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Research article

Generation of anti-SN38 antibody for loading efficacy and therapeutic monitoring of SN38-containing therapeutics

Tahereh Zarnoosheh Farahani [a,b], Mohammad-Reza Nejadmoghaddam [b,*], Soyar Sari [a], Ramin Ghahremanzadeh [b], Arash Minai-Tehrani [b]

[a] Department of Cellular and Molecular Biology, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

[b] Nanobiotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

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ABSTRACT

SN38, one of the most potent anti-tumor analogues of the camptothecins (CPTs), has limitations in its direct formulation as an anticancer agent due to its super toxicity and poor solubility in water and pharmaceutically approved solvents. However, it has garnered significant scientific interest as a payload in conjugated nanomedicine platforms (e.g., SN-38lip, NK012, SNB-101, and ADCs) to enhance their effectiveness and safety. The development of these platforms necessitates a convenient quantitative determination of SN38 in preclinical and clinical studies, a need that our study directly addresses, offering a practical solution to a pressing problem in cancer research and drug development. This study details the meticulous process of generating poly and monoclonal antibodies (pAb and mAb) against SN38 and their application to measure the SN38 in naked and conjugated forms of SN38-conjugated ADCs. For this purpose, two haptens of SN38 were synthesized by introducing the glycine or 4-amino-4-oxobutanyol(glycine) moiety as a conjugation functional group of the SN38. IR, NMR and mass spectrometric techniques confirmed the chemical modifications of the haptens. The haptens were then conjugated to each bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) protein. The SN38-KLH conjugates were meticulously examined for immunization and generation of pAb and mAb. The immunization efficiency, reactivity, binding affinity, specificity, and cross-reactivity of purified pAb and mAb against Irinotecan, a model for the emergence of an SN38 derivative in clinical settings, were evaluated using ELISA and western blotting (WB) techniques. Conjugation efficiency of the SN38 to the KLH was increased using 4-amino-4-oxobutanyol(glycine) moiety, as its immunization efficacy was more to generate pAb. Furthermore, only this hapten could immunized mice to generate mAb recognizing SN38 with nanomolar equilibrium affinity. Our recent findings strongly support the notion that the generated pAb employed in developing an ELISA effectively ascertains the presence of SN38 in SN38-conjugated ADC, with a test midpoint EC_{50} of 2.5 μg/mL.

Our study's unique contribution to the field lies in the development of specific antibodies against SN38 for measuring it on ADC, a feat that has not been achieved before. These immunoassays can be readily applied to detect other SN38-conjugate therapeutic platforms, thereby enhancing their clinical knowledge translation. The affinity of both pAb and mAb also meets the acceptance criteria for quantifying SN38 in fluidic material, as well as in Therapeutic drug monitoring (TDM) studies, a crucial aspect of personalized medicine. The potential applications of

* Corresponding author.
E-mail addresses: moghaddam@avicenna.ac.ir, m-nejadmoghaddam@alumnus.tums.ac.ir (M.-R. Nejadmoghaddam).

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the anti-SN38 antibodies extend to reducing SN38-induced systemic toxicity through an inverse targeting strategy, a novel approach that piques further interest in our findings.

1. Introduction

Camptothecin (NSC-100880, CPT, Campathecin, (S)-(+)-Camptothecin) derivatives are the most commonly used antineoplastic agent in terms of both conventional and novel targeted drug delivery systems. After two decade their water-soluble chemically prodrugs with generic names, camptosar (irinotecan), exatecan, lovotecan, camptotecan, topotecan and teniposide., etc. are still the safest and most cost–effective cytotoxic and adjuvant medicines of the FDA essential medicines list for treatment of various solid tumors metastatic colorectal, pancreatic and gastric cancers [1–3]. Owing to the body liver esterase enzyme activity, all the chemically water-soluble prodrugs are metabolized to the SN38 (7-ethyl-10-hydroxyl camptothecin), the efficient form of the camptothecin derivatives, 1000 times more antineoplastic activity [4].

Despite SN38's potent antineoplastic activity, its direct use as an anticancer therapeutic agent is challenging due to its Dose-limiting toxicity (DLT), poor solubility, and stability. To eliminate the limitations of the direct use of SN38, strategies are more attentive to using it as a payload in designing novel conjugate-based drug delivery systems with lower side effects, particularly those well suited for boosting cancer therapy efficacy [5]. Based on this, there are several nanomedicine designs [6,7] including but not limited to: SN-38lip [8] and LE-SN38 [9], two liposome encapsulated nanomedicine. NK012 [10,11] and SNB-101 [12] tow nano-formulation of SN38 based on core-shell polymeric micelle and EZN-2208, a polyethylene glycol drug conjugate of SN38 [13], all are in phase 2 clinical trials and commonly aimed at treatment choices for advanced cancers. In the same vein, the high potency of SN38 makes it also a great payload candidate for targeted cancer therapy with a rationally designed new generation of antibody-drug conjugation (ADCs) which currently are one of the fastest growing classes of oncology therapeutic [14,15]. On April 22, 2020 [16], and 13, 2021 [17] accelerated approval was granted to sacituzumab govitecan-hziy (under the trade name of Trodelvy made by Immunomedics, Inc.) as an ADC made up of an anti–Trop-2 antibody linked to the drug SN-38 [18]. This accelerated approval was based on successful clinical trial studies on effective results of the trodelvy respectively, for the treatment of metastatic triple-negative breast cancer (TNBC) [19] and locally advanced/metastatic urothelial cancer (mUC) [20]. Trodelv also enacted fast-track status for treating other diverse epithelial cancers, including metastatic non-small cell lung cancer (NSCLC) [21], small-cell lung cancer (SCLC) [22], pancreatic, prostate, brain metastases, and glioblastoma cancers [23–26]. In addition to the aforementioned FDA-approved ADC payload with SN38, there are also two other similar ADCs in phase 1 and 2 clinical trials, Labetuzumab Govitecan [27] and PEN-866 [28,29] which have been made up respectively using anti-CEACAM5 and anti HSP90 antibody linked to the drug SN38, and one in preclinical study phase, Epratuzumab–SN-38 conjugate (either anti-CD22 or anti-CD20 antibody linked to the SN38) [30]. We also recently started working on a novel ADC based on anti-PLAC1 antibody conjugated to the SN38 (Anti-PLAC1-ADC) for targeting prostate cancer cells in advanced steps [31]. These examples of early clinical success and enhanced development of the unprecedented number of clinical trials initiated every year over the past decade (Fig. 1) [32] are based on availability, affordability and FDA approval efficacy of the SN38 [31,33].

However, rapid and easy quantitative determination of SN38 during the early stages of SN38-based drug development, such as drug loading, efficient release maintenance, and therapeutic drug monitoring (TDM) in clinical trial phases, still need to be improved. Moreover, in line with the conception of personalized medicine, distinct toxicity risks of the SN38 posed by genetic polymorphism in the UGT1A1 gene [34,35] also highlight the necessity of SN38-level monitoring. The UGT1A encodes UDP-glucuronosyltransferases, converting the toxic form of SN38 to its nontoxic form, makes it able to be clearance from the body.

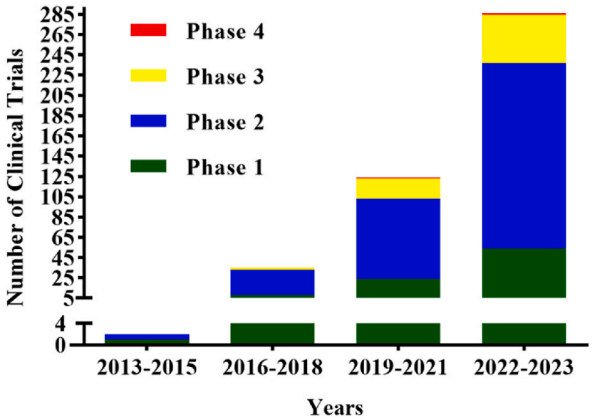


Fig. 1. Active, Recruiting, terminated, or completed clinical trials of SN38-based therapeutics over the past decade based on the last update: The number of clinical trials initiated every three years has steadily increased. Although a limited number of trials progress to phases 3 and 4, a marked increase is noticed in the phase 2 trial.

