Design, Synthesis, and Biological Evaluation of Antibody−Drug Conjugates Comprised of Potent Camptothecin Analogues

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Antibody−drug conjugates (ADCs) were prepared with potent camptothecin analogues attached to monoclonal antibodies (mAbs) via dipeptide or glucuronide-based linkers. Aniline-containing camptothecin analogues were employed to provide a site of linker attachment via carbamate bonds that would be stable in circulation. The camptothecin analogues, 7-butyl-10-amino-camptothecin and 7-butyl-9-amino-10,11-methylenedioxy-camptothecin, are generally 10−1000 times more potent than camptothecin. Dipeptide and glucuronide drug linkers were employed containing self-immolative spacers that release drug following lysosomal degradation upon ADC internalization into antigen-positive cell lines. The camptothecin drug linkers were conjugated to three antibodies: chimeric BR96, chimeric AC10, and humanized 1F6, which bind to the Lewis-Y antigen on carcinomas, CD30 on hematologic malignancies, and CD70 present on hematologic malignancies and renal cell carcinoma, respectively. ADCs bearing the potent camptothecin analogue, 7-butyl-9-amino-10,11-methylenedioxy-camptothecin, were highly potent and immunologically specific on a panel of cancer cell lines in vitro, and efficacious at well-tolerated doses in a renal cell carcinoma xenograft model.

INTRODUCTION

In an attempt to enhance activity and mitigate toxicity of cancer chemotherapy, there has been a great deal of interest in the use of monoclonal antibodies (mAbs) for the targeted delivery of cytotoxic agents to cancer cells (*1*−*3*). The principle behind this approach is that, by covalent attachment of a cytotoxic drug to a mAb that binds selectively to a tumor-specific antigen, the activity of the drug could be enhanced through high intratumoral concentrations while sparing normal, nontarget tissue from indiscriminant damage. Early-generation ADCs[1] have demonstrated that several parameters are important for efficient targeted cell kill, such as drug potency, drug linker stability, efficient on-target drug release, mAb immunogenicity, and normal tissue expression of the tumor antigen (*1*−*3*). More recently, significant progress in ADC design has led to the clinical advancement of two new immunoconjugates: SGN-35 for CD30-positive hematologic malignancies (*3*−*5*) and trastuzumab-DM1 for HER2-positive metastatic breast cancer (*6, 7*). In phase I clinical trials both ADCs induced tumor regressions at well-tolerated doses (*6, 8*−*10*), providing further validation of the use of ADCs as an emerging treatment modality.

We are interested in all classes of drugs that may be suitable for empowering mAbs. Early immunoconjugates used clinically approved cytotoxic agents due to their availability and known toxicological properties. This approach is exemplified by BR96-doxorubicin ADC targeted to the Lewis-Y antigen present on human carcinomas. Doxorubicin was attached to the mAb via an acid-sensitive hydrazone linker, and free drug was released upon ADC internalization into acidic lysosomes (*11*). In vivo efficacy was observed in tumor-bearing mice, albeit at elevated (>100 mg ADC/kg) dose levels. Clinical trials revealed low levels of activity in patients with Lewis-Y positive carcinomas (*11*−*13*). The limitations associated with this ADC included drug linker instability, antigen expression on normal tissues, and low drug potency. It has been proposed that optimized ADCs must address these issues to achieve maximal therapeutic efficacy (*1, 2*).

As part of our search for new chemotypes suitable for use in ADCs, we sought to evaluate conjugates consisting of potent camptothecin (CPT) analogues. Camptothecin (**1**) is a natural product that exerts cytotoxic activity through the inhibition of topoisomerase I, leading to cell death (*14*). As a consequence of its broad-spectrum antitumor activity and cytotoxic mechanism, there has been a substantial effort toward the development of clinical camptothecin analogues. To date, two water-soluble analogues have received FDA approval: topotecan and irinotecan. Topotecan is used primarily in the treatment of advanced ovarian cancer and non-small cell lung cancer and was recently approved for cervical cancer (*15*). Irinotecan is a prodrug that is converted into SN-38, a more potent CPT analogue, under the action of carboxylesterases (*16*) and is used primarily in the treatment of colorectal cancer (*15*). Clinical studies with irinotecan, topotecan, and other camptothecin analogues in development indicate that optimal efficacy is achieved with dosing regimens that provide for prolonged exposure to low levels of camptothecin (*17*). This suggests that camptothecin, or potent CPT analogues, may be ideally suited for mAb targeting, as antibodies often have extended circulation half-lives, ranging from many days to weeks (*1, 3*).

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[1]Abbreviations: ADC, antibody-drug conjugate; AUC, area under the curve; CPT, camptothecin; DIPEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DOX, doxorubicin; ES, electrospray; IC_{50}, concentration that inhibits 50% cell growth; mAb, monoclonal antibody; mc-val-cit, maleimidocaproyl-valine-citrulline; NHS, *N*-hydroxysuccinimide; PABA, *para*-aminobenzyl alcohol; PBS, phosphate-buffered saline; SCID, severe combined immunodeficient; THF, tetrahydrofuran.

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An ADC of CPT has already been described (*18*). It was found that BR96-CPT had a similar level of potency as BR96-DOX (*18, 19*). In addition, the CPT-containing ADC was relatively unstable, due to the use of a labile carbonate linker (*18*). Other camptothecin ADCs bearing SN-38 were more potent, but were also composed of labile linkers (*20*). On the basis of our previous work with optimized auristatin-containing ADCs (*4*), we felt that it was necessary to use camptothecin analogues with much higher potency than CPT, and to incorporate linkers that provide high serum stability.

Camptothecins have been subjected to extensive structure−activity relationship studies (*21*), which indicate that 7-position alkyl substituents and a methylenedioxy group spanning the 10 and 11 positions confer enhanced biochemical and cellular potency (*22, 23*). The selected camptothecin analogues, 7-butyl-10-amino-CPT (**2**) and the des-amino precursor (**12**) to 7-butyl-9-amino-10,11-methylenedioxy-CPT (**3**), are substantially more potent (1−3 logs) than camptothecin (*22, 24, 25*). These molecules are ideally suited for ADC application, not only because of their potencies, but also because they (**2** and **3**) contain functional groups that allow for stable mAb attachment. We now report the design, synthesis, and biological evaluation of ADCs comprising highly potent camptothecin analogues. This investigation resulted in CPT ADCs that were well-defined, potent, and immunologically specific in vitro and displayed good in vivo efficacy at well-tolerated doses.

EXPERIMENTAL PROCEDURES

General Methods and Materials. All commercially available anhydrous solvents were used without further purification and were stored under a nitrogen atmosphere. NMR spectral data were collected on a Varian Mercury 400 MHz spectrometer. Chemical shifts (δ) were reported in ppm and referenced off the deuterium solvent signal. Coupling constants (*J*) are reported in hertz. Mass spectral data were obtained on a ZMD Micromass mass spectrometer interfaced to an HP Agilent 1100 HPLC instrument configured for LC-MS analysis. 7-Butyl-10-amino-camptothecin (**2**) and 7-butyl-10,11-methylenedioxy-camptothecin (**12**) were prepared via Friedlander condensation as previously described (*23*).

Analytical HPLC was performed on two different HPLC systems. Data collected by "gradient A" were obtained on an HP Agilent 1100 HPLC equipped with a C12 Phenomenex Synergi 2.0 × 150 mm, 4 μm, 80 Å reverse-phase column. The acidic eluent consisted of a linear gradient of acetonitrile from 5% to 95% in 0.1% aqueous formic acid over 10 min, followed by isocratic 95% acetonitrile for 5 min (flow rate = 0.4 mL/min). Data collected by "gradient B" were obtained on a Waters 2690 HPLC interfaced with a Waters 996 PDA and Millenium[32] software. Samples were eluted over a C12 Phenomenex Synergi 2.0 × 150 mm, 4 μm, 80 Å reverse-phase column. The neutral eluent consisted of a linear gradient of acetonitrile from 10% to 90% in 5 mM ammonium phosphate pH 7 over 10 min, followed by isocratic 90% acetonitrile for 5 min (flow rate = 1.0 mL/min).

