxicity assays were performed with human 3677 melanoma cells expressing the melanotransferrin (p97) antigen. L-49-sFv-bL is a recombinant fusion protein comprised of the sFv fragment of the anti-melanoma mAb L49 fused to a mutated form of *E. cloacae* β-lactamase. The protein was expressed in *Escherichia coli* as described earlier[14] and purified by affinity chromatography. Cells were treated with the fusion protein and after washing off the unbound material various concentrations of **5** were added and incubated at 37 °C for 4 h. Incorporation of [^{3}H] thymidine was used as a measure of cytotoxic activity.

The prodrug **5** was found to be 12-fold less toxic than **1** on 3677 melanoma cells (Fig. 1). The fusion protein activated the prodrug to a level comparable to that of the free drug. When 3677 cells were first saturated with unconjugated L49 mAb prior to the addition of the fusion protein (blocking the antigenic sites), no activation of prodrug **5** took place, suggesting that the activation is immunologically specific. In this assay, **6** was 30 fold less toxic than **1** (IC_{50} for **6** = 388 nM, IC_{50} for **1** = 13 nM). However, the activation of **6** in combination with L-49-sFv-bL was poor (IC_{50} for L-49-sFv-bL + **6** = 189 nM) when compared with compound **5**.

In conclusion, we have prepared a series of cephalosporin derivatives of paclitaxel containing different self-immolative linkers. The rate limiting step for release of paclitaxel from these derivatives is the linker cyclization reaction, the kinetics of which is influenced by alkyl group substitution. Compound **5,** with the fastest rate of release of **1** in the presence of β-lactamase, was shown to be a prodrug which can be activated in an immunologically specific manner.

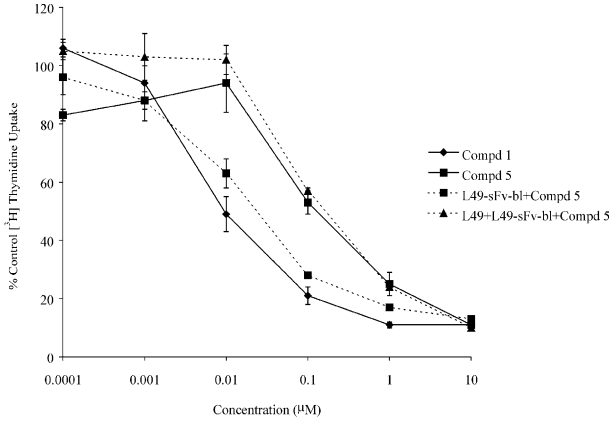


Figure 1. Cytotoxicity assay on 3677 melanoma cells demonstrating immunologically specific activation of **5**.

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15. Spectral characteristics of key intermediates and final compounds: In the assignment of NMR spectra of cephalosporin derivatives of **1**, the notation ceph for cephem nucleus, L for linker and T- for paclitaxel portion with appropriate numbering system was used. Compd **8**: MS: [M−H] = 658; IR (KBr) 3294 cm^{-1} (carboxyl), 1793 cm^{-1} (β-lactam), 1032 cm^{-1} (S=O); ^{1}H NMR (DMSO-d_6 + D_2O) δ 7.54–7.20 (m, 15H, ArH), 6.94 (s, 1H, Ph_2CH–), 5.91 (d, 1H, H-7, $J_{6,7}$ = 4.8 Hz), 5.00 (d, 1H, CH_{2A}–O–CO–N, J_{AB} = 13.4 Hz), 4.93 (d, 1H, H-6), 4.55 (d, 1H, CH_{2B}–O–CO–N), 3.69 (d, 1H, H-2A, J_{AB} = 14.0 Hz), 3.56 (d, 1H, H-2B), 3.42 (s, 2H, PhCH_2–), 2.96 (t, 2H, –CO–N–CH_2–, J_{vic}, = 7.1 Hz), 2.20 (t, 2H, –N–C–C–CH_2–CO–), 1.60 (m, 2H, N–C–CH_2–C–CO–).