The existing methods for SN38 determination in lab and company levels and also patient samples are mainly high-pressure liquid chromatography (HPLC) coupled with Ultraviolet (UV) [36], fluorescence [4,11,33] or mass spectrometric (LC-MS/MS) [37–39] detectors. Although these methods have high accuracy and sensitivity, they are high-cost, time-consuming, and labor intensive, have limited throughput and require a tedious extraction and concentration process of blood and tissue by solvent extraction. Moreover, the need for equipment is costly and needs to be amenable to routine clinical diagnostic laboratories. Therefore, it is necessary to employ alternative techniques while providing adequate selectivity and sensitivity that are simpler, low cost, and capable of

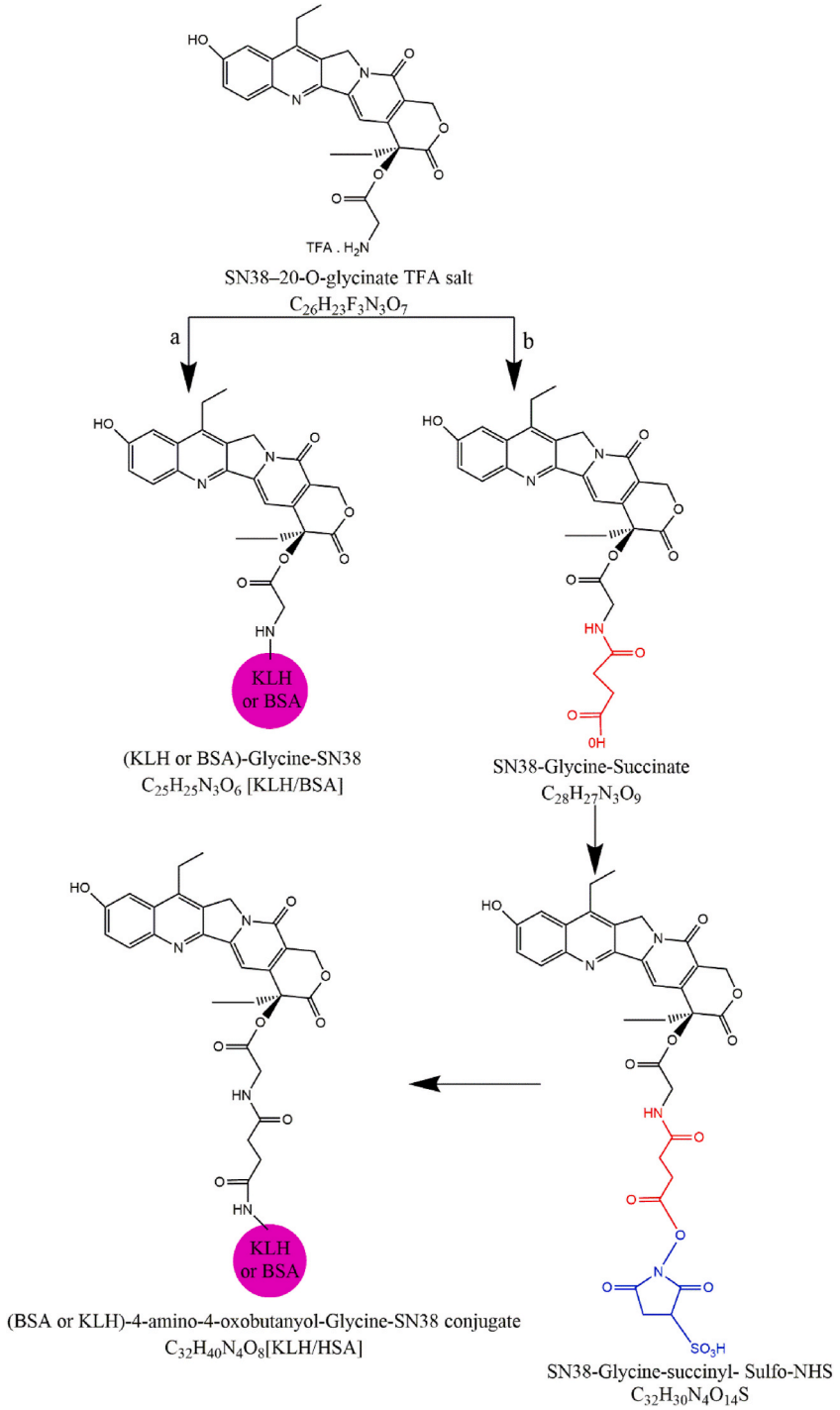


Fig. 2. SN38-BSA/KLH synthesis scheme. The process was divided into two steps: phenolic OH protection of SN38 (a) and adding five carbon atom spacers (b).

higher-throughput assay. some pH-mediated spectroscopies were introduced as uncomplicated, rapid and low-cost methods [40,41]; however, such methods fail to distinguish free SN38 from conjugated one to the carrier. In this regard, Immunoassays (e.g., ELISA) have been reported to be a better alternative in the clinical analysis field [42]. A variety of immunoassays have been established for the evaluation of TDM for different drugs [43]. It was based on the facts for being specific to the analyte while not requiring specific pretreatment for the specimens of the complex matrix (e.g., plasma, urine, etc.), They have high analytical throughputs, which make them suitable for clinical setting processing for a large number of samples, while being not expensive [44]. In literature, Two antibody production studies have been reported regarding camptothecin derivatives [45,46] Still no more studies have been focused on the generation of SN38 antibodies owing to SN38 immunogen preparation unless one was reported by Saita et al., in 2000 [47]. Saita et al. generated a polyclonal anti-*SN*38 antibody (pAb) by immunizing rabbits using SN38 directly conjugated with BSA via N-succinimidyl ester [47]. However, there has been no report for producing monoclonal anti-SN38 antibody (mAb) applicable to measure concentrations of SN38-conjugated therapeutic platforms, particularly in the form of SN38 based-ADCs. In this study, we present a novel approach to the determination of SN38 levels. We have prepared two different immunogens for SN38 to enhance its immunogenicity and have successfully generated both polyclonal and monoclonal antibodies. This marks the first-time establishment of an ELISA for determination of SN38 in both its free and conjugated forms in ADC samples. This innovative method opens up new possibilities for the accurate and efficient determination of SN38 levels, enhancing our understanding and control of its therapeutic effects.

2. Materials and methods

2.1. General methods and material

All mentioned materials and solvents were purchased from Merck, except 7-ethyl-10-hydroxycamptothecin (SN38) obtained from Gyro, Shanghai Puyi Chemical Co., Ltd (China). The purity determination of the chemical substrates and reaction monitoring was examined using thin layer chromatography (TLC) on handmade silica-gel plates. Avicenna Research Institute, Tehran, Iran, provided Sheep anti-Rabbit IgG and Sheep anti-mouse IgG HRP conjugate secondary antibodies.

The spectra of ^{1}HNMR and ^{13}CNMR were recorded on a Bruker DRX-400 spectrometer in DMSO-d_6 solvent. Chemical shifts (δ) are expressed in parts per million (ppm) and are given as s (singlet), d (doublet), t (triplet) and m (multiplet); coupling constant J is given in Hz (Hz). IR spectra were recorded on a PerkinElmer 781 spectrophotometer and an Impact 400 Nicolet FT-IR spectrophotometer as KBr particles. Mass spectrometry was performed on an Agilent.

Technologies 5975C VL MSD instrument (Agilent Technologies, USA) equipped with a triaxial detector. Analytical HPLC was done on a Smartline Knauer system (Knauer, Germany) equipped with a Grace C18 column (5 μm, 300 Å, 4.6 × 250 mm) using gradient elution including buffer A (0.3 % w/v ammonium acetate, pH 4.43), filtered through 0.45 μm) and B (9:1 v/v acetonitrile: buffer A). Other key tools are ELISA reader (BioTek, Winooski, VT, USA). shaking incubators for UV/Visible Biophotometer (Eppendorf Biophotometer Plus Hamburg, Germany) for protein assay at 280 nm, Eppendorf 5810R and 5415R refrigerated centrifuges, Peristaltic pump (GE Healthcare Bio-sciences AB, Made in Sweden) and HiTrap® Protein G High Performance column (Sigma)and Bio-Rad electrophoresis system for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, California, USA).