Preparative HPLC was carried out on a Varian ProStar 210 solvent delivery system configured with a Varian ProStar 330 PDA detector. Products were purified over a C12 Phenomenex Synergi 10.0 × 250 mm, 4 μm, 80 Å reverse-phase column eluting with 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B). Purification Method A consisted of the following gradient of solvent A to solvent B: 90:10 from 0 to 5 min; 90:10 to 40:60 from 5 to 45 min; 40:60 to 10:90 from 45 to 50 min; followed by isocratic 10:90 for 5 min. The flow rate was 4.6 mL/min with monitoring at 254 nm. Purification Method B consisted of the following gradient of solvent A to

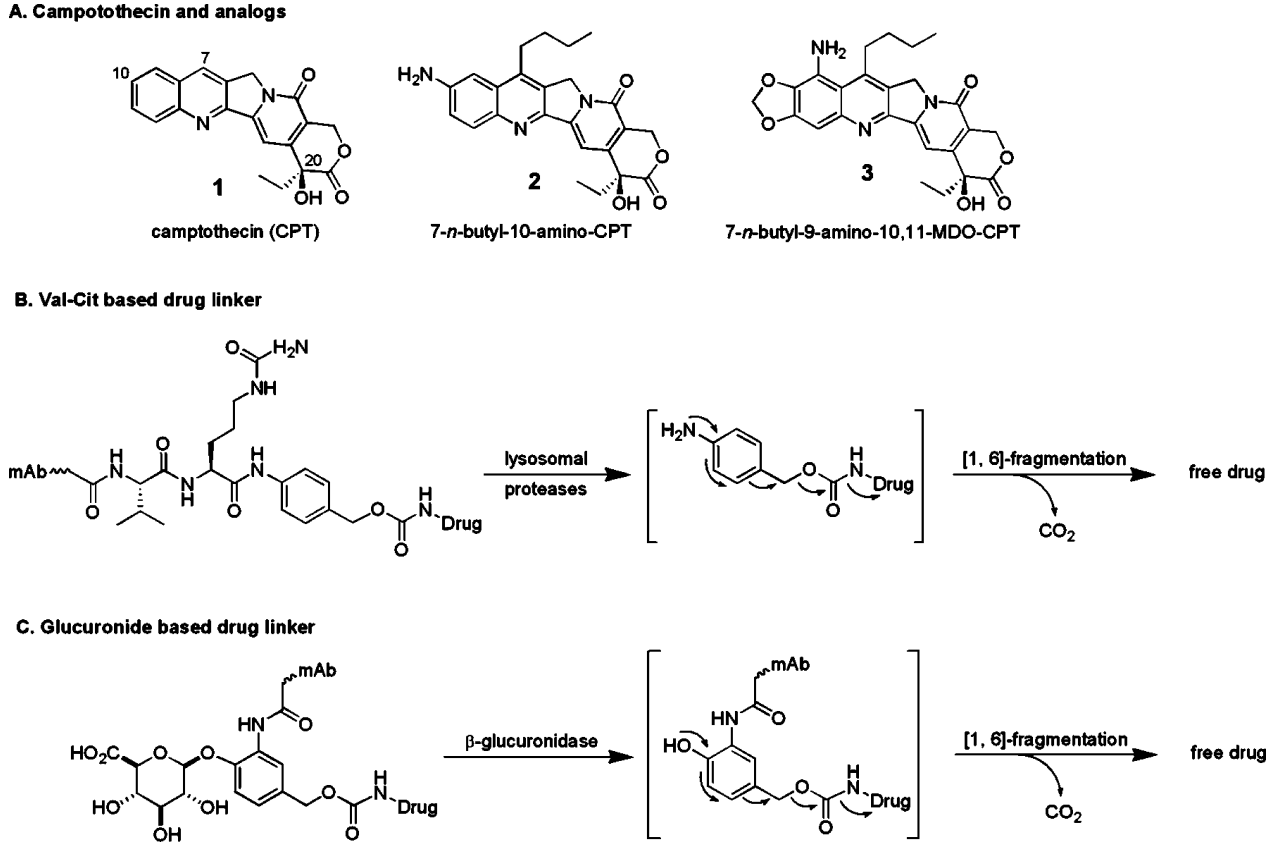


Figure 1. Structures and mechanism of release of active CPT drugs; (A) camptothecin and potent analogues prepared via Friedlander condensation; (B) val-cit dipeptide based drug linker; (C) glucuronide based drug linker.

solvent B: isocratic 90:10 from 0 to 5 min; 90:10 to 60:40 from 5 to 45 min; 60:40 to 10:90 from 45 to 50 min; followed by isocratic 10:90 for 5 min. The flow rate was 4.6 mL/min with monitoring at 254 nm.

Analytical thin layer chromatography was performed on silica gel 60 F_{254} aluminum sheets (EMD Chemicals, Gibbstown, NJ). Whatman 60 Å 230−400 mesh silica gel was used for flash chromatographic purification. Radial chromatography was performed on a Chromatotron instrument (Harris Research, Palo Alto, CA).

7-Butyl-9-amino-10,11-methylenedioxy-camptothecin (3). To a stirred solution of 7-butyl-10,11-methylenedioxy-camptothecin (**12**, 50 mg, 112 μmol) was added 2 mL of concentrated sulfuric acid. The reaction was cooled to 0 °C and sodium nitrate (14 mg, 166 μmol) was added. The reaction was warmed to room temperature and stirred for 30 min, at which time LC/MS revealed complete conversion to product. The reaction was then poured into ice-water and extracted with methylene chloride. The combined extracts were washed with water and concentrated under reduced pressure to provide the nitrated material (54 mg, 98%). UV λ_{max}: 225, 305, and 385 nm. LC-MS: *m/z* (ES^+) found 494.14 $(M+H)^+$, *m/z* (ES^-) found 492.10 $(M-H)^-$. The material was carried forward without further characterization. A stirred solution of the nitrated material (54 mg, 110 μmol) in ethanol was warmed to facilitate dissolution, and then the reaction vessel was purged with nitrogen. At room temperature, 10% palladium on carbon (100 mg) was added, and the reaction mixture was exposed to a hydrogen atmosphere. After 30 min, LC-MS indicated conversion to product. The reaction was filtered through a plug of Celite that was subsequently washed with 300 mL methanol. The filtrate was concentrated under reduced pressure and purified by radial chromatography on a 1 mm plate eluted with a CH_2Cl_2/MeOH mixture (97:3 to 95:5 CH_2Cl_2/MeOH) to provide **3** (29 mg, 57%). ^1H NMR ($CDCl_3$) δ (ppm): 1.02 (m, 6H), 1.57 (m, 3H, under HOD), 1.82 (m, 2H), 1.87 (m, 3H), 3.35 (m, 3H), 3.73 (m, 1H), 5.21 (s, 1H), 5.22 (s, 1H), 5.51 (AB, $J = 16.4$ Hz, 2H), 6.17 (d, $J = 2.0$ Hz, 2H). UV λ_{max} 229, 280, 335, and 381 nm. Analytical HPLC: gradient A, t_R = 12.56 min, $AUC_{255} = 97.9\%$; gradient B, $t_R = 8.35$ min, $AUC_{255} = 97.5\%$. LC-MS: *m/z* (ES^+) found 464.00 $(M+H)^+$, *m/z* (ES^-) found 461.97 $(M-H)^-$.

4-((*S*)-2-((*S*)-2-(6-(2,5-Dioxo-2*H*-pyrrol-1(5*H*)-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl-(7-butyl-10-aminocamptothecin)carbamate (4). An oven-dried flask was charged with a solution of **2** (10 mg, 23.8 μmol) in 0.5 mL anhydrous CH_2Cl_2. The solution was stirred at room temperature under nitrogen, and 0.4 mL of a 20% phosgene solution in toluene was added. The resultant brown suspension was stirred for 18 h at room temperature. The solution was then evaporated to dryness with a stream of nitrogen gas and further concentrated by rotary evaporation. To the residue obtained was added maleimidocaproyl-valine-citrulline-*para*-aminobenzyl alcohol (**13**, 20.5 mg, 35.7 μmol) (henceforth called mcVal-Cit-PABA (*26*)) dissolved in 0.5 mL anhydrous DMF. The solution was then heated to 45 °C under nitrogen. LC/MS revealed the formation of a product corresponding to the desired molecular weight. The reaction was diluted with 1.5 mL of DMSO and desired product isolated by preparative HPLC (Method A) to give **4** (3.6 mg, 15%). ^1H NMR (DMSO-d_6) δ (ppm): 0.87 (m, 9H), 0.97 (t, $J = 7.6$ Hz, 3H), 1.19 (m, 2H), 1.48 (m, 8H), 1.70 (m, 4H), 1.85 (m, 4H), 2.15 (m, 4H), 3.10 (m, 5H), 4.19 (m, 1H), 4.38 (m, 1H), 5.17 (s, 2H), 5.29 (s, 2H), 5.43 (s, 2H), 5.98 (m, 1H), 7.00 (s, 2H), 7.28 (s, 1H), 7.41 (d, $J = 8.6$ Hz, 2H), 7.65 (d, $J = 8.0$ Hz, 2H), 7.81 (d, $J = 9.2$ Hz, 1H), 7.90 (m, 1H), 8.10 (d, $J = 9.0$ Hz, 2H), 8.43 (s, 2H), 10.03 (s, 1H), 10.27 (s, 1H). UV λ_{max}: 225, 267, and 385 nm. Analytical HPLC: gradient A, $t_R = 12.38$ min, $AUC_{255} = 98.5\%$; gradient B, t_R = 7.81 min, $AUC_{255} = 93.9\%$. LC-MS: *m/z* (ES^+) found 1018.33 $(M+H)^+$, *m/z* (ES^-) found 1017.06 $(M-H)^-$.

