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ABSTRACT

Camptothecins exist in a pH dependent equilibrium between the active, closed lactone and the inactive open carboxylate forms. Several previous reports underscore the need for lactone stabilization in generating improved camptothecins, and indeed such designs have been incorporated into antibody-drug conjugates containing this drug. Here, we demonstrate that lactone stabilization is not necessary for camptothecin-based ADC efficacy. We synthesized and evaluated camptothecin SN-38 drug linkers that differed with respect to lactone stability, and released SN-38 or the hydrolyzed open-lactone form upon cleavage from the antibody carrier. An α-hydroxy lactone linked SN-38 drug linker preserved the closed lactone ring structure, while the phenol-linked version allowed conversion between the closed lactone and open carboxylate structures. The in vitro cytotoxicity, pharmacokinetic properties, and in vivo efficacy in L540cy Hodgkin's lymphoma model of the corresponding ADCs were found to be indistinguishable, leading us to conclude that camptothecin-based antibody-drug conjugates possess pronounced activity regardless of the lactone state of the bound drug. This is most likely due to ADC processing within acidic intracellular vesicles, delivering camptothecin in its active closed lactone form.

KEYWORDS: camptothecin, SN-38, antibody-drug conjugates, lactone, stability, drug delivery

INTRODUCTION

Camptothecin is a naturally occurring cytotoxic alkaloid derived from the plant Camptotheca acuminate that exhibits potent antitumor activity against a broad spectrum of tumors.[1-3] Drugs within this family exert their activities through the inhibition of topoisomerase I, an enzyme that catalyzes the relaxation of torsional strain in DNA. The potency and validation of its cytotoxic mechanism prompted substantial efforts for developing suitable camptothecin analogues for use in the clinic. These research efforts have led to three approved camptothecin analogues that are currently used in cancer chemotherapy, belotecan, irinotecan, and topotecan, which are used in the treatment of small cell lung, ovarian, and colorectal cancers.[4-6] Many more camptothecin analogues are in clinical development.

Early research studies have established that the α-hydroxy-δ-lactone ring moiety is critical for inhibition of topoisomerase I and its resulting antitumor activity.[7,8] Camptothecins exist in a pH-dependent reversible equilibrium between closed lactone, present in the parent molecule, and the hydrolyzed open carboxylate. At higher pH, the equilibrium of the lactone ring is shifted towards the less active carboxylate form, as the water-soluble open carboxylate sodium salt form was demonstrated to be 10-fold less potent in vivo compared to its active closed lactone counterpart.[9,10] Other issues that result from lactone ring instability include short half-life and severe drug toxicity.[11,12] Therefore, to address these issues, many camptothecin derivatives have been synthesized with a focus on enhancing stability of the lactone ring. Acyl derivatives at the α-hydroxy lactone group greatly stabilize the lactone ring against hydrolysis,[13] and derivatives synthesized from esterification at this position have been reported not only to enhance lactone stability, but to reduce toxicities and improve in vivo activities.[14,15]

Macromolecular conjugates of camptothecins have also incorporated design features to maximize lactone stability. Polymeric camptothecin constructs include poly(ethylene glycol) (PEG), poly[N-(2-hydroxypropyl)methacrylamide] (pHPMA), and poly-L-glutamate conjugates have been reported.[16-18] Antibody-drug conjugates have also been prepared linking camptothecins through the α-hydroxy lactone group. Early work done by Firestone et al. described the synthesis of immunoconjugates of camptothecin to the BR96 antibody through a labile carbonate linker.[19] More recently, an anti-Trop2 ADC was developed by Goldenberg et al., in which the camptothecin, SN-38, was linked through the α-hydroxy lactone position to stabilize the lactone ring in order to maintain drug efficacy.[20] This promising ADC, sacituzumab govitecan (IMMU-132), has been granted Breakthrough Therapy Designation from the U.S. FDA for the treatment of patients with triple-negative breast cancer.[21,22]

Based on the mechanism involved in the targeting and delivery of the payload in ADCs, we hypothesized that in the context of camptothecin ADCs, stabilization of the lactone ring in the closed structure may not be necessary to maintain efficacy. Binding of the ADC to the target receptor antigen typically triggers internalization of the ADC by receptor-mediated endocytosis. Intracellular trafficking of the ADCs results in accumulation in the lysosome, which is where delivery of the cytotoxic payload occurs by antibody degradation or by linker cleavage.[23-25] The acidic environment in this cellular compartment should drive the equilibrium of the lactone state towards the active closed form, independent of whether the lactone was open or closed before lysosomal delivery.

Here, we report a set of camptothecin drug linkers with differing lactone stabilities, and investigate how the molecular designs impact drug delivery within cells and ADC efficacy in vitro and in vivo. The drug linkers were synthesized in such a way to allow identical active

agents to be released, that differed in their intrinsic stabilities within the lactone structures. Our

results show that lactone stabilization is not required to maintain camptothecin ADC activity. A

rationale for these findings is presented.

EXPERIMENTAL SECTION

Materials and Methods

All commercially available anhydrous solvents were used without further purification. All chemicals and reagents were purchased from commercial suppliers (Sigma-Aldrich and Fisher) and used as received unless indicated otherwise.

Analytical techniques

Flash column chromatography was performed on a Biotage Isolera One flash purification system (Charlotte, NC). Analytical UPLC-MS was performed on a Waters SQ mass detector interfaced to an Acquity Ultra Performance LC equipped with an Acquity UPLC BEH C18 2.1 × 50 mm, 1.7 μm reversed-phase column. The acidic mobile phase (0.1% formic acid) consisted of a gradient of 3% acetonitrile/97% water to 100% acetonitrile (flow rate = 0.5 mL/min). Preparative HPLC was carried out on a Waters 2454 Binary Gradient Module solvent delivery system configured with a Waters 2998 PDA detector. Products were purified over a C12 Phenomenex Synergi 50.0 × 250 mm, 10 μm, 80 Å reverse phase column eluting with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The purification methods generally consisted of linear gradients of solvent A to solvent B, ramping from 90% aqueous solvent A to 10% solvent A. The flow rate was 60 mL/min with monitoring at 380 nm. HPLC for lactone state analysis was performed on a Waters 2690 Separations Module configured with a Waters 2998 PDA detector connected to an Acquity UPLC Fluorescence (FLR) Detector equipped with a XTerra C18 4.6 × 100 mm, 3.5 μm reversed-phase column. For SN-38 containing molecules, excitation at 360 nm and emission from 530 to 540 nm were used.

The mobile phase consisted of 1% v/v triethylamine in water adjusted to pH 5.5 with acetic acid (solvent A) and acetonitrile (solvent B). The method was a linear gradient starting from 80% solvent A to 3% solvent A. The flow rate was 1 mL/min. NMR spectra were obtained on a Bruker Ascend Aeon 400 MHz spectrometer (see Supporting Information).