White female rabbit aged 10–12 weeks and Male Balb/c mice aged 8–10 weeks were obtained from the Pasteur Institute of Iran (Tehran, Iran) for the generation of pAb and mAb, respectively. Experiments on all animals were performed after approval of the research ethics committee of Avicenna Research Institute (ARI), Approval ID IR.ACECR.AVICENNA.REC.1397.013.

2.2. Chemical modification of SN38 hapten

Chemical modification of SN38 to achieve an amine linkable functional group, SN38–20-*O*-glycinate TFA salt (SN38-glycinate), was done as previously described [31]. Afterward, it was used to be linked to the bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) proteins either directly or by introducing succinic acid moiety as a spacer into the chemical structure of SN38 (Fig. 2). For introducing succinic acid moiety, 75 mg (equivalent to 0.155 mmol) of SN38–glycinate, 27 mg (equivalent to 0.0027 mmol) of succinic anhydride, 35 μL Triethyl amine (TEA) (equivalent to 251 mmol), and 10 mL of anhydrous tetrahydrofuran (THF) (equivalent to 10 mmol) were mixed. The mixed contents were stirred for 5 days at room temperature (25 ± 2 °C). Aa rotary vacuum evaporator then evaporated the solvent, and the remaining salt was washed with HCl (0.001 N). The residue was further purified by filtration to obtain a Succinate-glycine-SN38 compound and confirmed by IR, NMR, and mass spectrometry.

Then 10 mg of the succinate-glycine-SN38 (equivalent to 0.0255 mmol), 6.8 mg 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (equivalent to 0.0357 mmol) and 7.8 mg N-hydroxy-sulfo-succinimide (sulfo-NHS) (equivalent to 0.00673 mmol) were solved in anhydrous dimethyl sulfoxide (DMSO) (1 mL) and mixed for 36 h under stirring at room temperature to prepare N-hydroxysuccinimid-glicinate-SN38 compound.

2.3. Carrier-hapten conjugation and immunogen preparation

A quantity (1 mg) of each BSA and KLH protein was dissolved in 1 mL of PBS buffer solution (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM, pH 7.4) to produce 1 mg/mL of carrier solutions. Then the solutions were used to prepare carrier-hapten conjugation directly (a) or through 5-atom spacers (b).

(KLH)-Glycine (SN38) (a)**:** 100 μL SN38–glycinate salt (10 mg/mL) was added to 200 μL of the carrier solutions (1 mg/mL) and then

mixed with three μL Glutaraldehyde 1 % (3 mM) for 1 h under stirring at room temperature. Next 1 μL ethanolamine 12.6 mM was added to make it 15 mM followed by incubation at room temperature on a roller mixer for 30 min to achieve stabilized secondary amine linkage (C–N).

[KLH]-4-amino-4-oxobutanyol-Glycine (SN38) (b)**:** To achieve the most conjugation efficacy, 500 μL of the KLH solutions (1 mg/mL) were added to the 12.5, 25, 50, and 100 μL solution N-hydroxysuccinimid-Glicinate-SN38. The reaction was incubated on a roller mixer at room temperature for fours h.

For both reactions (a & b), non-conjugated SN38 was removed by dialyzing against PBS at 4 °C and followed against stabilizer buffer (Na_2HPO_4 83 mM, NaCl 900 mM, Sorbitol 100 mM, pH at 7.2 and sterilized by passing through a 0.22 μm sterile syringe filter.) at the last exchanging buffer.

The dialyzed conjugates were assayed by ultraviolet–visible spectroscopy (UV/VIS) at the wavelength maximum absorbance's (λ max) of the SN38 (380 nm) and BSA/KLH (280 nm). The SN38 contents of the conjugates were estimated at a definite extinction coefficient ($\varepsilon SN38,380\ nm = 25{,}500\ mol^{-1}cm^{-1}$) [48] based on Beer-Lambert law. Because of the high immunogenicity of KLH [49], The KLH-SN38 conjugates were purposed for animal immunization to generate antibodies and the BSA-SN38 conjugates were used as a coating antigen all throughout ELISA's experiments. The SN38-KLH conjugate was characterized by ultraviolet–visible spectroscopy (UV/VIS).

2.4. Anti-SN38 pAb generation

An aliquot containing 1 mg [KLH]-glycine-SN38 or [KLH]-4-amino-4-oxobotanyol-glycine-SN38 complexes were emulsified with an equal volume of Freund's complete adjuvant. Two New Zealand white rabbits were intramuscularly injected at both sides of their legs. Booster injections were emulsified with an equal volume of Freund's incomplete adjuvant. They were then given four times at bi-weekly intervals, using same amount of the first immunization dose. The rabbits were bled from an ear vein before and after bi-weekly intervals of immunization. The sera (10 mL) were separated by centrifugation and heated at 55 °C for 30 min, followed by determination of antibody titers by ELISA. The sample that showed the highest avidity to SN38 (lower EC_{50} value) was used as the crude pAb for anti-SN38 antibody purification.

2.5. Anti-SN38 mAb generation

Three Male Balb/c mice were immunized intraperitoneally with [KLH]-4-amino-4-oxobutanyol-glycine (SN38) mixed with complete Freund's adjuvant, and four booster injections were given at 3-week intervals between the first and second injections, and at 2-week intervals between subsequent injections, using incomplete Freund's adjuvant. Blood was collected from the retro orbital sinus and ELISA determined the antibody titers ten days after the last immunization. Based on obtained ELISA results, the mouse with the highest titer was selected and injected intravenously with 20 μg of antigen (without any adjuvant). cell fusion was performed three days later, as described elsewhere [50]. Splenocytes from the immunized mouse were harvested and fused with SP2/zero at a 5:1 ratio using Polyethylene Glycol 1500 (Sigma, Milwaukee, WI, USA). Hybridoma cells were cultured in RPMI supplemented with 20 % FBS and MEM Non-essential Amino Acids (1x), Sodium Pyruvate (1 mM) and Hypoxanthine-Aminopterin-Thymidine selective medium (HAT medium). Ten to twelve days after fusion, supernatants of hybridomas were screened by indirect ELISA as described above. Particular antibody-generating hybridomas (tremendous hybridoma cells) have been cloned four times by limiting dilution to select the stable hybridomas. The isotype of each mAb in culture supernatant was determined by a mouse mAb isotyping kit (IsoStrip) (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Ascites fluid was prepared after an intraperitoneal injection of [KLH]-glycine-SN38 or [KLH]-4-amino-4-oxobutanyol- glycine (SN38) hybridoma cells.

2.6. Affinity chromatography and purification confirms

The rabbit sera and ascites fluid of the mouse inoculated with the hybridoma that showed the highest response and gave the best affinity to SN38 were collected respectively, as crude anti-SN38 pAb and mAb samples. To purify pAb and mAb IgG antibodies, both HiTrap® Protein G HP Columns and hand-made columns affinity chromatography by coupling of SN38 to Sepharose 4B. Purification by a HiTrap protein G HP affinity chromatography column (GE Healthcare, Uppsala, Sweden) was done according to the manufacturer's instructions. However, columns affinity chromatography over the coupling of SN38 to Sepharose 4B was done based on the method has previously been described method [51] with some modification briefly described below.

2.5 g of dry CNBr-Activated Sepharose-4B resin were washed three times using 500 mL of cold activation buffer (1 mM HCl) for 6 h intervals incubation on a roller mixer at 4 °C. All activation buffer were removed from the swollen resin and replaced with 12.5 mL coupling buffer (Sodium bicarbonate buffer 0.1 M, pH 8.3 that is containing 0.5 M NaCl) containing 5 mg/mL of the SN38-glycinate salt in a 50 mL falcon tube on a roller mixer for 120 min at room temperature followed at 4 °C for overnight. The coupled resin was poured into the funnel column (0.9 × 160 cm) and washed with 50 mL coupling buffer followed washing with 100 mL quenching buffer (100 mM Tris–HCl, pH 8.0) to ensure no unreacted CNBr sites have remained.