4-((*S*)-2-((*S*)-2-(6-(2,5-Dioxo-2*H*-pyrrol-1(5*H*)-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl-(7-butyl-9-amino-10,11-methylendioxycamptothecin)carbamate (5). Drug linker **5** was prepared from CPT **3** (24 mg, 51.8 μmol) in an analogous reaction to that described for **4**. The crude reaction material was purified by flash chromatography on silica gel eluted with MeOH in CH_2Cl_2 (98:2 to 95:5 to 90:10 CH_2Cl_2/MeOH) to yield **5** (11.2 mg, 20%). ^1H NMR (DMF-d_7) δ (ppm): 0.96 (m, 9H), 1.28 (m, 3H), 1.56 (m, 8H), 2.00 (m, 3H), 2.16 (m, 1H), 2.29 (m, 2H), 3.08 (q, $J = 6.2$ Hz, 1H), 3.26 (m, 3 H), 3.46 (t, $J = 6.8$ Hz, 2H), 4.36 (t, $J = 7.6$ Hz, 1H), 4.63 (bt, 1H), 5.20 (s, 2H), 5.31 (s, 2H), 5.51 (s, 2H), 5.66 (m, 1H), 6.27 (bt, 1H), 6.37 (s, 1H), 6.49 (s, 1H), 7.02 (s, 2H), 7.37 (s, 1H), 7.45 (d, $J = 8.0$ Hz, 1H), 7.55 (s, 1H), 7.84 (d, $J = 8.0$ Hz, 1H), 7.90 (d, $J = 8.4$ Hz, 1H), 8.16 (d, $J = 7.6$ Hz, 1H), 9.37 (s, 1H), 10.11 (s, 1H). UV λ_{max}: 227, 253, and 383 nm. Analytical HPLC: gradient A, $t_R = 12.11$ min, $AUC_{255} = 96.2\%$; gradient B, $t_R = 7.41$ min, $AUC_{255} = 94.9\%$. LC-MS: *m/z* (ES^+) found 1062.45 $(M+H)^+$, *m/z* (ES^-) found 1060.29 $(M-H)^-$.

(2*S*,3*S*,4*S*,5*R*,6*S*)-Methyl 6-(2-(3-(((9*H*-fluoren-9-yl)methoxy)-carbonylamino)propanamido)-4-(((7-butyl-10-aminocamptothecin)carbamoyloxy)methyl)phenoxy)-3,4,5-triacetoxy-tetrahydro-2*H*-pyran-2-carboxylate (6). A flame-dried flask was charged with the known (*27*) protected glucuronide linker fragment (**14**, 28.5 mg, 38.1 μmol) dissolved in 1.5 mL anhydrous CH_2Cl_2. Anhydrous pyridine (37 μL, 457 μmol) was added, and the solution was cooled to −78 °C under a nitrogen atmosphere. Diphosgene (5.5 μL, 46 μmol) was added; the light yellow, limpid solution was stirred for 3 h at −78 °C. A solution of **2** (16.0 mg, 38.1 μmol) in 1.5 mL anhydrous THF was then added, and the reaction was warmed to 0 °C over 1 h. Analytical HPLC at 1 h revealed the formation of product. The reaction was poured into 50 mL EtOAc, and the organic phase was washed with 20 mL water, then 20 mL brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue so obtained was purified by flash chromatography on silica gel eluted with a CH_2Cl_2/MeOH mixture (98:2 to 97:3 CH_2Cl_2/MeOH) to provide **6** (28.8 mg, 64%). ^1H NMR ($CDCl_3$) δ (ppm) 0.95 (t, $J = 7.6$ Hz, 3H), 0.99 (t, $J = 7.2$ Hz, 3H), 1.46 (m, 3H), 1.66 (m, 3H), 1.86 (m, 3H), 2.03 (s, 3H), 2.05 (s, 6H), 2.73 (m, 2H), 3.05 (q, $J = 8.0$ Hz, 1H), 3.57 (m, 2H), 3.72 (s, 3H), 4.12 (t, $J = 7.2$ Hz, 1H), 4.31 (m, 4H), 5.15 (m, 7H), 5.42 (m, 1H), 5.59 (m, 1H), 5.76 (bt, 1H), 6.95 (d, $J = 8.4$ Hz, 1H), 7.04 (d, $J = 8.0$ Hz, 1H), 7.21 (m, 1H), 7.22 (s, 1H), 7.32 (t, $J = 7.2$ Hz, 2H), 7.52 (d, $J = 7.2$ Hz, 2H), 7.59 (d, $J = 8.4$ Hz, 1H), 7.68 (m, 3H), 8.06 (m, 2H), 8.29 (s, 1H), 8.41 (s, 1H). UV λ_{max}: 267 and 385 nm. LC-MS: *m/z* (ES^+) found 1194.84 $(M+H)^+$, *m/z* (ES^-) found 1192.57 $(M-H)^-$.

(2*S*,3*S*,4*S*,5*R*,6*S*)-Methyl 6-(2-(3-(((9*H*-fluoren-9-yl)methoxy)-carbonylamino)propanamido)-4-(((7-butyl-9-amino-10,11-methylendioxycamptothecin)carbamoyloxy)methyl)phenoxy)-3,4,5-triacetoxy-tetrahydro-2*H*-pyran-2-carboxylate (7). A flame-dried flask was charged with **3** (10 mg, 21 μmol) dissolved in 0.5 mL anhydrous CH_2Cl_2. The solution was stirred at room temperature under nitrogen, and 0.4 mL of a 20% phosgene solution in toluene was added. The reaction was stirred for 18 h at room temperature; a small aliquot of the reaction was quenched with methanol, and the methyl carbamate (indicating formation of the isocyanate) was detected by LC-MS. The solution was then evaporated to dryness with a stream of nitrogen gas and further concentrated by rotary evaporation. To the residue obtained was added the known (*27*) protected glucuronide linker fragment (**14**, 30 mg, 40 μmol) dissolved in 0.2 mL anhydrous DMF. The reaction was stirred at room

temperature for 2 h, after which time LC-MS revealed conversion to product. The reaction was then concentrated, redissolved in minimal CH_2Cl_2, and purified by radial chromatography eluting CH_2Cl_2/MeOH (97:3 to 95:5 CH_2Cl_2/MeOH) to give **7** (10 mg, 38%). ^{1}H NMR ($CDCl_3$) δ (ppm): 0.88 (m, 3H), 1.00 (t, J = 7.6 Hz, 3H), 1.26 (s, 2H), 1.42 (m, 3H), 1.87 (m, 2H), 2.06 (s, 9H), 2.74 (m, 2H), 2.99 (bs, 1H), 3.18 (bs, 1H), 3.61 (m, 2H), 3.74 (s, 3H), 4.19 (q, J = 6.8 Hz, 2H), 4.39 (q, J = 10 Hz, 2H), 5.16 (m, 9H), 5.43 (q, J = 8.8 Hz, 1H), 5.65 (d, J = 16.4 Hz, 1H), 5.85 (s, 1H), 6.17 (s, 3H), 6.96 (m, 3H), 7.22 (s, 1H), 7.32 (t, J = 6.8 Hz, 2H), 7.45 (s, 1H), 7.59 (m, 3H), 7.67 (d, J = 7.2 Hz, 2H), 8.14 (s, 1H), 8.47 (s, 1H). UV λ_{max}: 229, 263, and 383 nm. LC-MS: m/z (ES^+) found 1237.95 $(M+H)^+$, m/z (ES^-) found 1236.85 $(M-H)^-$.