(2S,3R,4S,5S,6S)-2-(2-(2-(((((9H-fluoren-9-yl)methoxy)carbonyl)(methyl)amino)acetamido)-4-(((((chloromethyl)(2-(methylsulfonyl)ethyl)carbamoyl)oxy)methyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (4): In an oven dried flask, compound **3** (1235 mg, 1.38 mmol) was dissolved in anhydrous dichloromethane (5 mL), followed by the addition of paraformaldehyde (13.8 mg, 0.46 mmol) and TMSCl (1.0 mL, 7.88 mmol). The solution was stirred at room temperature for 30 min with monitoring by quenching with methanol and observing the formation of the methanol adduct by LC/MS. The reaction was filtered, washed with anhydrous toluene and dichloromethane, and dried *in vacuo* for 3 cycles to yield crude **4**, which was used in the subsequent reaction without further purification. MS (*m/z*) calculated for the methanol adduct 942.29 (M+H)$^+$, found 942.28.

(2S,3R,4S,5S,6S)-2-(2-(2-(((((9H-fluoren-9-yl)methoxy)carbonyl)(methyl)amino)acetamido)-4-((((((((S)-4,11-diethyl-4-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-9-yl)oxy)methyl)(2-(methylsulfonyl)ethyl)carbamoyl)oxy)methyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (6): An oven dried flask was charged with SN-38 **5** (90 mg, 0.23 mmol). Compound **4** was dissolved in anhydrous

dichloromethane (6 mL) and DIPEA (359 µL, 2.06 mmol), and added to the flask containing SN-38. The reaction was capped and stirred at 40 °C for 18 h. The reaction mixture was purified over silica *via* Biotage flash column chromatography (CH_2Cl_2/MeOH, 0-10%) to provide **6** (94.6% yield). t_R = 1.51 min. MS (*m/z*) calculated 1302.40 (M+H)$^+$, found 1302.36.

(2S,3S,4S,5R,6S)-6-(4-(((((((S)-4,11-diethyl-4-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-9-yl)oxy)methyl)(2-(methylsulfonyl)ethyl)carbamoyl)oxy)methyl)-2-(2-(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-methylpropanamido)acetamido)phenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (1): Compound **6** (282.6 mg, 0.22 mmol) was dissolved in THF and MeOH and cooled to 0 °C in an ice bath. LiOH (91.1 mg, 2.17 mmol) was dissolved in H_2O and added dropwise. The reaction was stirred at room temperature and was complete within 45 min. The reaction was neutralized with acetic acid, concentrated, and directly purified by preparative HPLC to afford the deprotected intermediate (80% yield). DMF was added followed by DIPEA and 3-maleimidopropinoic acid N-hydroxysuccimide ester **7**. The reaction was stirred for 3 h until completion as monitored by LC/MS. The reaction mixture was neutralized with acetic acid and directly purified by preparative HPLC to provide **1** (35% yield). t_R = 1.40 min. MS (*m/z*) calculated 1091.31 (M+H)$^+$, found 1091.47.

(S)-4,11-diethyl-4-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-9-yl acetate (8): SN-38 **5** (76.0 mg, 0.19 mmol) was dissolved in dichloromethane, followed by addition of triethylamine (128 µL, 0.92 mmol) and DMAP (2.60 mg, 0.02 mmol). Mixture was cooled to 0 °C in an ice bath, followed by

dropwise addition of acetyl chloride (15.9 µL, 0.22 mmol). The reaction mixture was stirred at room temperature for 16 h. The reaction was diluted with dichloromethane, washed with saturated NH_4Cl, water, and brine. The organic phase was then dried over $MgSO_4$, filtered, concentrated and purified over silica *via* Biotage flash column chromatography (CH_2Cl_2/MeOH 0-15%) to provide acetylated SN-38 **8** (34% yield). t_R = 1.17 min. MS (*m/z*) calculated 435.15 (M+H)$^+$, found 435.07.

(2S,3R,4S,5S,6S)-2-(2-(2-(((((9H-fluoren-9-yl)methoxy)carbonyl)(methyl)amino)acetamido)-4-(((((((S)-9-acetoxy-4,11-diethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)methyl(2-(methylsulfonyl)ethyl)carbamoyl)oxy)methyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (9): In an oven dried flask, compound **3** (1116 mg, 1.24 mmol) was dissolved in anhydrous dichloromethane (5 mL), followed by the addition of paraformaldehyde (12.4 mg, 0.41 mmol) and TMSBr (300 µL, 1.68 mmol). The solution was stirred at room temperature for 10 min with monitoring by quenching with methanol and observing the formation of the methanol adduct by LC/MS. The reaction was filtered, washed with anhydrous toluene and dichloromethane, and dried *in vacuo* for 3 cycles to yield the crude product, which was used in the subsequent reaction without further purification. The brominated compound was redissolved in anhydrous dichloromethane and added to **8** (90.0 mg, 0.21 mmol), followed by 1,2,2,6,6-pentamethylpiperidine (390.2 µL, 1.86 mmol). The reaction was capped and stirred at 40 °C for 18 h. The reaction mixture was purified over silica *via* Biotage flash column chromatography (CH_2Cl_2/MeOH, 0-10%) to provide **9** (99% yield). t_R = 1.68 min. MS (*m/z*) calculated 1344.41 (M+H)$^+$, found 1344.46.

(2S,3S,4S,5R,6S)-6-(4-(((((((S)-4,11-diethyl-9-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)methyl)(2-(methylsulfonyl)ethyl)carbamoyl)oxy)methyl)-2-(2-(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-methylpropanamido)acetamido)phenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (2): Compound **9** (290.0 mg, 0.22 mmol) was dissolved in THF and MeOH and cooled to 0 °C in an ice bath. LiOH (90.5 mg, 2.16 mmol) was dissolved in H_2O and added dropwise. The reaction was stirred at room temperature and was complete within 45 min. The reaction was neutralized with acetic acid, concentrated, and directly purified by preparative HPLC to yield the deprotected intermediate. DMF was added followed by DIPEA and 3-maleimidopropinoic acid N-hydroxysuccimide ester. The reaction was stirred for 3 h until completion as monitored by LC/MS. The reaction mixture was neutralized with acetic acid and directly purified by preparative HPLC to provide **2** (25% yield). t_R = 1.42 min. MS (*m/z*) calculated 1091.31 (M+H)$^+$, found 1091.31.