Compd **9**: MS: [M−H] = 686; IR (KBr) 3288 cm^{-1} (carboxyl), 1790 cm^{-1} (β-lactam), 1391 cm^{-1} (gem di Me), 1065 cm^{-1} (S=O); ^{1}H NMR (DMSO-d_6 + D_2O) δ 7.49–7.27 (m, 15H, ArH), 6.84 (s, 1H, Ph_2CH–), 5.85 (d, 1H, H-7, $J_{6,7}$ = 4.8 Hz), 4.94 (d, 1H, CH_{2A}–O–CO–N, J_{AB} = 13.5 Hz), 4.84 (d, 1H, H-6), 4.53 (d, 1H, CH_{2B}–O–CO–N), 3.58 (AB q, 2H, H-2A, J_{AB} = 14.3 Hz), 2.90 (t, 2H, –CO–N–CH_2–C–,

J_{vic} = 8.2 Hz), 1.56 (t, 2H, –C–*CH*$_2$–C(Me)$_2$–CO–), 1.05 (s, 6H, 2 × *gem*-CH_3). Compd **10**: MS: [M−H] = 686; IR (KBr) 3288 cm^{-1} carboxyl), 1794 cm^{-1}, 1384 cm^{-1} (gem di Me), 1040 cm^{-1} (S=O); ^{1}H NMR (DMSO-d_6 + D_2O) δ 7.54–7.29 (m, 15H, ArH), 6.91 (s, 1H, Ph_2*CH*–), 5.91 (d, 1H, H-7, $J_{6,7}$ = 4.8 Hz), 5.00 (d, 1H, CH_{2A}–O–CO–N, J_{AB} = 13.2 Hz), 4.92 (d, 1H, H-6), 4.60 (d, 1H, CH_{2B}–O–CO–N), 3.81 (s, 2H, $PhCH_2$–), 3.68 (d, 1H, H-2A, J_{AB} = 14.0 Hz), 3.57 (d, 1H, H-2B), 2.92 (s, 2H, –CO–N–*CH*$_2$–C(Me)$_2$–), 2.08 (s, 2H, –C–C(Me)$_2$–*CH*$_2$–CO–), 0.89 (s, 6H, 2 × *gem*-CH_3).

Compd. **11**: MS: [M−H] = 729; IR (KBr) 3289 cm^{-1} (carboxyl), 1793 cm^{-1}, 1388 cm^{-1} (gem di Me), 1074 cm^{-1} (S=O); ^{1}H NMR (DMSO-d_6 + D_2O) δ 7.54–7.18 (m, 15H, ArH), 6.92 (s, 1H, Ph_2*CH*–), 5.90 (d, 1H, H-7, $J_{6,7}$ = 4.8 Hz), 5.04 (d, 1H, CH_{2A}–O–CO–N, J_{AB} = 13.6 Hz), 4.94 (d, 1H, H-6), 4.61 (d, 1H, CH_{2B}–O–CO–N), 3.99 (q, 1H, D-alanyl–*CH*(Me)–CO–, J_{vic} = 7.1 HZ), 3.70 (d, 1H, H-2A, J_{AB} = 14.0 Hz), 3.56 (d, 1H, H-2B), 3.41 (s, 2H, $PhCH_2$–) 1.35 and 1.33 (2 × s, 6H, 2 × *gem*-CH_3), 1.16 (d, 3H, D-alanyl–CH_3).

Compd. **3**: HRMS: $[M + Na]^+$ = 1351.4257 (found), $[M]^+$ = 1328.44 (calcd); IR (KBr) 1781 cm^{-1} (β-lactam); ^{1}H NMR (DMSO-d_6) δ 8.31–7.22 (m, 23H, ArH and amide), 6.29 (s, 1H, T-10), 5.87 (t, 1H, T-13, $J_{13,14}$ = 9.0 Hz), 5.58 (dd, 1H, ceph-7, $J_{NH,7}$ = 8.6 Hz, $J_{6,7}$ = 4.4 Hz), 5.53 (dd, 1H, T-3′, $J_{NH,3'}$ = $J_{2',3'}$ = 9.0 Hz), 5.40 (d, 1H, T-2, $J_{2,3}$ = 7.1 Hz), 5.34 (d, 1H, T-2′), 5.04 (d, 1H, ceph-CH_{2A}–OCO–, J_{AB} = 12.3 Hz), 4.93 (m, 1H, T-5), 4.69–4.60 (m, 3H, ceph-CH_{2B}–OCO–, ceph-6, NH or OH), 4.10 (m, 1H, T-7), 4.00 (m, 1H, T-20), 3.68 (d, 1H, ceph-2A, J_{AB} = 14.3 Hz), 3.60–3.50 (d and m, ceph-$PhCH_2$–CO–, ceph-2B and T-3), 2.98 (m, 2H, L-NH–*CH*$_2$–), 2.42 (t, 2H, L-CH_2–CO, J_{vic} = 7.1 Hz), 2.25 (m and 2 × s, T-6A, 2 × $COCH_3$), 1.78 (s, 3H, T-18), 1.49 (s, 3H, T-angular CH_3), 1.01 and 0.99 (2 × s, 6H, T-16 and T-17).