(2*S*,3*S*,4*S*,5*R*,6*S*)-6-(2-(3-Aminopropanamido)-4-(((7-butyl-10-aminocamptothecin)carbamoyloxy)methyl)phenoxy)-3,4,5-trihydroxy-tetrahydro-2*H*-pyran-2-carboxylic Acid (8). A flask charged with **6** (24.0 mg, 20.1 μmol) dissolved in a solvent mixture of 2.5 mL MeOH, 3.0 mL THF, and 2.5 mL water, was cooled to 0 °C. To the stirred solution was added LiOH monohydrate (8.6 mg, 212 μmol) and the reaction was warmed to room temperature. After 3 h, LC-MS revealed complete conversion to product. Glacial acetic acid (12.7 μL, 222 μmol) was added, and the reaction was evaporated to dryness. Purification of residue by preparative HPLC using Method B gave **8** (12.7 mg, 67%). ^{1}H NMR (CD_3OD) δ (ppm) 0.96 (t, J = 7.6 Hz, 3H), 0.99 (t, J = 7.2 Hz, 3H), 1.49 (q, J = 7.2 Hz, 2H), 1.65 (m, 2H), 1.89 (m, 2H), 2.62 (s, 1H), 2.86 (t, J = 6.0 Hz, 2H), 2.95 (bt, 2H), 3.53 (m, 3H), 3.93 (m, 1H), 4.84 (d, J = 7.6 Hz, 1H), 4.91 (s, 2H), 5.11 (s, 2H), 5.34 (AB, J = 16.0 Hz, 2H), 7.20 (m, 2H), 7.40 (s, 1H), 7.60 (m, 1H), 7.82 (d, J = 8.8 Hz, 1H), 8.12 (s, 1H), 8.22 (s, 1H), 9.63 (s, 1H). UV λ_{max}: 227, 267, 385 nm. LC-MS: m/z (ES^+) found 832.20 $(M+H)^+$, m/z (ES^-) found 830.27 $(M-H)^-$.

(2*S*,3*S*,4*S*,5*R*,6*S*)-6-(2-(3-Aminopropanamido)-4-(((7-butyl-9-amino-10,11-methylendioxycamptothecin)carbamoyloxy)-methyl)phenoxy)-3,4,5-trihydroxy-tetrahydro-2*H*-pyran-2-carboxylic Acid (9). Drug linker **9** was prepared from **7** (25.0 mg, 25.2 μmol) in the same manner as that described for **8**. The reaction gave **9** (17.6 mg, 92%). UV λ_{max}: 223 and 383 nm. LC-MS: m/z (ES^+) found 875.94 $(M+H)^+$, m/z (ES^-) found 873.95 $(M-H)^-$. The material was carried forward without further characterization.

(2*S*,3*S*,4*S*,5*R*,6*S*)-6-(2-(3-(6-(2,5-Dioxo-2*H*-pyrrol-1-(5*H*)-yl)hexanamido)propanamido)-4-(((7-butyl-10-aminocamptothecin)carbamoyloxy)methyl)phenoxy)-3,4,5-trihydroxy-tetrahydro-2*H*-pyran-2-carboxylic Acid (10). To a solution of **8** (23.0 mg, 27.6 μmol) dissolved in 1.5 mL anhydrous DMF was added maleimidocaproyl NHS ester (12.8 mg, 41.4 μmol), followed by DIPEA (14.4 μL, 82.8 μmol). The reaction was stirred at room temperature under nitrogen for 4 h, at which time LC-MS revealed conversion to product. The crude reaction was diluted in DMSO and purified by preparative HPLC (Method A) to give **10** (20.5 mg, 72%). ^{1}H NMR (DMF-d_7) δ (ppm): 0.99 (m, 6H), 1.26 (p, J = 8.4 Hz, 2H), 1.57 (m, 6H), 1.81 (p, J = 7.2 Hz, 2H), 2.01 (m, 2H), 2.16 (t, J = 7.2 Hz, 2H), 2.68 (t, J = 6.8 Hz, 2H), 3.21 (t, J = 8.0 Hz, 2H), 3.57 (m, 10H), 4.10 (d, J = 9.6 Hz, 2H), 5.03 (m, 1H), 5.23 (s, 2H), 5.36 (s, 2H), 5.52 (d, J = 2.4 Hz, 2H), 6.52 (bs, 1H), 7.01 (s, 2H), 7.19 (dd, J = 8.4, 2.0 Hz, 1H), 7.27 (d, J = 8.4 Hz, 1H), 7.43 (s, 1H), 7.94 (t, J = 5.6 Hz, 1H), 8.09 (dd, J = 9.2, 2.0 Hz, 1H), 8.18 (d, J = 9.2 Hz, 1H), 8.48 (s, 1H), 8.57 (s, 1H), 9.36 (s, 1H), 10.37 (s, 1H). UV λ_{max}: 267 and 385 nm. Analytical HPLC: gradient A, t_R = 11.93 min, AUC_{255} = 94.7%; gradient B, t_R = 5.76 min, AUC_{255} = 97.1%. LC-MS: m/z (ES^+) found 1025.40 $(M+H)^+$, m/z (ES^-) found 1023.29 $(M-H)^-$.

(2*S*,3*S*,4*S*,5*R*,6*S*)-6-(2-(3-(6-(2,5-Dioxo-2*H*-pyrrol-1-(5*H*)-yl)hexanamido)propanamido)-4-(((7-butyl-9-amino-10,11-methylendioxycamptothecin)carbamoyloxy)methyl)phenoxy)-3,4,5-trihydroxy-tetrahydro-2*H*-pyran-2-carboxylic Acid (11). Drug linker **11** was prepared from **9** (16.3 mg, 18.6 μmol) in the same manner as that described for **10**. The reaction gave **11** (12.9 mg, 65%). ^{1}H NMR (DMF-d_7) δ (ppm): 0.93 (t, J = 7.2 Hz, 3H), 0.99 (t, J = 8.0 Hz, 3H), 1.27 (m, 2H), 1.56 (m, 8H), 1.70 (m, 2H), 1.98 (m, 2H), 2.17 (t, J = 7.6 Hz, 2H), 2.69 (m, 2H), 3.52 (m, 10H), 4.10 (d, J = 9.2 Hz, 2H), 5.03 (d, J = 6.4 Hz, 2H), 5.19 (s, 2H), 5.30 (s, 2H), 5.51 (d, J = 2.8 Hz, 2H), 6.37 (m, 3H), 7.02 (d, J = 3.6 Hz, 2H), 7.18 (d, J = 8.4 Hz, 1H), 7.28 (d, J = 8.4 Hz, 1H), 7.37 (s, 1H), 7.55 (s, 1H), 8.50 (s, 1H), 9.36 (s, 1H), 9.43 (s, 1H). UV λ_{max}: 223 and 383 nm. Analytical HPLC: gradient A, t_R = 11.70 min, AUC_{255} = 97.2%; gradient B, t_R = 5.47 min, AUC_{255} = 97.7%. LC-MS: m/z (ES^+) found 1069.44 $(M+H)^+$, m/z (ES^-) found 1067.37 $(M-H)^-$.

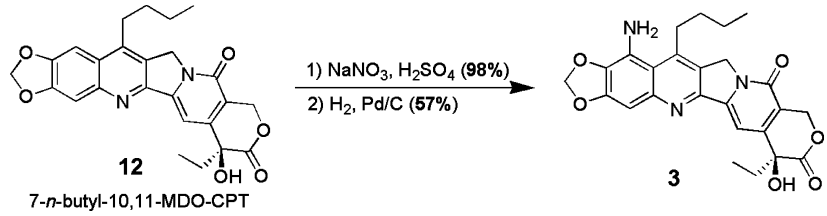
Antibody−Drug Conjugate Preparation. The immunoconjugates were prepared as previously described (*4, 28*). Briefly, the mAbs (4−5 mg/mL) in PBS containing 50 mM sodium borate at pH 7.4 were reduced with tris(carboxyethyl)phosphine hydrochloride at 37 °C. The progress of the reaction, which reduces interchain disulfides, was monitored by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) and allowed to proceed until the desired level of thiols/mAb was achieved. The reduced antibody was then cooled to 0 °C and alkylated with 1.5 equiv of maleimide drug linker per antibody thiol. After 1 h, the reaction was quenched by the addition of 5 equiv of *N*-acetyl cysteine (NAC). Quenched drug linker was removed by gel filtration over a PD-10 column. The ADC was then sterile-filtered through a 0.22 μm syringe filter. Protein concentration and drug loading were determined by spectral analysis at 280 and 375 nm, respectively, with correction for the contribution of drug absorbance at 280 nm. Size-exclusion chromatography was used to determine the extent of antibody aggregation, and RP-HPLC confirmed the absence of remaining NAC-quenched drug linker.

ADC Binding Assay. Karpas 299 cells were treated with serial dilutions of cAC10 or cAC10-**10** in PBS. cBR96-**10** was also included as a nonbinding control. After 30 min on ice, the cells were washed and resuspended in 4 °C PBS. Secondary goat antibody-FITC was added to detect bound mAb or ADC. The cells were then washed, fixed with paraformaldehyde, and analyzed by flow cytometry.