The column was washed three times alternately with 30 mL of acidic (NaOAc 100 mM, NaCl 500 mM, pH 4.0) and alkaline buffers (Tris–HCl 100 mM, NaCl 500 Mm, pH 8.0), and then followed with 20 mL PBS containing 0.1 % sodium azide to remove unbound SN38 from the resin and prepare it for long-term storage.

The amount of SN38 coupled to each gel was taken as the difference between the amount recovered in the washings and the quantity initially added to the reaction mixture. For this purpose, a standard SN38 curve (0-0·1 mg/mL) was calibrated at 380 nm [52].

The sera (10 mL) diluted in cooled PBS (1:5) and the supernatants were isolated after centrifugation at 10,000 g at 4 °C for 10 min, then filtered using a 0.45 μm syringe filter. The filtered sera were passed through the column (flow rate 18 mL/h, duration 30 min) and then washed with 5 column volumes of PBS to clear resin from unspecified serum components. The immobilized Anti-SN38 antibody was eluted using elution buffer (Glycine-HCL 0.2 M, pH 2.5). The eluent was collected in 1 mL fractions into 1.5 mL tubes containing 50 μL of 1 mol/L Tris–HCl buffer (pH 9.0), and evaluated for protein by determining the absorbance of the eluted fractions at 280 nm. The pooled fractions were dialyzed overnight against five changes of PBS (~4 h intervals). The antibody concentration of the dialysate was examined at 280 nm spectroscopically [C_{Ab}(mg/mL) = OD_{280nM} × dilution/1 × 0.7] and the purity of the antibody was assessed by SDS-PAGE as described elsewhere [53].

2.7. Enzyme linked immunosorbent assay (ELISA) procedures

Four types of ELISA were set up: i. for titration of rabbit sera and mouse sera (for screening of hybridomas), ii. reactivity assessment of purified pAb and mAbs, iii. quantitation of SN38 in free and iv. conjugated form of ADC samples.

In brief, ELISA plates were coated with 50 μL of the BSA-Glycine-SN38 (for i,ii, and iii) or Anti-SN38 pAb (for iv) prepared in PBS (10 μg/mL) and incubated at 37 °C for 1 h followed by overnight incubation at 4 °C. With 100 μL of the 3 % skim milk at 37 °C for 120 min, Blocking was then done. Hybridoma supernatants or serial dilutions of mouse or rabbit sera (for i), purified rabbit anti-SN38 pAb and mouse anti-SN38 mAbs (for ii), pre-incubated SN38 (50 μM or nM) with anti-SN38 pAb (8 nM) or mAb (22 nM) (for iii), and anti-PLAC1-ADC (for iv) were subsequently added and allowed to bound with specific coated antigens by incubation for 90 min at 37 °C. Specific binding Signals were developed by successive addition of 50 μL appropriate secondary antibodies (sheep anti-rabbit IgG or sheep anti-mouse IgG HRP conjugates; diluted 1:1000 in PBS) followed incubation at 37 °C for 90 min and then using tetramethylbenzidine(TBM) substrates for HRP. After 15 min incubation at room temperature in the dark, the reaction stopped by adding 15 μl of stop solution (20 % H2SO4) and optical density (OD) was measured at 450 nm. Following each step, plate was washed five times with PBS containing 0.05 % (v/v) Tween 20 (PBS/T). The cross-reactivity was calculated with the following formula: Cross-reactivity= (EC_{50} of SN38/EC_{50} of irinotecan) × 100 %.

2.8. Immunoreactivity assessment of pAb and mAb by western blot analysis

[BSA]-glycine-SN38 (10 μg/mL) was electrophoresed in 10 % SDS-PAGE gel and transferred to the polyvinylidene difluoride (PVDF) membrane (Roche, Mannheim, Germany). Membrane blocking was done using washing buffer (PBS-T) containing 5 % skim milk overnight at 4 °C. Following washing, the membrane was incubated with purified anti-SN38 pAb & mAb at concentrations ranging from 50 to 500 ng/mL for 90 min at 37 °C. Anti-BSA antibody and preimmunized rabbit or mouse Immunoglobulin G (IgG) were used as positive and negative primary antibody

controls, respectively. After washing, membranes were incubated for 60 min with Sheep anti-rabbit IgG or sheep anti-mouse IgG HRP conjugate. The bands were finally imagined with an enhanced chemiluminescence substrate. The activity of the anti-SN38 mAb in Western blot analysis was also tested using transferred BSA-SN38 conjugate and naked BSA (either 5 or 10 μg/mL) to the PVDF membrane as positive and negative controls, respectively. In this case, all methods and incubation times were according to mentioned above, except anti-SN38 mAb and sheep anti-mouse IgG HRP conjugate were used as the primary and secondary antibodies, respectively.

2.9. Binding affinity (Kd) assessment of the pAb and mAb

The method for binding affinity (Kd) assessment has previously been described [31]. Briefly, a 96 wells microplate plates were coated with [BSA]-SN38 conjugate at 10 or 5 μg/mL. The wells were washed five times with PBS-T and then blocked with 3 % skimmed milk in PBS-T at 37 °C for 90 min. After washing with PBS-T, rabbit anti-SN38 pAb or mouse anti-SN38 mAb were serially diluted 1:2 and added in triplicate, yielding a concentration range from 130 to 0.25 nM and incubated for 90 min at 37 °C. The wells were washed, as mentioned earlier, sheep anti-rabbit IgG or sheep anti-mouse IgG HRP conjugated secondary antibodies (1:100 dilution) for 90 min at 37 °C and signal development with TMB. Data were plotted as specific binding (OD450) versus anti-SN38 pAb and mAb concentration and fitted to a One Site-Total and nonspecific binding [specific = Bmax × χ/(χ+Kd), nonspecific = NS × χ+ Background] using GraphPad Prism software.

2.10. Statistical analysis

All of the experiments were repeated at least two or three times with the same settings. Graphs were organized using GraphPad Prism 8 software (Advanced Graphics Software, CA). Data were expressed as mean ± SEM. The graph's weight or goodness-of-fit for regression analysis was determined by the R-squared (R^2) coefficient of determination. Statistical difference was analyzed by either *t*-test when two groups were considered or two-way ANOVA multiple comparisons, and the $P < 0.05$ or less was considered to be a significant difference.

3. Result

3.1. Confirmation of modified SN38 and immunogens preparation

SN38 is a small chemical molecule whose immunogenic property cannot stimulate the animal immune system against itself. Thus, we had to linked SN38 to routinely used KLH or BSA highly immunogenic carriers. SN38 modification for having functional groups were successfully performed respectively via a glycine or 4-amino-4-oxobutanyol(glycine) spacers. The structures of these products were confirmed by ^{1}H NMR and mass spectrometry that gave a molecular weight of 522; ^{1}H NMR (300 MHz, DMSO) δ ppm 8.45 (s, 1H, 8.05 (d, $J = 9$ Hz, 1H), 7.41 (s, 1H), 7.05 (s, 1H), 5.5 (s, 2H), 5.28 (s, 2H), 4.18 (d, $J = 14$ Hz, 1H), 4.01 (d, $J = 17$ Hz, 1H), 3.08 (d, $J = 7.5$ Hz, 3H), 2.38–2.43 (m, 4H), 2.16 (d, $J = 7.2$ Hz), 1.29 (t, $J = 7.2$ Hz, 3H), 0.922 (t, $J = 7.2$ Hz, 3H).