Antibody conjugations

Anti-CD30 and anti-CD71 antibodies, cAC10 and cOKT9, respectively, were used. ADCs were prepared by reduction of antibody interchain disulfides as previously described.[26] Briefly, full reduction of the four interchain disulfides was accomplished by addition of 20 equivalents of tris(2-carboxyethyl)-phosphine (TCEP) to an antibody solution (1-10 mg/mL in PBS pH 7.4). The extent of antibody reduction was monitored by reverse-phase chromatography and additional TCEP was added as needed to complete the reaction. Purification of the reduced antibody was achieved by three rounds of ultrafiltration (10-fold dilution into PBS pH 7.4

containing 1 mM EDTA, centrifugation at 4000 × g through a 30 kDa MWCO filter). Reduced

mAbs prepared in propylene glycol/PBS pH 7.4 with EDTA were mixed with 25% excess of the

drug linker as a propylene glycol/DMSO solution to a final propylene glycol concentration of

50%. The resulting solution was left at room temperature for 30 min and conjugation progress

was assessed by reverse-phase chromatography. Additional drug linker was added as needed.

The mixture was purified to remove excess free drug by the addition of charcoal (1 mg of

charcoal per 1 mg of ADC) for 30 min, which was then filtered. The solution was buffer

exchanged using a PD-10 desalting column into 5% trehalose in PBS buffer. The resulting ADCs

were sterile-filtered and stored at -80 °C until use. Final ADC concentration was determined by

UV-Vis with a correction factor based on 280nm/360nm absorbance ratio to account for the

additional 280nm absorbance from the drug.

Lactone analysis

SN-38 pH stability

A 1 mM DMSO stock solution of SN-38 was diluted 50-fold into PBS at varying pHs at 37 °C.

At specific time points, an aliquot of the solution was diluted 100-fold into PBS pH 7.4 at a final

concentration of 200 nM and immediately analyzed by HPLC. Detection of the lactone status

and the ratio of the closed lactone and open carboxylate forms was determined by retention times

and area under the curve based from SN-38 standards. Concentrations of closed lactone or open

carboxylate forms were calculated by fitting to a standard curve of known concentrations of

100% closed lactone or 100% carboxylate samples.

SN-38 drug linker pH stability

The SN-38 drug linkers were fully reacted with N-acetylcysteine (NAC) and evaluated by LC/MS to ensure reaction has gone to completion. The reaction was then purified by preparatory HPLC. The NAC drug linker adduct was prepared as a 10 mM DMSO stock solution. The samples were diluted 50-fold into PBS pH 3.0 or pH 10.0 and equilibrated at room temperature for 1 h. The samples were diluted 100-fold into PBS pH 7.4 at a final concentration of 2000 nM for HPLC analysis.

Drug release assays

Release of free drug from enzyme treatment of drug linkers **1** and **2**: The NAC drug linker adduct as a 10 mM DMSO stock was diluted 50-fold into PBS pH 3.0 or pH 10.0 and equilibrated at room temperature for 1 h. The samples were neutralized to pH 7.4 and diluted 10-fold with PBS pH 7.4 containing 120 kU/mL β-glucuronidase. After a 5 min digestion, the samples were diluted 100-fold in PBS pH 7.4 and immediately analyzed by HPLC.

Release of free drug from enzyme treatment of SN-38 ADCs: Conjugates of the non-binding IgG to drug linkers **1** and **2** were prepared with a DAR of 8. IgG-**1** and IgG-**2** were incubated with β-glucuronidase in PBS pH 7.4 at 37 °C and time points were taken over the course of 1 h. At the specified time point, an aliquot from the mixture was precipitated in ice cold methanol and kept at -20 °C for 20 min. The sample was then centrifuged at $16,000 \times g$ for 5 min, the supernatant was collected and immediately injected for HPLC analysis.

Intracellular SN-38 lactone state

L540cy cells were grown in RPMI-1640 media supplemented with 20% FBS. L540cy cells were seeded at a density of 5×10^5 cells/mL for a total of 2.5×10^7 cells and dosed with cOKT9-

1 or cOKT9-2 at a final concentration of 500 ng/mL. After incubation at 37 °C/5% CO_2 for 24 h, the cells were pelleted, washed twice with PBS, and stored at -20 °C until further processing. At the time of analysis, the pellets were thawed, and the free drug was extracted with ice cold methanol at -20 °C for 30 min. The samples were centrifuged at 16,000 × g for 5 min, the supernatant was collected and immediately injected for HPLC analysis.

ADC plasma stability

ADCs were prepared with a drug to antibody ratio (DAR) of 8 on a non-binding h00 IgG antibody. The conjugates were incubated in citrated mouse (BALB/c) plasma at a final concentration of 0.25 mg/mL for 7 days at 37 °C. At each time point, an aliquot of each ADC was removed and kept frozen at -80 °C until further processing. Upon completion of the time course, ADCs were captured on IgSelect resin. After elution, the samples were deglycosylated with PNGase, reduced with DTT, and analyzed by LC/MS.

In vitro cytotoxicity assays

In vitro potency was assessed on multiple cancer cell lines: L540cy and Ramos. Cells cultured in log-phase growth were seeded for 24 h in 96-well plates containing 150 μL RPMI-1640 supplemented with 20% FBS for L540cy cells, and RPMI-1640 with 10% FBS for Ramos cells. Serial dilutions of the compounds in cell culture media were prepared at 4x working concentrations, and 50 μL of each dilution was added to the 96-well plates. Following addition of the test articles, cells were incubated for 4 days at 37 °C, after which growth inhibition was assessed by CellTiter-Glo (Promega). The IC_{50} value, determined in duplicate, is defined as the concentration that achieves half maximal growth inhibition over the course of the titration curve.

ADC pharmacokinetics

Pharmacokinetic experiments were performed using radiolabeled antibody or ADC. To a solution of antibody or ADC in PBS supplemented with an additional 50 mM potassium phosphate (pH 8.0) and 50 mM sodium chloride was added 55 µCi N10 succinimidyl propionate, [propionate-2,3-^{3}H]- (Moravek Biochemicals, Brea, CA, 88 Ci/mmol, 1 mCi/mL, 9:1 hexane:ethyl acetate solution) per mg of antibody or ADC. The resulting mixture was vortexed and left at room temperature for 2 h. The mixture was centrifuged at $1,000 \times g$ for 1 min and the lower aqueous layer was removed and loaded onto equilibrated PD-10 desalting columns (GE Healthcare, Cat. No. #17-0851-01). These columns removed unconjugated radioactivity, and radiolabeled test article was eluted using PBS pH 7.4. Eluate was diluted 5-fold in PBS pH 7.4 and then concentrated in Amicon Ultra-15 Centrifugal Filter Units (Millipore, Cat. No.: UFC903024, 30 kDa MWCO) to achieve final concentration of approximately 1 mg/mL. The resulting products were filtered through sterile 0.22 µm Ultrafree-MC Centrifugal Filter Units (Millipore, Billerica, MA) and the final antibody or ADC concentration was measured spectrophotometrically. The specific activity (µCi/mg) of each product was determined by liquid scintillation counting.