Stability Assay. The stability of cBR96-**10** was assessed by incubation in citrated CD-1 mouse plasma. cBR96-**10** was incubated at 37 °C in plasma over the course of two weeks. Aliquots were withdrawn as a function of time and protein precipitated through the addition of 5 volumes ice-cold methanol containing 0.1% formic acid and spiked with a camptothecin reference standard. The protein was then removed by centrifugation for 5 min at 16 100 *g*; the supernatant was collected and analyzed by LC-MS (ES^+ mode) using gradient A for the detection of counts at m/z 420.2 $(M+H)^+$ at a retention time of 11.78 min, corresponding to free drug **2**. A standard calibration curve spanning the range 1−100% drug release was employed to convert the m/z 420.2 AUC at 11.78 min to a concentration value.

In Vitro Growth Inhibition. The cytotoxic activity of the immunoconjugates was assessed using a resazurin (Sigma, St. Louis, MO, USA) reduction assay (*4*). Karpas 299 (2000 cells/well), H3396 (2000 cells/well), L540cy (7000 cells/well), and Caki-1 (1000 cells/well) cells cultured in log-phase growth were seeded for 24 h in 96-well plates containing 150 μL RPMI 1640 supplemented with 20% FBS. Serial dilutions of ADC in cell culture media were prepared at 4× working concentration; 50 μL of each dilution was added to the 96-well plates. Following

Scheme 1



addition of ADC, the cells were incubated with test articles for 4 or 8 d at 37 °C. Resazurin was then added to each well to achieve a 50 μM final concentration, and the plates were incubated for an additional 4 h at 37 °C. The plates were then read for the extent of dye reduction on a Fusion HT plate reader (Packard Instruments, Meridien, CT, USA) with excitation and emission wavelengths of 530 and 590 nm, respectively. The IC_{50} value, determined in triplicate, is defined here as the concentration that results in a 50% reduction in cell growth relative to untreated controls.

In Vivo Therapy Experiments. All experiments were conducted in concordance with the Animal Care and Use Committee in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. In vivo tolerability was first assessed to ensure that the conjugates were tolerated at clinically relevant doses. BALB/c mice were treated with escalating doses of ADC formulated in PBS with 0.01% Tween 20. Mice were monitored for weight loss following drug treatment; those that experienced 20% weight loss or other signs of morbidity were euthanized.

The efficacy experiment was conducted in a renal cell carcinoma xenograft model. Caki-1 tumor fragments were implanted into the right flank of Nude mice. Mice were randomized to study groups ($n = 5$) on day nine with each group averaging around 100 mm^3 tumor volume. The ADC or controls were dosed ip according to a q4dx4 schedule. Tumor volume as a function of time was determined using the formula ($L \times W^2$)/2. Animals were euthanized when tumor volumes reached 1000 mm^3. Mice showing durable regressions were terminated on day 96 post-implant.

RESULTS

Drug Linker Design. There are two significant challenges associated with designing an immunoconjugate with camptothecin: lack of a suitable functional group for conjugation, and hydrophobicity, which can cause antibody aggregation. The first obstacle requires designing a drug linker that combines a high level of stability in circulation with rapid drug release upon internalization into targeted cells. The only readily accessible functional group in camptothecin is the C20 tertiary hydroxyl. The use of ester or carbonate linkages to assemble conjugates with aliphatic alcohols is common (*18, 20, 29*−*31*). However, as cited earlier, this can be a liability, as mouse plasma contains high levels of esterase activity and readily cleaves ester and carbonate functional groups (*32*).

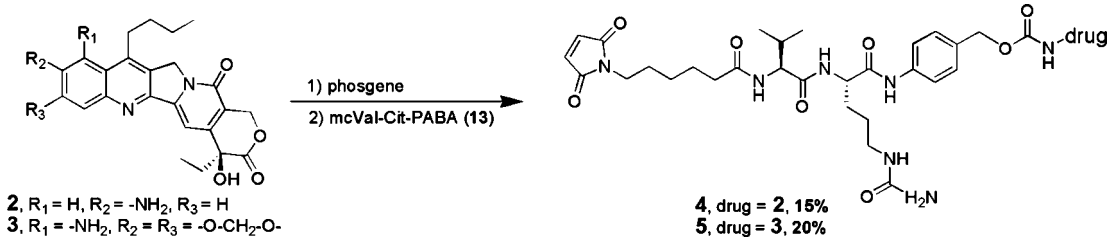
To address drug linker stability, we envisioned employing a carbamate bond to an amine-containing camptothecin such as 7-butyl-10-amino-CPT (**2**) shown in Figure 1. Carbamate bonds typically provide superior stability relative to ester and carbonate bonds with respect to hydrolysis (*33*). Alternatively, we installed an amine into the known (*23, 24*) analogue 7-butyl-10,11-methylenedioxy-CPT (**12**, Scheme 1). In both cases, the presence of the amine provided for the use of a cathepsin B-cleavable valine-citrulline-PABA dipeptide linker (val-cit) system (Figure 1B). The val-cit dipeptide linker system is designed to achieve circulation stability and rapid lysosomal drug release upon internalization into the targeted cancer cells (*4, 34*).

A second obstacle associated with this class is manifest in the hydrophobic nature of these compounds. Camptothecins possess a rigid, extended aromatic ring system that confers limited aqueous solubility and can induce significant antibody aggregation. To address this issue, hydrophilic glucuronide-based CPT drug linkers (Figure 1C) were pursued in addition to dipeptide drug linkers. The glucuronide linker system is characterized by its ability to provide monomeric ADCs with a high degree of circulation stability and rapid drug release in the presence of endogenous β-glucuronidase at the target site (*27*).

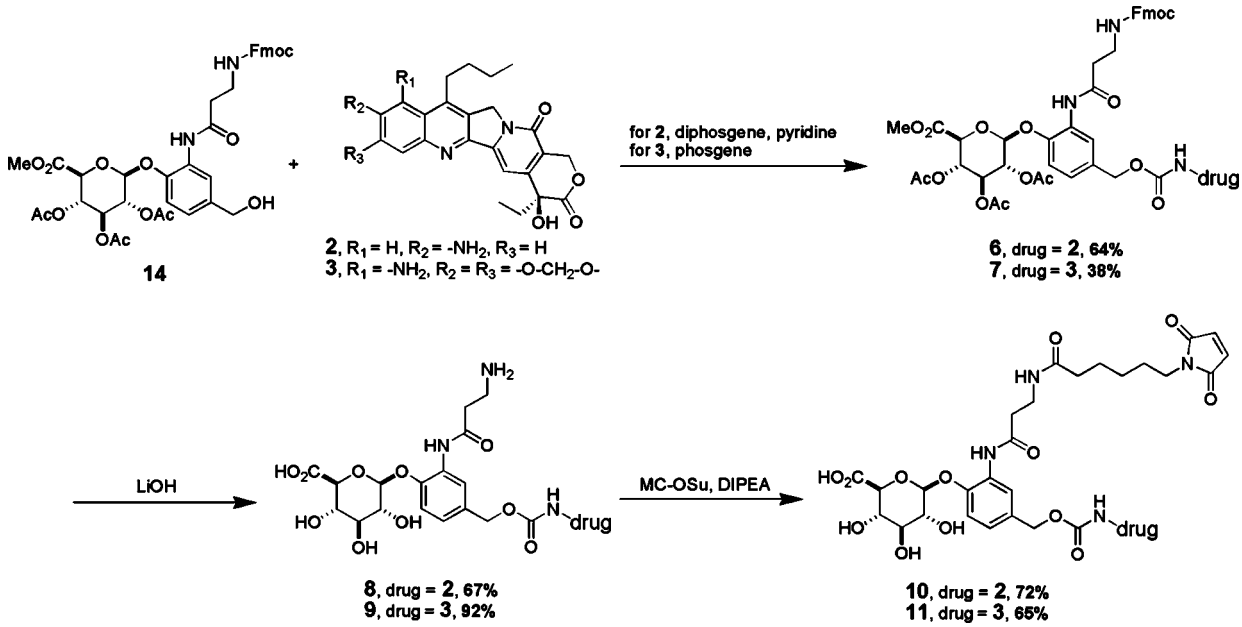
Drug Linker Synthesis. The syntheses of CPT analogues **2** and **12** were achieved by the well-precedented Friedlander condensation route (*23, 25*). Regioselective nitration and subsequent hydrogenation of **12** provided the 9-amino analogue **3** in 56% overall yield, as shown in Scheme 1. The protease-cleavable dipeptide drug linkers were prepared in two synthetic steps (Scheme 2). Camptothecins **2** and **3** were treated with phosgene to convert their respective amines to isocyanates in situ. The isocyanates were subsequently trapped by benzyl alcohol linker **13** (*26*) (mcVal-Cit-PABA), forming the carbamate linkages in drug linkers **4** and **5**, respectively.