SN38 was amenable to conjugate with KLH or BSA from functional amine and N-hydroxy succinimide groups respectively involved in SN38-glycinate (Fig. 2a) and NHS-modified SN38-glycinate salts (Fig. 2b). Conjugation efficacy of immunogen was examined by UV detection at 380 nm of the conjugates, SN38, and the unconjugated KLH or BSA under the same conditions. The spectra obtained in the case of the KLH-SN38 conjugates are illustrated in Fig. 3. It has been clearly shown that the conjugates resulted in higher absorbances than unconjugated proteins at their maximum absorption peaks. These hyperchromic effects while moving toward to SN38 wavelength in the absorption spectra of the conjugates compared with the unconjugated proteins were apparent showing the successful conjugation of SN38 with the KLH or BSA and formation of the conjugates. However, NHS-modified SN38-glycinate demonstrated greater conjugation efficiency to KLH or BSA (Fig. 3c and d) as we were able to attain an optical density of 10.892 at 380 nm in comparison to SN38-glycinate that could not reach above 2.191 (Fig. 3a, b), a loading capacity five times greater than SN38-glycine.

3.2. Immunization efficiency and antiserum screening for anti-SN38 pAb & mAb

The best/optimum working concentration of the BSA-SN38 conjugate required for SN38 immobilization onto the ELISA plate microwells was determined as a prerequisite for antiserum titration of the rabbit and mouse immunized animals by both [KLH]-glycine-SN38 and [KLH]-4-amino4-oxobotanyol-glycine-SN38 conjugates. The efficient [BSA]-glycine-SN38 conjugate was used to

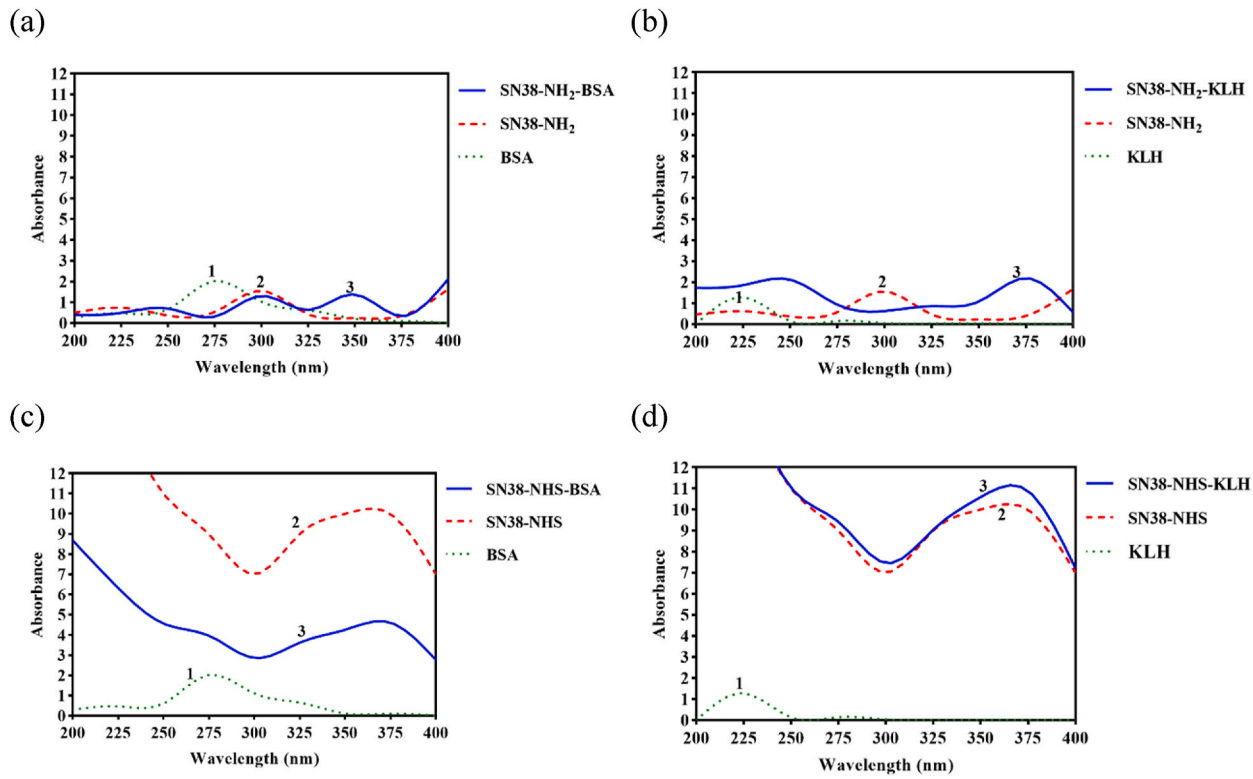


Fig. 3. Immunogen's preparation and analysis: UV absorption spectra were utilized for the characterization of conjugates compromising [BSA]-glycine-SN38 and [KLH]-glycine-SN38 abbreviated to SN38–NH_2-BSA (a) and SN38–NH_2-KLH (b), respectively. Likewise, the conjugates of [BSA]-4-amino 4-oxobotanyol-glycine (SN38) and [KLH]-4-amino 4-oxobotanyol-glycine-SN38 were abbreviated as SN38–NHS-BSA (c) and SN38–NHS-KLH (d). Spectra were unconjugated KLH or BSA (1), free SN38–NH_2 or SN38–NHS (2), and [BSA or KLH]-SN38 conjugates to SN38–NH_2 or SN38–NHS (3). The concentrations of SN38–NH_2 and SN38–NHS were maintained at 100 μg/mL and equal concentrations of [KLH/BSA]-NH_2SN38, [KLH/BSA]–NHS–SN38, and KLH/BSA (1 μg/mL) were prepared in 50 mM PBS (pH 7.4).

coat microwell plates at 5, 10, and 20 μg/mL concentrations. Although, no substantial difference was found between them, the optimum concentrations of coating antigen were designated at 10 μg/mL (Fig. 4a). These coating concentrations were used in all further Enzyme-linked immunosorbent assays. Furthermore, substantial enhancement in reactivity towards the immobilized SN38 ([BSA]-glycine-SN38) was discerned in the hyperimmunized serum when compared to the pre-immunization state (Fig. 4a). It was found that antibody responses obtained using [KLH]-4-amino4-oxobotanyol-glycine-SN38 immunogen were significantly more than immunization by [KLH]-glycine-SN38 as it gave the highest reactivity for immobilized SN38 at all four steps regular boosts and bleeding (Fig. 4b); therefore, the total antisera obtained from rabbits who had immunized by [KLH]-4-amino-4-oxobotanyol-glycine-SN38 was collected and applied as the anti-SN38 pAb in the development of the ELISA explained herein for SN38. Our first mice immunization attempt via [KLH]-glycine-SN38 immunogen failed. Meanwhile, mice immunization by [KLH]-4-amino-4-oxobotanyol-glycine-SN38 immunogen could generate an anti-SN38 immune response (Fig. 4c).

To select anti-SN38 hybridoma for mAb production, The mouse with the highest titer of anti-SN38 antibody was used for cell fusion (Fig. 4c). The supernatants of all growing hybridomas were examined to find a specific antibody against the SN38 immunogen. After four times cloning's, one final clone named 1D7E10 was achieved. After limiting dilution and several additional screenings, the isotypes 1D7E10 were IgG1/κ.