The PK properties of the unconjugated antibody or ADC were examined in female nude mice. In this experiment, the radiolabeled antibody control or ADC were injected via the tail vein at a dose of 1 mg/kg. Each test article was dosed once in replicate animals. Blood was drawn into K_2EDTA tubes via the saphenous vein or by cardiac puncture for terminal bleeds at various time points. Plasma was isolated by centrifugation at $10,000 \times g$ for 10 min. A 10-150 µL sample of plasma from each time point was added to 4 mL Ecoscint-A liquid scintillation cocktail

(National Diagnostics) and the total radioactivity was measured by liquid scintillation counting. The resulting disintegrations per minute values were converted to µCi and the specific activity of the radiolabeled test articles was used to calculate the concentration of antibody or ADC remaining in the plasma at each time point.

In vivo xenograft study

All experiments were conducted in concordance with the Institutional Animal Care and Use Committee in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The efficacy experiment was conducted using a L540cy xenograft model. L540cy cells were implanted subcutaneously into severe combined immunodeficiency (SCID) mice (Harlan, Indianapolis, IN). Upon tumor engraftment, mice were randomized to study groups (n = 6) with each group averaging around 100 mm^3 tumor volume. The test articles were dosed intraperitoneal according to a q4dx3 schedule. Tumor size was monitored at least twice weekly and tumor volume as a function of time was determined using the formula (L × W^2)/2.

RESULTS

Design of SN-38 drug linkers and synthesis

Glucuronide methylene alkoxy carbamate (MAC) linked SN-38 drug linkers were synthesized through linkages at the two different alcohols on SN-38 (Figure 1). SN-38 was chosen as the target drug because identical drug linkers could be synthesized differing only by the site of linker attachment. It was expected based on what has previously been reported for camptothecin derivatives that the two different sites would differ dramatically in maintaining lactone stability, with the α-hydroxy lactone linked SN-38 being resistant to hydrolysis[13] and the corresponding phenolic hydroxyl linked SN-38 having a large proportion of drug in the hydrolyzed state.

The MAC glucuronide SN-38 drug linkers were prepared as shown in **Scheme 1** and **Scheme 2**. The reactivities of the two alcohol moieties were exploited for selective attachment of the MAC linker at the different positions. The steric hindrance of the α-hydroxy lactone makes it far less reactive, and thus does not require protection when preparing the phenol-linked drug linker. The MAC glucuronide linker intermediate used was prepared from previously described work.[27] Synthesis of the phenol-linked SN-38 drug linker started with conversion of the protected glucuronide MAC compound **3** to the chloromethyl adduct **4** through reaction with chlorotrimethylsilane and paraformaldehyde. Reaction with SN-38 provided the phenol-linked SN-38 intermediate **6**. Saponification and simultaneous Fmoc-deprotection, followed by maleimide incorporation using the N-hydroxysuccinimide ester of maleimidopropionic acid 7, yielded the phenol-linked SN-38 drug linker **1**. Synthesis of the tertiary alcohol linked SN-38 drug linker commenced by acetylation of the phenolic hydroxyl group using acetyl chloride to provide the acetylated SN-38 **8**. The protected SN-38 was coupled to the bromomethyl adduct of

the MAC glucuronide intermediate **3** to provide the α-hydroxy lactone linked acetylated SN-38 compound **9**. Saponification and Fmoc-deprotection, followed by maleimide incorporation yielded the α-hydroxy lactone linked SN-38 drug linker **2**.

Lactone state analysis

An HPLC-based method utilizing fluorescence detection for camptothecins was adapted to analyze the state of the lactone.[28] Briefly, simultaneous separation and detection of the closed lactone and open carboxylate form was achieved using a mobile phase consisting of a triethylamine acetate buffer and acetonitrile. Initial validation of the method and the influence of pH on the lactone state was evaluated with the free drug SN-38. SN-38 was incubated in PBS at varying pH values and the ratio of closed lactone to open carboxylate forms was analyzed over time (**Figure 2**). As expected, the closed lactone is preferred in acidic conditions, while the open carboxylate form is dominant at higher pH.[29,30]

Linker attachment site on SN-38 and its effect on lactone stability was characterized by determining the lactone state of the SN-38 drug linkers **1** and **2**. The drug linkers were reacted with N-acetylcysteine (NAC) and the resulting adducts were then equilibrated at different pH values to observe any changes in the lactone states. Retention time shifts were observed depending on the pH for the phenol-linked SN-38 drug linker, indicating that the lactone can convert between the open and closed forms (Figure 3a). Stabilization against lactone ring opening was observed with the α-hydroxy lactone linked drug linker (Figure 3b).

The lactone state of the released free drug from NAC-**1** and NAC-**2** was evaluated to further support the difference in lactone stability between the two drug linkers. The NAC drug linker adducts were first equilibrated to the closed lactone or open carboxylate form, and then treated

with β-glucuronidase to release the free drug. For NAC-**1**, the open carboxylate drug linker (Figure 3a, green chromatogram) released the corresponding open carboxylate SN-38 form, and the closed lactone drug linker (Figure 3a, red chromatogram) released the corresponding closed lactone SN-38 form (Figure 3c). With the α-hydroxy lactone linked NAC-**2**, even when equilibrated in either pH 3.0 or pH 10.0 conditions, the closed lactone is the only form released (Figure 3d). This set of data indicates that the lactone state of the released free drug is representative of the lactone state on the drug linker.

Next, similar free drug release experiments were performed on non-binding h00 IgG antibody conjugates of drug linkers **1** and **2**. The resulting ADCs were treated with β-glucuronidase and the lactone state of the released drug was tracked over the course of only 1 hour, as longer incubation times influence the equilibrium of the lactone and carboxylate forms. Partial free drug release from the ADCs was observed, with about 10% of the payload released after 1 hour. The free drug released from both IgG-**1** and IgG-**2** was primarily in the closed lactone form (>90%). The ratio of the closed lactone to open carboxylate form remained constant as additional SN-38 was released from the ADC over a 1 hour time course (Figure 4a). This indicates that the released camptothecin drug from both ADCs is almost exclusively in the closed lactone form.

In order to better characterize the state of the lactone and more effectively show relevance to its efficacy, the SN-38 ADCs were analyzed in an in vitro cell-based assay. L540cy cells were treated with cOKT9-**1** or cOKT9-**2** for 24 hours and the intracellular free drug SN-38 was extracted for analysis. The majority of intracellular SN-38 was in the closed lactone form with 97.1% and 96.1% for cOKT9-**1** and cOKT9-**2**, respectively (Figure 4b). Regardless of attachment site on SN-38, the released free drug equilibrates into the closed lactone form. The released SN-38 being primarily in the closed lactone form is consistent with the mechanism of

ADC internalization, where digestion and release of the payload occurs in the lysosome and its acidic environment drives camptothecins toward the active closed lactone version of the drug.