The glucuronide drug linkers were prepared according to the synthetic sequence in Scheme 3. The synthesis commenced by converting the protected glucuronide benzyl alcohol **14** (*27*) to the chloroformate with diphosgene, which was then trapped in situ with camptothecin **2** to provide intermediate **6**. The more sterically hindered amine in **3** was converted to the isocyanate with phosgene and trapped with glucuronide benzyl alcohol **14**. Exhaustive saponification and simultaneous Fmoc-deprotection of **6** and **7** yielded free amines **8** and **9**, respectively. The synthetic sequence was then completed via the coupling of amines **8** and **9** with the NHS ester of maleimidocaproic acid

Scheme 2



Scheme 3



(MC-OSu), providing drug linkers **10** and **11**, respectively, in moderate overall yield.

Preparation of Antibody−Drug Conjugates. Conjugates comprising antibodies cBR96, cAC10, and h1F6 and drug linkers **4**, **5**, **10**, and **11** were prepared following standard protocols (*4, 28*). Reduced interchain disulfides were alkylated with drug linker, forming Michael adducts between the mAb cysteines and drug linker maleimide. Upon completion of the conjugation reaction, excess drug linker was quenched with *N*-acetylcysteine and removed by gel filtration, which was confirmed by HPLC. Drug loading was assessed by chromatographic analysis and UV absorption of the ADCs with correction for the contribution of the camptothecin chromophore at 280 nm.

The extent of ADC aggregation was determined by size-exclusion chromatography. Glucuronide drug linkers **10** and **11** provided ADCs with 4 drugs or 8 drugs per antibody with minimal (<5%) aggregation (Tables 1 and 2). The dipeptide drug linkers **4** and **5** led to antibody aggregation to a greater extent than the more hydrophilic glucuronide drug linkers. ADCs loaded with 4 drugs per antibody contained 4−16% aggregate. ADCs comprising **4** or **5** loaded with 8 drugs/mAb gave much higher levels of aggregation. For example, cAC10 loaded with drug linker **4** was >80% aggregated (Table 1). Highly aggregated (>20%) ADCs were excluded from the biological evaluation.

ADC Binding. As previously shown (*4*), alkylation of interchain disulfides typically has negligible effect on ADC antigen binding activity. To ensure that this was the case with the camptothecins, cAC10-**10** (7 drug/mAb) was assayed for binding affinity relative to unconjugated mAb with CD30+ Karpas 299 cells. The results, shown in Figure 2, indicate that conjugation did not significantly impact the antigen binding. Antigen-negative cBR96 displayed no appreciable binding.

Drug Linker Stability. Val-Cit-PAB and glucuronide carbamate drug linkers have been previously shown to be highly stable toward hydrolysis in various matrices, including plasma (*4,28*). To corroborate these observations with the camptothecin series, cBR96-**10** (7 drug/mAb) was incubated in mouse plasma, and the appearance of free drug was assessed by a calibrated LC/MS assay. After 14 d at 37 °C, the free drug (**2**) signal was below the limit of detection of the assay, which is <1% of the theoretical maximum drug release. This suggests that the carbamate linkage is stable to hydrolysis in mouse plasma. Control experiments established efficient recovery of free drug from the matrix.

In Vitro Biological Evaluation. The activities of ADCs containing 7-butyl-10-amino-CPT (**2**) incorporated via dipeptide (**4**) or glucuronide (**10**) drug linker were assessed on Karpas 299 (CD30+) ALCL and H3396 (LeY+) breast carcinoma cell lines following 4 or 8 d treatment (Table 1). ADCs comprising dipeptide drug linker **4** displayed negligible activity following a 4 d treatment (data not shown). When the treatment time was extended to 8 d, modest immunologically specific activity was observed. ADCs bearing glucuronide drug linker **10** were weakly

Table 1. Cytotoxic Activities of ADCs Containing 2

					IC_{50} values (nM)	
cytotoxic agent	treatment time (d)	antigen	drug/mAb ratio	percent aggregate	Karpas 299[a] (CD30+, LeY−)	H3396[a] (CD30−, LeY+)
cAC10−**4**		CD30	8	>80	nt	nt
cBR96−**4**		LeY	8	>80	nt	nt
cAC10−**4**	8	CD30	3.7	9	26	no effect
cBR96−**4**	8	LeY	4.2	16	110	13
cAC10−**10**	4	CD30	7	<5	160	>500[b]
cBR96−**10**	4	LeY	7	<5	>500[b]	22
cAC10−**10**	8	CD30	7	<5	1.8	460
cBR96−**10**	8	LeY	7	<5	170	21
1	4	–	–	–	9.7	7.4
2	4	–	–	–	1.0	0.9

[a] Cells were treated with test articles for 4 or 8 d, and cell viability was determined by resazurin conversion. The IC_{50} values represent the concentrations (nM) of the drug component of the ADC. [b] IC_{50} values are greater than the highest concentration tested.

Table 2. Cytotoxic Activities of ADCs Containing 3 Following 4 d Treatment

				IC_{50} (nM)		
cytotoxic agent	antigen	drug/mAb ratio	percent aggregate	Karpas 299[a] (CD30+, CD70−)	L540cy[a] (CD30+, CD70−)	Caki-1[a] (CD 30−, CD70+)
cAC10−**5**	CD30	3.8	8	4.4	0.32	>150[b]
h1F6−**5**	CD70	4.2	4	>190[b]	no effect	6.2
cAC10−**11**	CD30	4.4	3	0.91	0.23	>210[b]
h1F6−**11**	CD70	4.5	1	>220[b]	190	1.5
cAC10−**11**	CD30	8	2	0.89	0.41	510
h1F6−**11**	CD70	8	1	650	280	3.3
1	–	–	–	9.7	7.4	7.0
3	–	–	–	0.06	0.03	0.05

[a] Cells were treated with test articles for 4 d, and cell viability was determined by resazurin conversion. The IC_{50} values represent the concentrations (nM) of the drug component of the ADC. [b] IC_{50} values are greater than the highest concentration tested.

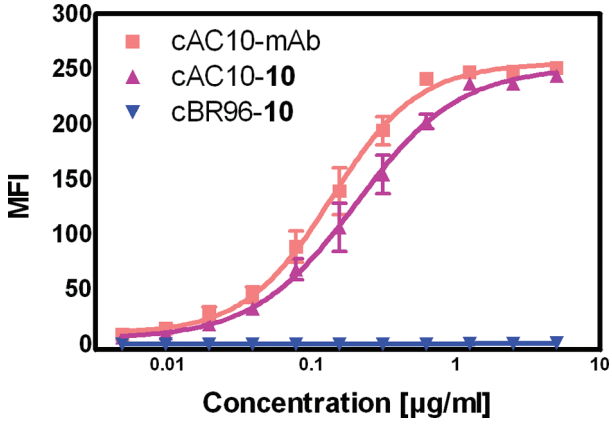


Figure 2. FACS analysis of antigen binding of glucuronide drug linker cAC10-**10** relative to cAC10 mAb and nonbinding cBR96-**10** on CD30+ Karpas 299 cells.

active, albeit more potent than mAb−**4** conjugates, over the course of a 4 d drug treatment with IC_{50} values of 160 nM and 22 nM on Karpas 299 and H3396, respectively. When drug treatment was extended to 8 d, cAC10−**10** displayed a substantial increase in potency on Karpas 299. An analogous increase in potency on H3396 cells was not observed. The activities of conjugates comprising h1F6 or cAC10 and **5** and **11** were tested on Karpas 299, L540cy (Hodgkin lymphoma), and Caki-1 (renal cell carcinoma) following a 4 d treatment (Table 2). The conjugates were immunologically specific and highly potent on the three cell lines, with IC_{50} values ranging from 0.23 to 4.4 nM across the panel.

In Vivo Biological Evaluation. In light of the superior in vitro potency of ADCs armed with camptothecin analogue **3** relative to **2**, in vivo evaluation focused on ADCs bearing drug linkers **5** and **11**. ADCs were initially evaluated for mouse tolerability prior to efficacy experiments. BALB/c mice were injected once ip with cAC10−**5** (4 drug/mAb) or cAC10−**11** (4 drug/mAb) and evaluated for weight loss and morbidity. Both the glucuronide (cAC10−**11**) and the val-cit dipeptide (cAC10−**5**) conjugates were well-tolerated at 50 mg/kg. At 100 mg/kg, cAC10−**5** resulted in moderate weight loss (5−10%) and cAC10−**11** was toxic. From these results, the maximum tolerated doses (MTD) for cAC10−**5** and cAC10−**11** are approximately 100 mg/kg and 50 mg/kg, respectively.