The titer of both pAb and mAb antisera was defined as the dilution of antiserum exhibiting a minimum 4-fold increase (1:128000) in absorbance compared to the control before immunization. It is important to note that the mAb, unlike the pAb, failed to recognize

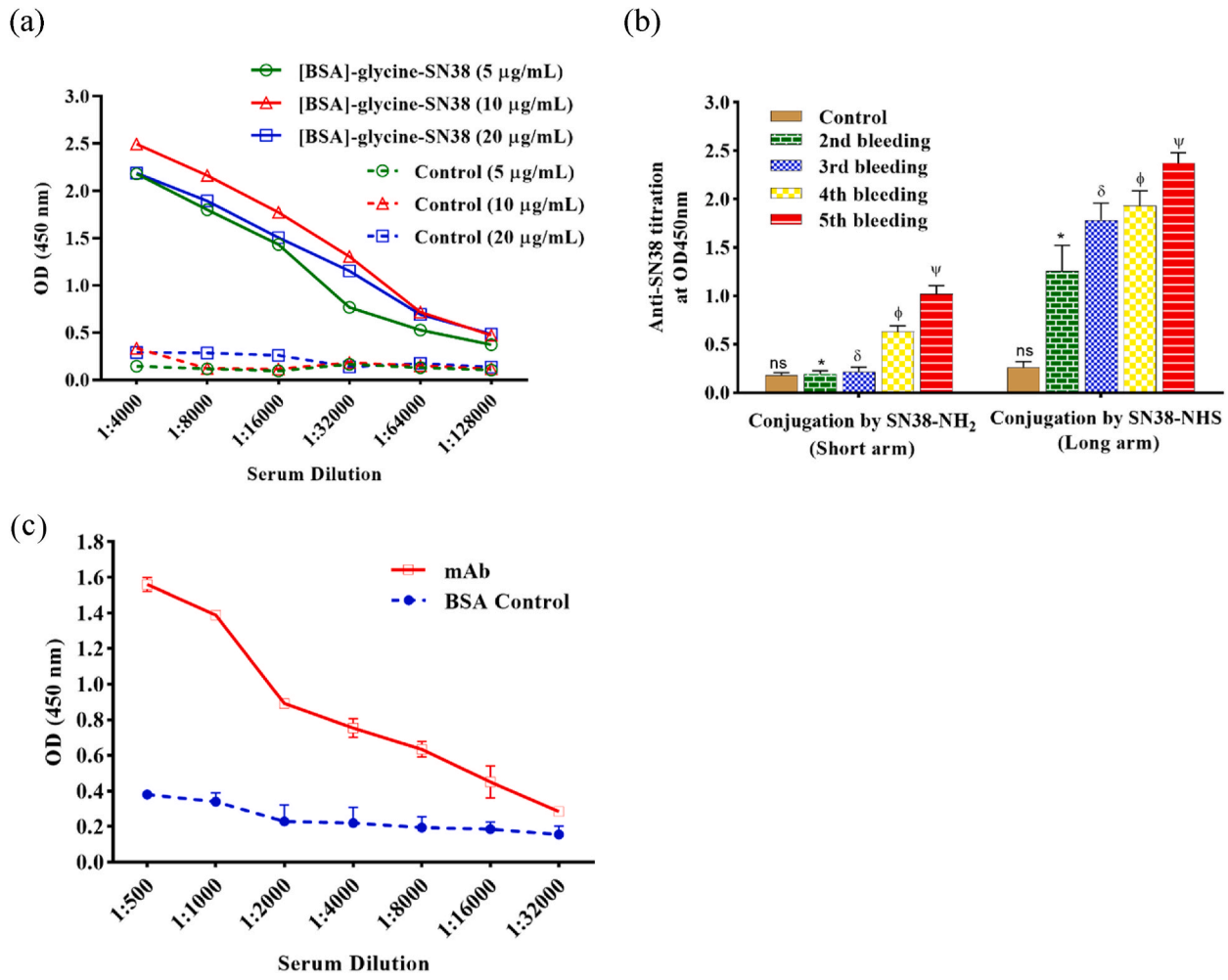
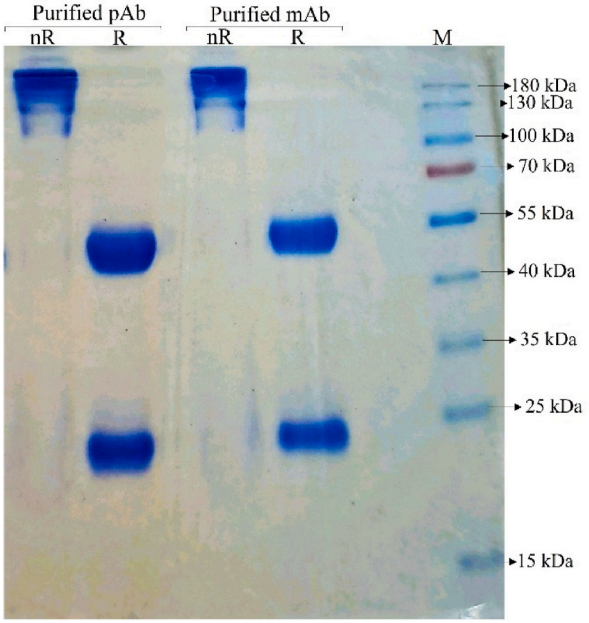
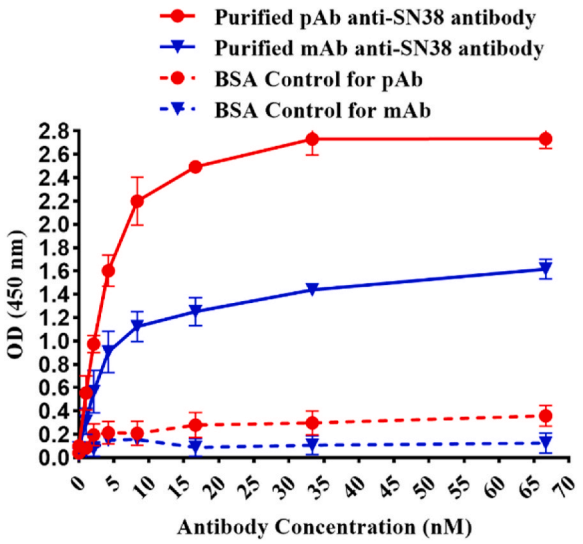


Fig. 4. Titration of anti-SN38 antibody in immunized rabbit serum by indirect ELISA.
Hyperimmunized serum was serially titrated on a 5, 10, and 20 μg/mL of the [BSA]-glycine-SN38 coated plate microwells to determine optimum coating antigen for anti-SN38 detection, preimmune serums were used as a control (a)., Comparison of immune response of Rabbit immunized with [KLH]-glycine-SN38 (short arm) and [KLH]-4-amino 4-oxobotanyol-glycine-SN38 (long arm) was done using serum dilution at 1:4000, Controls in each group were compared together (ns, i.e., nonsignificant), 2nd, 3rd, 4th, fifth bleedings in each group were compared together (*, δ, φ, and ψ $p < 0.0001$ respectively) (b)., Titration of anti-SN38 antibody in immunized mouse serum by indirect ELISA. Hyperimmunized serum was serially titrated on a [BSA]-glycine-SN38 coated plate (c). For all titration assays, BSA coated wells was used as control to check possible nonspecific linking of the serum dilution to the BSA consisting of the target antigen ([BSA]-glycine-SN38). The values plotted are mean ± SEM of three determinations.

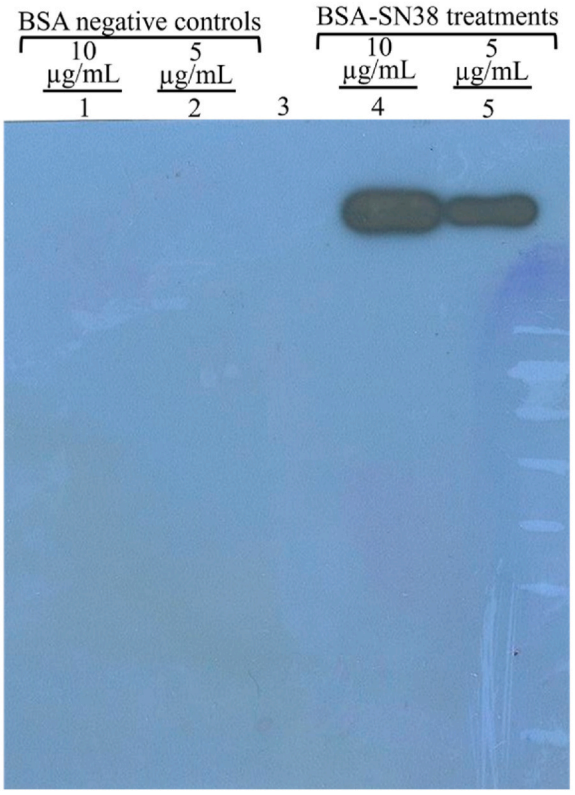
(a)



(b)



(c)



(caption on next page)

Fig. 5. Purification and reactivity assessment of purified anti-SN38 antibodies.
10 % SDS-PAGE gel analysis of purified anti-SN38 IgG, M: Protein ladder, nR: purified mAb under non-reduced condition, R: purified antibodies under reduced condition(a). Reactivity evaluation of purified pAb and mAb anti-SN38 by indirect ELISA, purified antibodies using protein G ligand were serially diluted on a BSA-SN38-coated plate (b). Use of anti-SN38 mAbs for Western blot analysis of SN38, Lane 1 and 2 represent the negative controls with high (10 μg/mL) and low concentrations (5 μg/mL) of BSA, respectively. Lane 3 is left empty. Lane 4 and 5 correspond to the treatment with high (10 μg/mL), and low (5 μg/mL) concentration of BSA-SN38 conjugate, respectively. Samples were separated on 10 % SDS-PAGE and transferred to the PVDF membrane. Each lane was incubated with purified anti-SN38 mAb as the primary antibody, followed by sheep anti-mouse IgG HRP conjugate as the secondary antibody (c).