ADC stability

The plasma stability from non-binding h00 IgG antibody conjugates of drug linkers **1** and **2** was assessed. IgG-**1** and IgG-**2** (DAR 8) were incubated in citrated mouse plasma at 37 °C for a period of 7 days. Assessment of drug loss was monitored by LC/MS. Apart from the initial drug loss, presumably due to fragmentation of the maleimide thioether adduct,[31] both conjugates and the MAC linkage at the different alcohol positions exhibited stability against SN-38 release from the drug linker in mouse plasma (Figure 5).

In vitro cytotoxicity

The cytotoxic activity of the SN-38 ADCs were evaluated in a panel of cell lines including L540cy and Ramos (Figure 6). SN-38 as the free drug is cytotoxic across all the cell lines tested with IC_{50} values in the 1-5 nM range. Consistent with this was the finding that the cOKT9 conjugates of drug linkers **1** and **2** displayed IC_{50} values in L540cy cells of 113 and 99 ng/mL, respectively, and 67 and 105 ng/mL, respectively, in Ramos cells. With DARs of 8, as used in these studies, 100 ng/mL mAb contains 5 nM drug. Conjugates of **1** and **2** displayed immunological specificity as the nonbinding IgG-**1** and IgG-**2** were inactive on these cell lines. Importantly, no significant difference in cytotoxic activity was observed between the two conjugates, and any benefit of lactone stabilization through construct design was not apparent.

In vivo biological evaluation

The pharmacokinetic properties of the SN-38 ADCs were determined using the non-binding IgG conjugates of **1** and **2** with a DAR of 8. Nude mice were administered a single intravenous dose of 1 mg/kg and the circulating antibody was monitored over time (Figure 7). Both IgG-**1** and IgG-**2** had similar clearance profiles. Since the different SN-38 linker attachment sites has no effect on its clearance profile, we then investigated any resulting effects in efficacy solely due to stabilizing the closed lactone ring structure.

The in vivo activities of the two SN-38 ADCs evaluated in L540cy Hodgkin lymphoma xenograft model are shown in Figure 8. Conjugates of cAC10 and the non-binding IgG antibody to drug linkers **1** and **2** were prepared with an average DAR of 4. SCID mice bearing L540cy tumors were treated every 4 days for a total of 3 times with the ADCs once the average tumor volume reached 100 mm^3. Both cAC10-**1** and cAC10-**2** dosed at 10 and 30 mg/kg induced tumor growth delay out to 49 days after the initial dose. In contrast, the non-binding control ADCs dosed at 30 mg/kg were both inactive and did not slow the growth rate, indicating a high degree of immunological specificity. More significantly, no substantial differences in potency were observed between the SN-38 ADCs, suggesting that stabilization of the closed lactone form is not a requirement in the context of camptothecin ADCs. Regardless of how SN-38 was linked and conjugated to the antibody, internalization and release of the payload resulted in delivery of the closed lactone form of SN-38.

DISCUSSION

Stabilizing the closed lactone ring structure of camptothecins has been demonstrated to be a necessary feature for its antitumor activity. This acceptance has figured prominently in the areas of camptothecin ADCs and polymeric constructs. However, given the mechanism of how ADCs traffic, we believed that lactone stabilization may not be necessary or even beneficial for internalized camptothecin payloads. Thus, in the context of camptothecin ADCs, we sought to explore the potential role of stabilizing the closed lactone ring structure on its therapeutic efficacy. A set of MAC glucuronide SN-38 drug linkers were synthesized differing by the alcoholic linkage position where the resulting biological differences between the two drug linkers would primarily be due to lactone stability. The phenol-linked SN-38 drug linker allowed the lactone ring to be able to convert between the closed lactone and open carboxylate forms, while the α-hydroxy lactone linked drug linker preserved the closed lactone ring structure. Antibody conjugates prepared from the SN-38 drug linkers were identical in regards to their in vitro and in vivo activities. It should be pointed out that our results were obtained on hematological tumors, which have been shown to be more sensitive to ADCs than solid tumors.[32] Further studies are needed to assess the generality of our findings in other tumor types.

The pharmacokinetic profiles of the phenol-linked and α-hydroxy lactone linked SN-38 ADCs were quite similar and clearance was similar to what has previously been described for auristatin-based ADCs. Based on previous reports, systemic clearance rates of the lactone are higher than that of the carboxylate form likely due to the increased binding affinity for plasma proteins to the carboxylate over the lactone form.[33,34] Because of this, we anticipated the possibility that the α-hydroxy lactone linked SN-38 linker, which stabilizes the closed lactone form, might exhibit a

shorter circulation half-life. However, similar pharmacokinetic profiles between the two ADCs, demonstrated that the antibody backbone had a larger influence on circulation half-life than the state of the bound drug.

The in vitro and in vivo activities of camptothecin-based ADC differing in lactone stability were indistinguishable. In addition, the amount of active, free drug within cells that were treated with either ADC resulted in the generation of the same amount of free active camptothecin in the closed lactone form. Our results indicate that active camptothecin-based ADCs do not require provisions for enhanced lactone stability.

Supporting Information

NMR spectra of compounds.

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Scheme 1. Synthesis of phenol-linked SN-38 drug linker **1**.