Compd. **5**: HRMS: $[M + Na]^+$ = 1379.4570 (found), $[M]^+$ = 1356.4672 (calcd); IR (KBr) 1778 cm^{-1} (β-lactam); ^{1}H NMR (DMSO-d_6) δ 8.27–7.17 (m, 21H, ArH and amide), 6.29 (s, 1H, T-10), 5.87 (t, 1H, T-13, $J_{13,14}$ = 9.0 Hz), 5.57–5.53 (2 × dd, 2H, ceph-7and T-3′, $J_{NH,7}$ = 8.4 Hz, $J_{6,7}$ = 4.4 Hz, $J_{2',3'}$ = 5.1 Hz), 5.42–5.35 (m, 2H, T-2′ and T-2), 5.09 (d, 1H, ceph-CH_{2A}–OCO–, J_{AB} = 12.2 Hz), 4.94 (m, 2H, T-5 and NH or OH), 4.67–4.30 (m, 3H, ceph-CH_{2B}–OCO–, ceph-6, NH or OH), 4.12 (m, 1H, T-7), 4.01 (m, 1H, T-20), 3.68 (d, 1H, ceph-2A, J_{AB} = 14.1 Hz), 3.58 (m, 1H, T-3), 3.53 (d, 1H, ceph-2B), 2.89 (s, 2H, L-NH–*CH*$_2$–), 2.40–2.20 (m and s, 6H, T-6A, T-$COCH_3$, L-CH_2CO–), 2.10 (s, 3H, T-$COCH_3$), 1.99–1.77 (m and s, 4H, T-14 and T-18), 1.66–1.49 (m and s, 4H, T-6B, T-angular CH_3), 1.07 (s, 1H, OH), 1.01 and 0.99 (2 × s, 6H, T-16 and T-17), 0.88 and 0.84 (2 × s, 6H, L-*gem*-CH_3).

Compd. **6**: HRMS: $[M + Na]^+$ = 1422.4628 (found), $[M]^+$ = 1399.4730 (calcd); IR (KBr) 1780 cm^{-1} (β-lactam); ^{1}H NMR (DMSO-d_6) δ 8.37–7.15 (m, 23H, ArH and amide), 6.28 (s, 1H, T-10), 5.81 (t, 1H, T-13, $J_{13,14}$ = 9.3 Hz), 5.57–5.53 (m, 2H, ceph-7 and T-3′), 5.42–5.36 (m, 2H, T-2′ and T-2), 5.06 (d, 1H, ceph-CH_{2A}–OCO–, J_{AB} = 11.9 Hz), 4.91 (m, 2H, T-5), 4.67–4.30 (m, 2H, ceph-CH_{2B}–OCO–, ceph-6), 4.10 (m, 1H, T-7), 4.00 (m, 1H, T-20), 3.94 (m, 1H, L-*CH*–Me), 3.68 (d, 1H, ceph-2A, J_{AB} = 14.0 Hz), 3.62–3.50 (m and d, 3H, T-3 and ceph-2B), 2.30–2.20 (m and s, 4H, T-6A, T-$COCH_3$), 2.10 (s, 3H, T-$COCH_3$), 1.79–1.72 (m and s, 4H, T-14 and T-18), 1.59 (m, 1H, T-6B), 1.49 (s, 3H, T-angular CH_3), 1.40 and 1.35 (2 × s, 6H, L-*gem*-CH_3), 1.13 (d, 3H, L-CH–*CH*$_3$), 1.01 and 0.99 (2 × s, 6H, T-16 and T-17).