SN38 (BSA-SN38) at a dilution of 1:16,000.

3.3. Purification and reactivity evidence of the anti-SN38 pAb and mAb

Anti-SN38 pAb and mAb were purified, from hyper-immunized rabbit serum and ascetic fluid of 1D7E10 clone through affinity chromatography columns contained either immobilized SN38 or protein G ligands. Although protein G affinity chromatography was

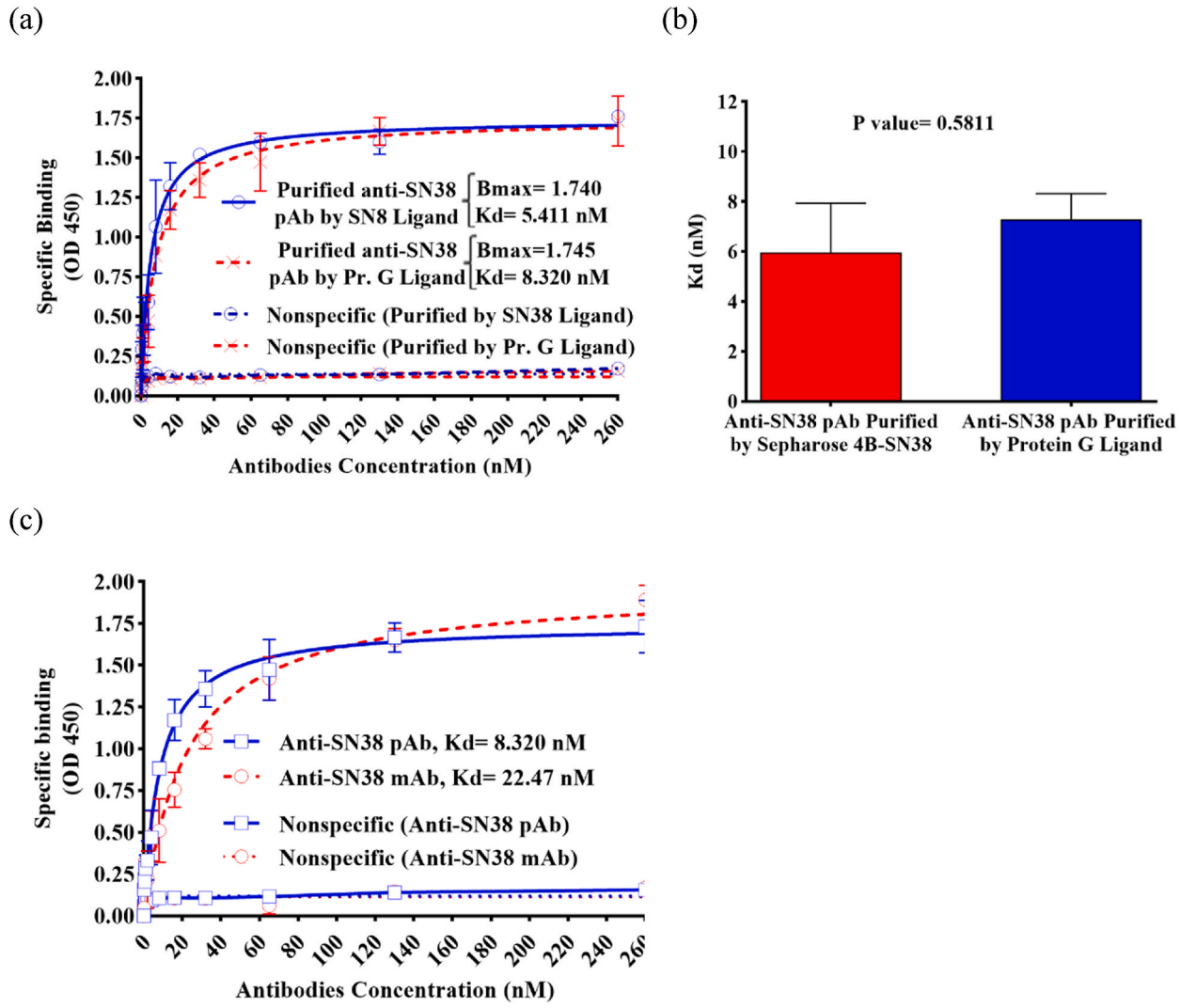


Fig. 6. Antibodies' affinity constant evaluations: The saturation binding curve of anti-SN38 antibody was generated using the Prism™ software and the equation for one-site binding model [Y = Bmax*X/(Kd + X)], Comparative graphs depicting the Kd values (equilibrium dissociation constant) of purified anti-SN38 pAb through affinity chromatography columns which contained either immobilized SN38 (R^2 = 0.9254) or protein G ligands (R^2 = 0.9586) (a), describing the equilibrium binding of the purified anti-SN38 antibody from both purification method does not differ significantly (b). Apparent Kd of anti-SN38 pAb and mAb to the SN38 were compared and plotted, R^2 = 0.9586 and 0.9415 respectively (c). Data were generated from three independent experiments.

more effective than SN38-Sepharose 4B columns at obtaining polyclonal antibodies, both yielded 2.35 mg/mL versus 1.29 mg/mL, respectively. Moreover, the final concentration of purified pAb in the SN38-Sepharose 4B affinity chromatography elution buffer was trivial about 0.2 mg/mL. However, the final concentration of purified pAb and mAb in the protein G affinity chromatography elution buffer was typically 0.8–1.5 mg/mL. SDS-PAGE assessed the purity of purified anti-SN38 antibodies. Both Anti-SN38 pAb and mAb migrated as clear, single bands, apparently free from high and low molecular weight contaminants (Fig. 5a). Estimated IgG molecular weights were consistent with expectations for non-reduced (~150 kDa) and reduced conditions (approximately distinct bands at 50 and 25 kDa, respectively are representative of the light and heavy chains of the antibody) (Fig. 5a) (full length gel in supplementary data file, Figs. S–4). The purified pAb and mAb demonstrated excellent reactivity against SN38, as indicated by the obtained p-value of 0.0859 (Fig. 5b). The antibodies showed a similar pattern of reactivity reaching to plateau at as low an antibody concentration as15-20 nM. This indicates reasonable affinities for produced antibodies (Fig. 5b). We also conducted a Western blot analysis utilizing the anti-SN38 mAb to assess the antibody's activity in Western blot analysis. For this purpose, we employed transferred BSA-SN38 conjugate and naked BSA as positive and negative controls, respectively. Notably, a distinct and concentrated band corresponding to the BSA-SN38 conjugate lanes was observed, indicating specific binding of the mAb antibody as the negative control lanes containing BSA exhibited no band (Fig. 5c) (full length blot in supplementary data file, Figs. S–5).

3.4. Comparative determination of affinity constant of anti-SN38 pAb and mAb

The affinity constant of pAbs in both purification methods were investigated and compared based on nonlinear regression of a one-site specific binding model of the GraphPad Prism software (Fig. 6a). Binding data from affinity constant experiments showed no significant difference in the specificity of the antibody purified by both purification methods (Fig. 6b). Based on this, the affinity constant of anti-SN38 pAb and mAb were shown to be 8.32 ± 2.64 and 22.47 ± 8.96 nM respectively (Fig. 6c). Therefore, these concentrations were used in all further testing.