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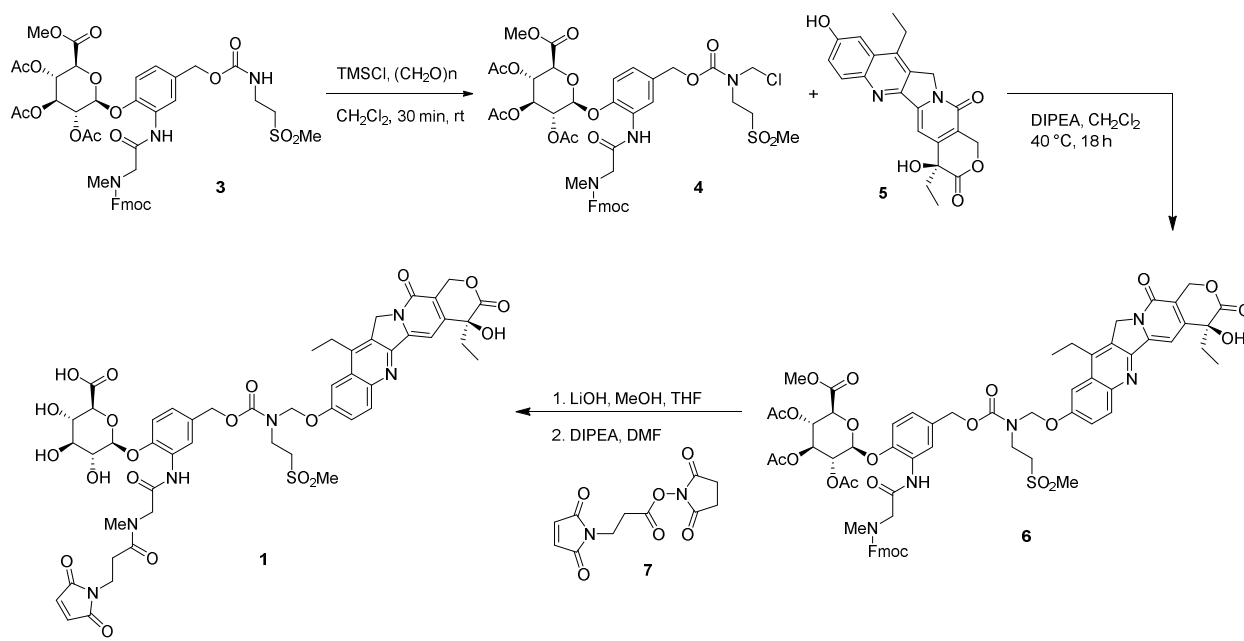
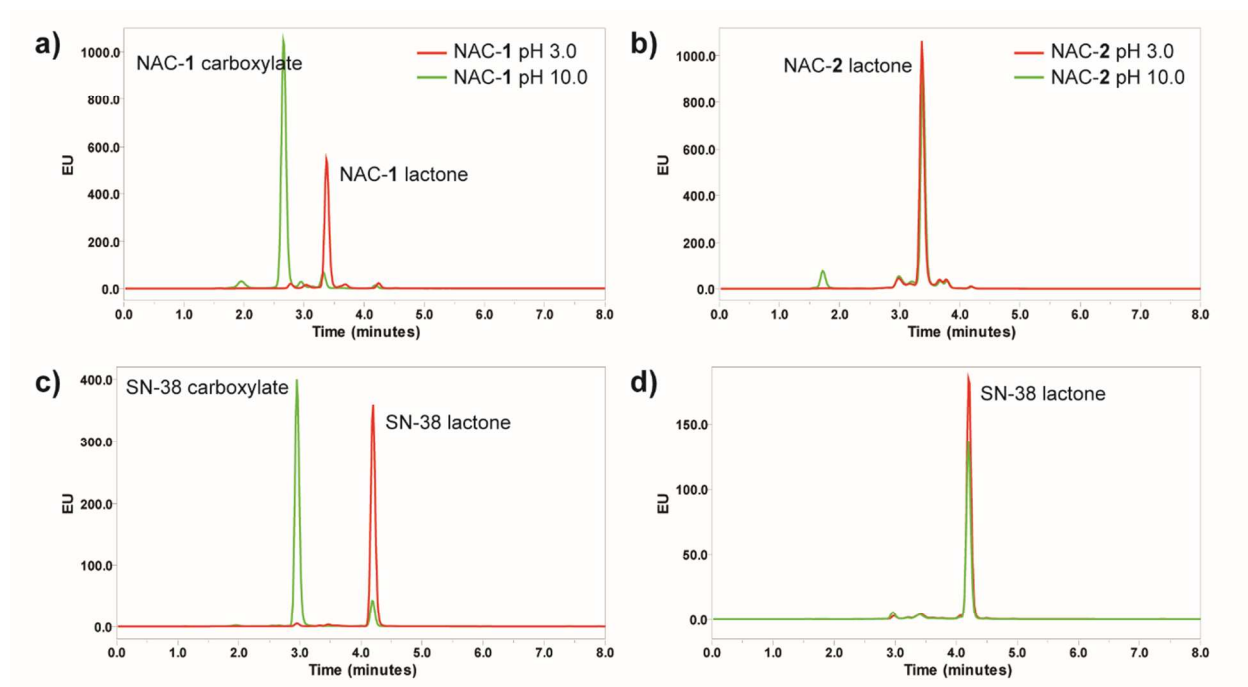**Scheme 2.** Synthesis of α-hydroxy lactone-linked SN-38 drug linker **2**.
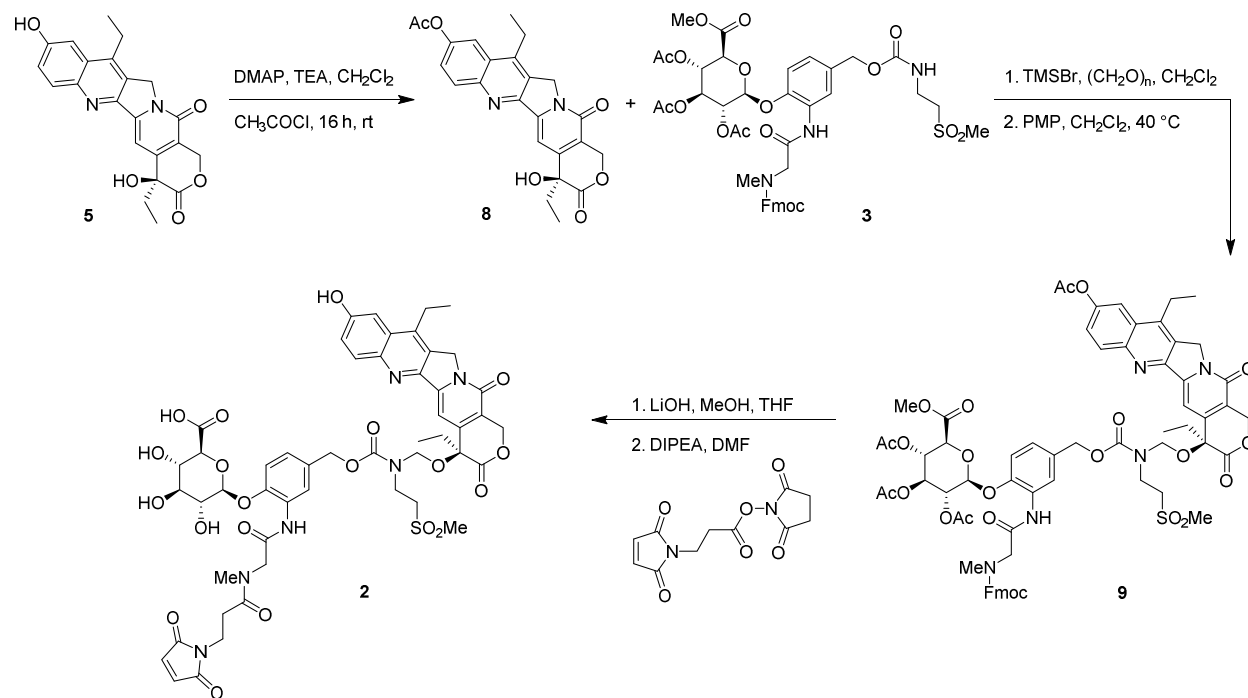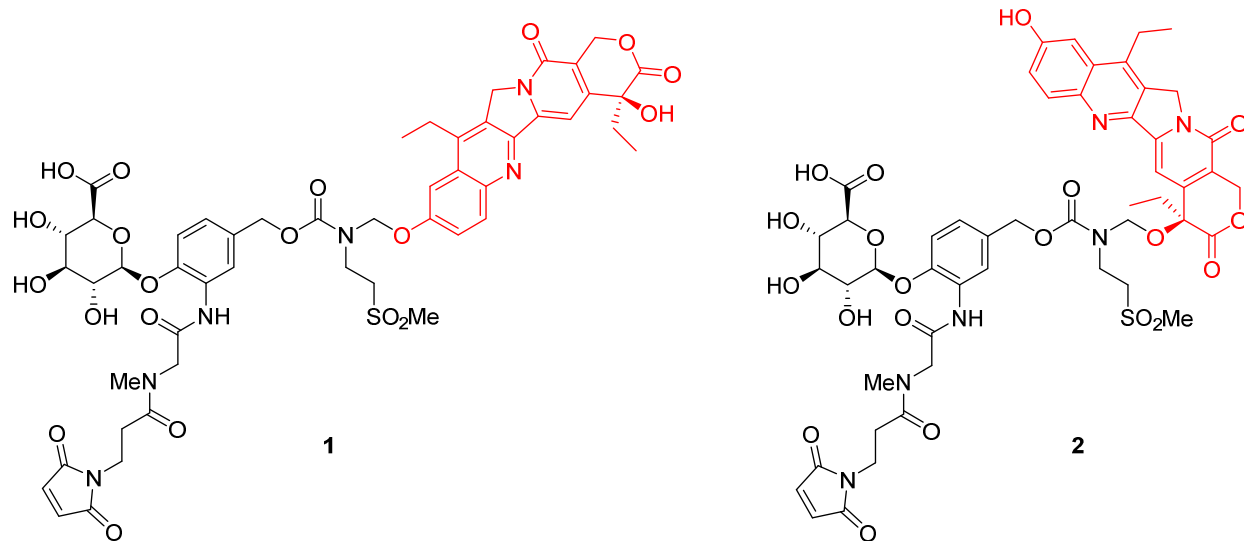