An efficacy experiment with immunoconjugates h1F6−**5** and h1F6−**11** was carried out in the Caki-1 renal cell carcinoma model. Nude mice bearing Caki-1 tumors were treated four times every 4 d (q4d × 4) with ADCs once the average tumor size was approximately 100 mm^3 (Figure 3). At 3 mg/kg, the val-cit immunoconjugate (h1F6−**5**) had no effect on tumor growth. In contrast, the glucuronide conjugate (h1F6−**11**) induced substantial tumor growth delay. At 10 mg/kg, the glucuronide conjugate again

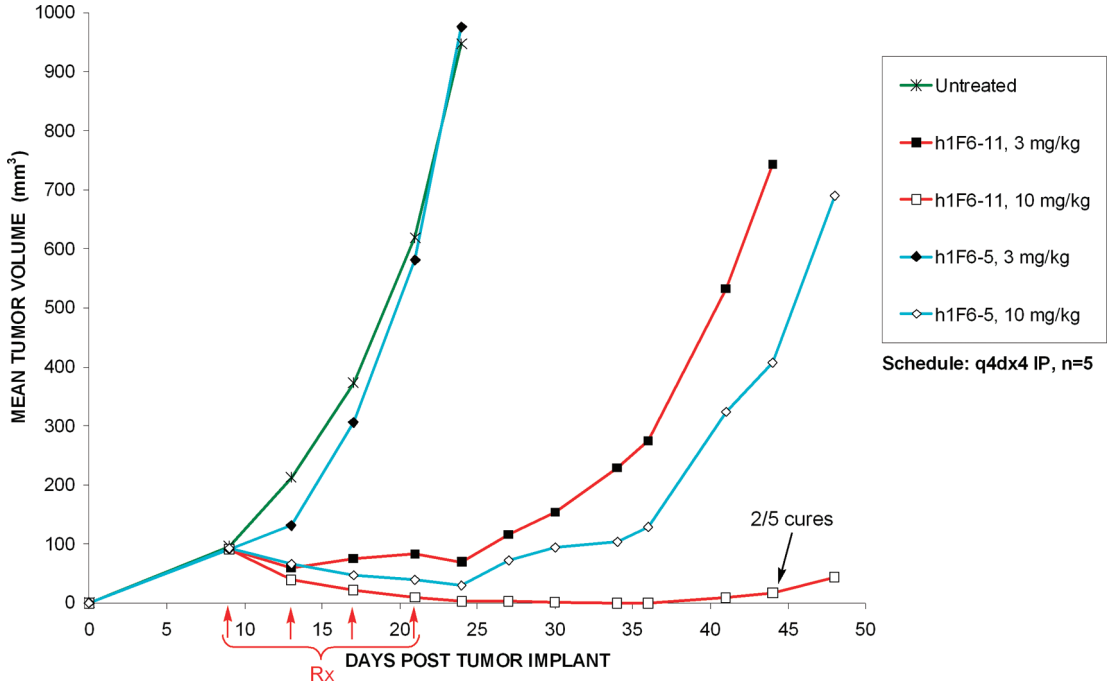


Figure 3. In vivo efficacy of camptothecin ADCs in a Caki-1 renal cell carcinoma xenograft model; all ADCs contain 4 dr/mAb.

outperformed the val-cit conjugate. Mice treated with h1F6−**5** achieved 5/5 partial regressions, while mice treated with h1F6−**11** had 5/5 complete regressions with 2/5 mice cured.

DISCUSSION

Early work on ADCs established drug potency as an important parameter contributing to the conjugate activity. The camptothecin analogues employed here are substantially more potent than CPT or related clinical analogues, irinotecan and topotecan. Among the two CPT analogues evaluated, the more potent analogue (**3**) provided significantly more potent ADCs (Tables 1 and 2). ADCs bearing **3** provided in vitro potencies 20- to 400-fold greater than CPT ADCs (*18*). More recently, ADCs bearing SN-38 were reported to be active at the single-digit nM level (*20*). Specificity controls were not included, however, and it is possible that nonspecific drug release from the carbonate or ester drug linker significantly contributed to the observed conjugate potency. The ADCs bearing **3** led to high levels of immunologically specific cell kill at subsaturating doses (Table 2). Immunological specificity implies that the linker is stable since the cells were continuously exposed to the ADCs for 96 h. The binding ADCs were less potent than the respective free drugs (Tables 1 and 2). It is possible that free drug accumulation via passive cellular uptake over the course of the 96 h treatment exceeds the intracellular drug concentration achieved through antigen-mediated delivery. This has been previously observed with other ADCs (*5, 35, 36*).

Camptothecins are an especially compelling drug class for ADCs because they can be produced synthetically, modified to give analogues with high potency, and have been shown to possess pronounced clinical activity. However, aqueous insolubility presents a significant challenge in utilizing the CPT drug class. Clinical camptothecins, irinotecan and topotecan, were designed to overcome this by inclusion of solubilizing amine groups (*15*). From the standpoint of ADC development, upon conjugation, CPT hydrophobicity can lead to significant mAb aggregation. The glucuronide linker system is ideal in addressing this issue. The glucuronide drug linkers consistently provided monomeric ADCs when loaded at 4 and 8 drug/mAb, whereas the dipeptide drug linkers tended to provide ADCs with a greater degree of aggregation (Tables 1 and 2). This is consistent with our previous work with hydrophobic minor groove binders (*37*).

We have described the preparation and biological evaluation of ADCs empowered with camptothecin analogues that were selected on the basis of the presence of an amine functional group and enhanced cellular potency. The amine functional group was deemed important because it facilitated the use of carbamate-based linkers such as the val-cit dipeptide and the glucuronide linker systems. On the basis of these results, immunoconjugates comprising the more potent analogue (**3**) linked via val-cit dipeptide or glucuronide drug linker systems are well-suited for further development.

LITERATURE CITED

(1) Carter, P. J., and Senter, P. D. (2008) Antibody-drug conjugates for cancer therapy. *Cancer J. 14*, 154–169.

(2) Chari, R. V. J. (2008) Targeted cancer therapy: conferring specificity to cytotoxic drugs. *Acc. Chem. Res. 41*, 98–107.

(3) Wu, A. M., and Senter, P. D. (2005) Arming antibodies: prospects and challenges for immunoconjugates. *Nat. Biotechnol. 23*, 1137–1146.

(4) Doronina, S. D., Toki, B. E., Torgov, M. Y., Mendelsohn, B. A., Cerveny, C. G., Chace, D. F., DeBlanc, R. L., Gearing, R. P., Bovee, T. D., Siegall, C. B., Francisco, J. A., Wahl, A. F., Meyer, D. L., and Senter, P. D. (2003) Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol. 21*, 778–784.

(5) Francisco, J. A., Cerveny, C. G., Meyer, D. L., Mixan, B. J., Klussman, K., Chace, D. F., Rejniak, S. X., Gordon, K. A., DeBlanc, R. L., Toki, B. E., Law, C.-L., Doronina, S. D., Siegall, C. B., Senter, P. D., and Wahl, A. F. (2003) cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. *Blood 102*, 1458–1465.

(6) Beeram, M., Krop, I. E., Modi, S., Tolcher, A. W., Rabbee, N., Girish, S., Tibbitts, J., Holden, S. N., Lutzker, S. G., and Burris, H. A. (2007) A phase I study of trastuzumab-MCC-DM1 (T-DM1), a first-in-class HER2 antibody-drug conjugate (ADC), in patients (pts) with HER2+ metastatic breast cancer (BC). *J. Clin. Oncol.* 25 Abstract 1042.

(7) Phillips, G. D. L., Li, G., Dugger, D. L., Crocker, L. M., Parsons, K. L., Mai, E., Blattler, W. A., Lambert, J. M., Chari, R. V. J., Lutz, R. J., Wong, W. L. T., Jacobson, F. S., Koeppen, H., Schwall, R. H., Kenkare-Mitra, S. R., Spencer, S. D., and Sliwkowski, M. X. (2008) Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-drug conjugate. *Cancer Res. 68*, 9280–9290.

(8) Krop, I. E., Beeram, M., Modi, S., Rabbee, N., Girish, S., Tibbitts, J., Holden, S. N., Lutzker, S. G., and Burris, H. A. 30th Annual San Antonio Breast Cancer Symposium, 2007, Abstract 310.

(9) Younes, A., Forero-Torres, A., Bartlett, N. L., Leonard, J. P., Lynch, C., Kennedy, D. A., and Sievers, E. L. American Society of Hematology National Meeting, 2008a, Abstract 1006.