Figure 1. Structures of the SN-38 drug linkers. Phenol-linked **1** and α-hydroxy linked lactone **2**, with SN-38 drug depicted as red.
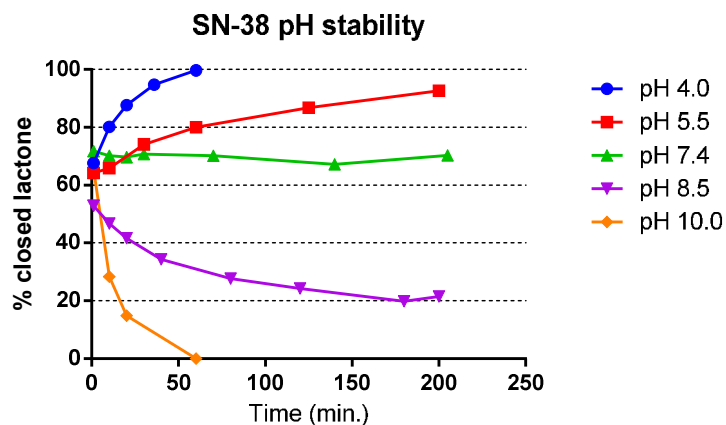
**Figure 2.** Equilibrium of open carboxylate and closed lactone forms of SN-38 as a function of pH.

Figure 3. HPLC chromatograms of lactone states; a) N-acetylcysteine-**1** (NAC-**1**) drug linker in pH 3.0 (red) and pH 10.0 (green) buffers, and b) NAC-**2** drug linker in pH 3.0 (red) and pH 10.0 (green) buffers. Determination of the lactone state of released SN-38 after β-glucuronidase digestion of: c) NAC-**1** drug linker when equilibrated in pH 3.0 (red) and pH 10.0 (green) buffers, and d) NAC-**2** drug linker when equilibrated in pH 3.0 (red) and pH 10.0 (green) buffers.
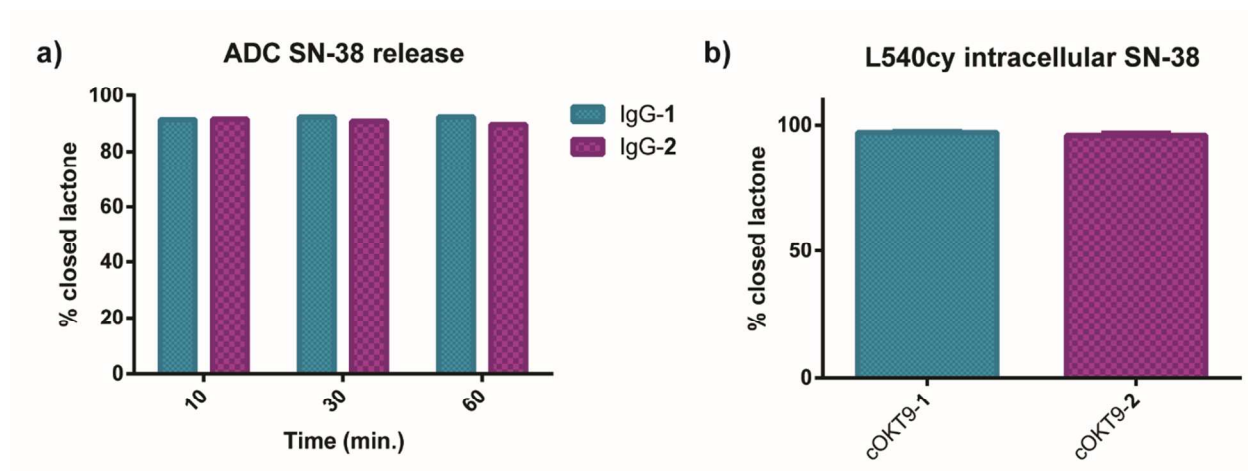
Figure 4. Lactone analysis of released free drug from SN-38 ADCs; a) released SN-38 from β-glucuronidase treatment of non-binding IgG antibody conjugates of drug linkers **1** and **2**, and b) intracellular SN-38 from SN-38 ADC treatment of L540cy cells.
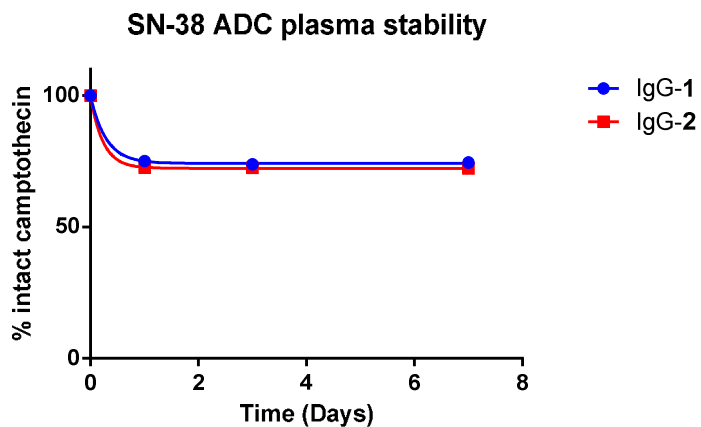
Figure 5. Plasma stability of SN-38 ADCs, IgG-**1** and IgG-**2**. Measurement of the amount of drug loss from the ADC when incubated in citrated mouse (BALB/c) plasma over the course of 7 days.
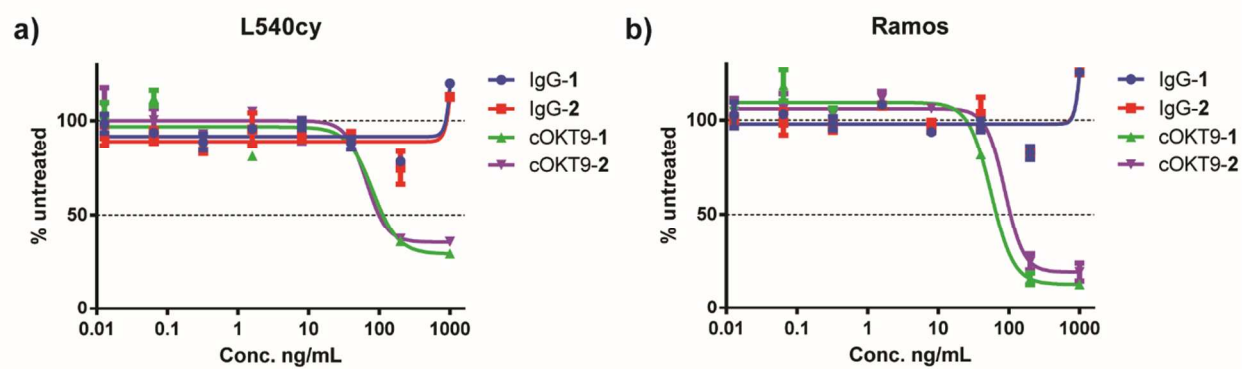
Figure 6. In vitro cytotoxicity of SN-38 ADCs on L540cy Hodgkin's lymphoma (left) and
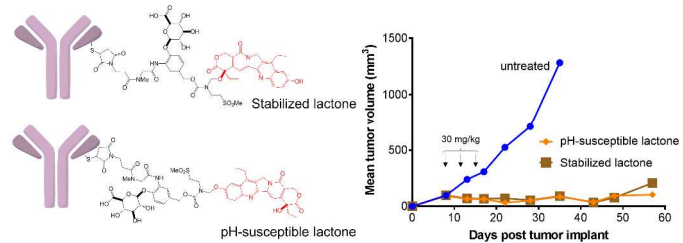
Ramos Burkitt's lymphoma (right) cell lines.
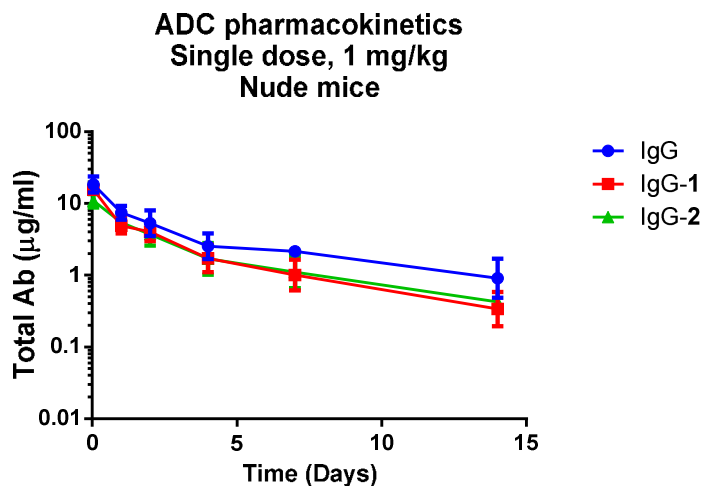
Figure 7. Pharmacokinetic profiles of SN-38 ADCs assessed in female nude mice following a single 1 mg/kg dose of IgG antibody, IgG-**1** or IgG-**2** conjugate.
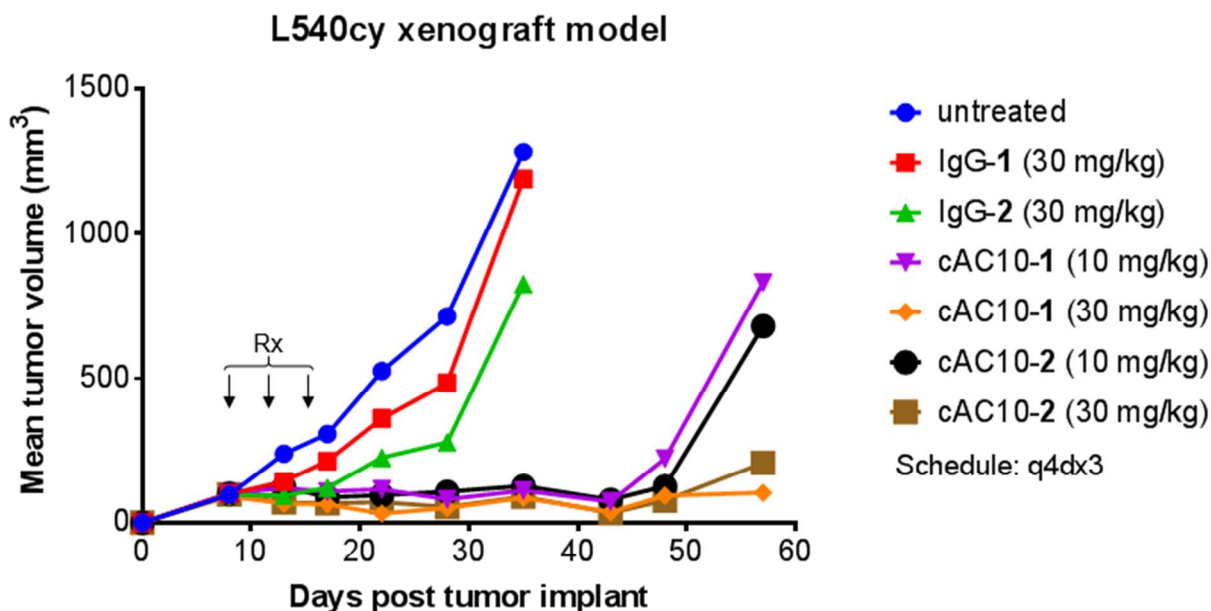
Figure 8. In vivo efficacy of SN-38 ADCs in L540cy xenograft model. SCID mice were implanted with L540cy cells, and treated when the tumor volume reached 100 mm^3 (n = 6 per group). Animals were dosed ip according to a q4dx3 schedule with non-binding IgG-**1** or IgG-**2** at 30 mg/kg or cAC10-**1** or cAC10-**2** at 10 mg/kg and 30 mg/kg.

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