(10) Younes, A., Forero-Torres, A., Bartlett, N. L., Leonard, J. P., Rege, B., Kennedy, D. A., Lorenz, J. M., and Sievers, E. L. American Society of Clinical Oncology, 2008b, Abstract 8526.

(11) Trail, P. A., Willner, D., Lasch, S. J., Henderson, A. J., Hofstead, S., Casazza, A. M., Firestone, R. A., Helllstrom, I., and Hellstrom, K. E. (1993) Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. *Science 261*, 212–215.

(12) Saleh, M. N., Sugarman, S., Murray, J., Ostroff, J. B., Healey, D., Jones, D., Daniel, C. R., LeBherz, D., Brewer, H., Onetto, N., and LaBuglio, A. F. (2000) Phase I trial of the anti-Lewis Y drug immunoconjugate BR96-doxorubicin in patients with Lewis Y-expressing epithelial tumors. *J. Clin. Oncol. 18*, 2282–2292.

(13) Tolcher, A. W., Sugarman, S., Gelman, K. A., Cohen, R., Saleh, M., Isaacs, C., Young, L., Healey, D., Onetto, N., and Slichenmyer, W. (1999) Randomized phase II study of BR96-doxorubicin conjugate in patients with metastatic breast cancer. *J. Clin. Oncol. 17*, 478–484.

(14) Pommier, Y. (2006) Topoisomerase I inhibitors: camptothecins and beyond. *Nat. Rev. Cancer 6*, 789–802.

(15) Garcia-Carbonero, R., and Supko, J. G. (2002) Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins. *Clin. Cancer Res. 8*, 641–661.

(16) Satoh, T., Hosokawa, M., Atsumi, R., Suzuki, W., Hakusui, H., and Nagai, E. (1994) Metabolic activation of CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, a novel antitumor agent, by carboxylesterase. *Biol. Pharm. Bull. 17*, 662–664.

(17) Gerrits, C. J. H., de Jonge, M. J. A., Schellens, J. H. M., Stoter, G., and Verweij, J. (1997) Topoisomerase I inhibitors: the relevance of prolonged exposure for present clinical development. *Br. J. Cancer 76*, 952–962.

(18) Walker, M. A., Dubowchik, G. M., Hofstead, S. J., Trail, P. A., and Firestone, R. A. (2002) Synthesis of an immunoconjugate of camptothecin. *Bioorg. Med. Chem. Lett. 12*, 217–219.

(19) Willner, D., Trail, P. A., Hofstead, S. J., King, H. D., Lasch, S. J., Braslawsky, G. R., Greenfield, R. S., Kaneko, T., and Firestone, R. A. (1993) (6-Maleimidocaproyl)hydrazone of doxorubicin - A new derivative for the preparation of immunoconjugates of doxorubicin. *Bioconjugate Chem. 4*, 521–527.

(20) Moon, S. J., Govindan, S. V., Cardillo, T. M., D'Souza, C. A., Hansen, H. J., and Goldenberg, D. M. (2008) Antibody conjugates of 7-ethyl-10-hydroxycamptothecin (SN-38) for targeted cancer chemotherapy. *J. Med. Chem. 51*, 6916–6926.

(21) Thomas, C. J., Rahier, N. J., and Hecht, S. M. (2004) Camptothecin: current perspectives. *Bioorg. Med. Chem. 12*, 1585–1604.

(22) Adams, D. J., Webba da Silva, M., Flowers, J. L., Kohlhagen, G., Pommier, Y., Colvin, O. M., Manikumar, G., and Wani, M. C. (2006) Camptothecin analogs with enhanced activity against human breast cancer cells. I. Correlation of potency with lipophilicity and persistence in the cleavage complex. *Cancer Chemother. Pharmacol. 57*, 135–144.

(23) Wani, M. C., Manikumar, G., Wall, M. E., and Wall, M. A. (2004) (US PTO, Ed.), US 2004/0266803 A1, Research Triangle Institute, USA.

(24) Vladu, B., Woynarowski, J. M., Manikumar, G., Wani, M. C., Wall, M. A., Von Hoff, D. D., and Wadkins, R. M. (2000) 7- and 10-substituted camptothecins: dependence of topoisomerase I-DNA cleavable complex formation and stability on the 7- and 10-substituents. *Mol. Pharmacol. 57*, 243–251.

(25) Wall, M. A., Wani, M. C., Nicholas, A. W., Manikumar, G., Tele, C., Moore, L., Truesdale, A., Leitner, P., and Besterman, J. M. (1993) Plant antitumor agents. 30. Synthesis and structure activity of novel camptothecin analogs. *J. Med. Chem. 36*, 2689–2700.

(26) Dubowchik, G. M., Firestone, R. A., Padilla, L., Willner, D., Hofstead, S. J., Mosure, K., Knipe, J. O., Lasch, S. J., and Trail, P. A. (2002) Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and antigen-specific in vitro anticancer activity. *Bioconjugate Chem. 13*, 855–869.

(27) Jeffrey, S. C., Andreyka, J. B., Bernhardt, S. X., Kissler, K. M., Kline, T., Lenox, J. S., Moser, R. F., Nguyen, M. T., Okeley, N. M., Stone, I. J., Zhang, X., and Senter, P. D. (2006) Development and properties of beta-glucuronide linkers for monoclonal antibody-drug conjugates. *Bioconjugate Chem. 17*, 831–840.

(28) Jeffrey, S. C., Torgov, M. Y., Andreyka, J. B., Boddington, L., Cerveny, C. G., Denny, W. A., Gordon, K. A., Gustin, D., Haugen, J., Kline, T., Nguyen, M. T., and Senter, P. D. (2005) Design, synthesis, and in vitro evaluation of dipeptide-based antibody minor groove binder conjugates. *J. Med. Chem. 48*, 1344–1358.

(29) de Groot, F. H. M., Busscher, G. F., Aben, R. W. M., and Scheeren, H. W. (2002) Novel 20-carbonate linked prodrugs of camptothecin and 9-aminocamptothecin designed for activation by tumour-associated plasmin. *Bioorg. Med. Chem. Lett. 12*, 2371–2376.

(30) de Groot, F. H. M., Loos, W. J., Koekkoek, R., van Berkom, L. W. A., Busscher, G. F., Seelen, A. E., Albrecht, C., de Bruijn, P., and Scheeren, H. W. (2001) Elongated multiple electronic cascade and cyclization spacer systems in activatible anticancer prodrugs for enhanced drug release. *J. Org. Chem. 66*, 8815–8830.

(31) Lerchen, H. G., Baumgarten, J., von dem Bruch, K., Lehmann, T. E., Sperzel, M., Kempka, G., and Fiebig, H. H. (2001) Design and optimization of 20-O-linked camptothecin glycoconjugates as anticancer agents. *J. Med. Chem. 44*, 4186–4195.

(32) Liederer, B. M., and Borchardt, R. T. (2006) Enzymes involved in the bioconversion of ester-based prodrugs. *J. Pharm. Sci. 95*, 1177–1195.

(33) Huang, T. L., Szekacs, A., Uematsu, T., Kuwano, E., Parkinson, A., and Hammock, B. D. (1993) Hydrolysis of carbonates, thiocarbonates, carbamates, and carboxylic esters of alpha-naphthol, beta-naphthol, and para-nitrophenol by human, rat, and mouse liver carboxylesterases. *Pharm. Res. 10*, 639–648.

(34) Dubowchik, G. M., and Firestone, R. A. (1998) Cathepsin B-sensitive dipeptide prodrugs. 1. A model study of structural requirements for efficient release of doxorubicin. *Bioorg. Med. Chem. Lett. 8*, 3341–3346.

(35) Jeffrey, S. C., Nguyen, M. T., Andreyka, J. B., Meyer, D. L., Doronina, S. D., and Senter, P. D. (2006) Dipeptide-based highly potent doxorubicin antibody conjugates. *Bioorg. Med. Chem. Lett. 16*, 358–362.

(36) Tassone, P., Gozzini, A., Goldmacher, V., Shammas, M. A., Whiteman, K. R., Carrasco, D. R., Li, C., Allam, C. K., Venuta, S., Anderson, K. C., and Munshi, N. C. (2004) In vitro and in vivo activity of the maytansinoid immunoconjugate huN901-N2′-deacetyl-N2′-(3-mercapto-1-oxopropyl)-maytansine against CD56+ multiple myeloma cells. *Cancer Res. 64*, 4629–4636.

(37) Jeffrey, S. C., Nguyen, M. T., Moser, R. F., Meyer, D. L., Miyamoto, J. B., and Senter, P. D. (2007) Minor groove binder antibody conjugates employing a water soluble beta-glucuronide linker. *Bioorg. Med. Chem. Lett. 17*, 2278–2280.

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