3.5. Anti-SN38 antibodies efficacy to detect naked and conjugated SN38 (ADC)

The purified anti-SN38 pAb and mAb were used for their efficacy evaluation to find SN38 in free and conjugated forms (ADC). The best coating antigen ([BSA]-glycine-SN38) concentration for optimum binding with the purified antibodies was the same 10 μg/mL based on checkerboard assay [54] (S-1) as it needs to set competitive ELISA. Based on competitive ELISA, SN38 was serially diluted (1.56–50 nM) in PBS and mixed with a pre-determined amount of the anti-SN38 pAb (8 nM) and mAb (22 nM), a 50 μL of the mixed solution was transferred into the microwells that are already coated with [BSA]-glycine-SN38. The competitive binding reaction yielded an inverse curve relative to the SN38 concentration (Fig. 7a). The curve showed an excellent coefficient of determination based on Y= YIntercept + X*Slope, using GraphPad Prism software. The Limit of detection (LOD) and limit of quantitation (LOQ) were found to be 7 nM and 21 nM and also 8 nM and 25 nM respectively for anti-SN38 pAb and mAb.

We could assess only the anti-SN38 pAb capability to detect SN38 in ADC structure, as the antibody's component of the ADC is a mouse IgG, i.e., anti-PLAC1 ADC with drug to antibody ratio (DAR) about 6 that has been reported elsewhere [31]. Based on sandwich indirect ELISA, a standard curve for anti-SN38 pAb was obtained by plotting the absorbency rate against the concentration of either ADC (acDrug) or its naked (tAb) form resulting in an asymmetric sigmoidal curve which was generated using nonlinear regression analysis in a GraphPad prism (Fig. 7b). The standard curve shows optical absorbance of the ADC increases under ADC concentration as is proportional to the presence of SN38 conjugated with the antibody. However, there is no such trend in naked antibodies, confirming

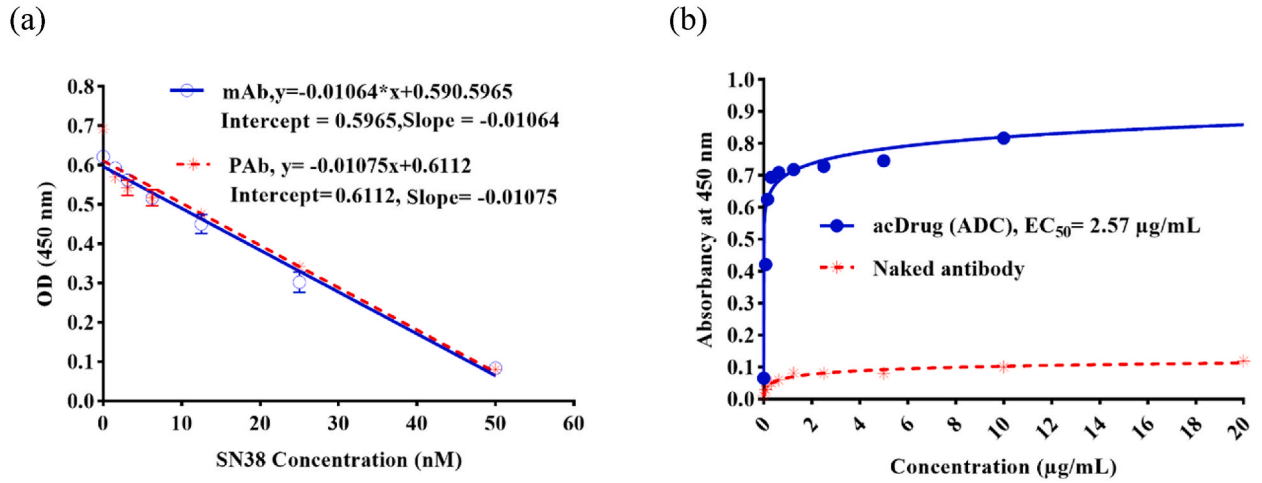


Fig. 7. Antibodies' efficacy in SN38 detection: The calibration curves for the competitive ELISA of SN38 were obtained for pAb ($R^2 = 0.9754$) and mAb ($R^2 = 0.951$), respectively. Each point represents the mean ± SEM of 3 replicates (a). Binding reactivity between anti-SN38 pAb and ADC was analyzed via enzyme-linked immunosorbent assay (ELISA). The EC50 value was determined to be 2.5 μg/mL (b).

the ability of our produced antibody to identify SN38 in ADCs. The ADC could bind to anti-SN38 pAb at test midpoint EC50 of 2.5 μg/mL (Fig. 7b).

3.6. Comparative cross-reactivity of anti-SN38 pAb and mAb

The antibody specificity was determined based on the same competitive ELISA have explained in section 3.5 by the displacement of SN38 by Irinotecan (Campto®), a well-known camptothecin (CPT) prodrug derivative has been given as a chemotherapy drug for treating various malignancies for many years (Fig. 8), Values of the cross-reactivity was defined as the ratio of irinotecan to SN38 in the concentrations required for 50 % inhibition of SN38 or Irinotecan binding to the anti-SN38 pAb and mAb antibodies with the following formula:

$$\text{Cross-reactivity} = (EC_{50} \text{ of SN38} / EC_{50} \text{ of irinotecan}) \text{ x100\%}.$$

The anti-SN38 pAb and mAb showed about 65 % and 30 % cross-reaction with irinotecan (Table 1). A statistical analysis comparing the EC50 values of pAb and mAb antibodies to both SN38 and Irinotecan and assessing the cross-reactivity of pAb and mAb to Irinotecan showed notable differences. Specifically, significant disparities were found between the EC50 values of pAb and mAb antibodies for both SN38 ($P < 0.0001$) and Irinotecan ($P = 0.0317$) (Figs. S–2), along with significant discrepancies in cross-reactivity to Irinotecan ($P = 0.0005$) (Figs. S–3) within the micromolar concentration range. These findings underscore the high specificity of Anti-SN38 pAbs and mAbs against SN38, as evidenced by the cross-reactivity to Irinotecan at micromolar rather than nanomolar levels.

4. Discussion

The design of immunogen utilizing the small molecule SN38 has led to the successful development of specific and affine pAb and mAb antibodies. These antibodies can detect SN38 in its free form and, when conjugated to antibodies within an SN38-based ADC through immunoassay-based methods. The motivation for this endeavor lies in the fact that SN38 is the active metabolite of all camptothecin-derived drugs, and its measurement instead of its prodrugs is judiciously one for therapeutic drug monitoring (TDM) as the foundation of personalized medicine. Moreover, the promise of the therapeutic platforms containing SN38 to treat complex diseases is mounting [5].

The chemical modification of the SN38 as a small molecule [55] to integrate it into an immunogenic carrier is an integral part of the project's implementation.

The reason is that this small molecule, on the one hand, has no proprietary reactive functional groups to bind to immunogen carriers. on the other hand, it is highly toxic, poorly soluble, and relatively unstable via lactone ring hydrolysis at a physiological pH [46,56]. We designed haptens based on introducing an amine activation group on the C-20 position of the SN38 molecule to stabilize the lactone form of the SN38 [56]., which was previously used for SN38 conjugation to the antibody [31,57]. The resulting aminated SN38 was conjugated to BSA and KLH by two methods, including Schiff base formation and introducing succinic acid moiety as a spacer to prevent the possible steric hindrance and promote more SN38 binding to the carriers, which has been reported as essential for immunization activity [56].

The modification provides controlled conjugation of SN38 to the carrier's primary amine functional groups without opening the lactone ring of SN38 and changing its structure. The conjugation methods are simple, mild, and reproducible and do not result in extensive (inter-or intramolecular) self-coupling of SN38 or KLH. KLH/BSA-SN38, thus prepared, can easily and rapidly be purified by dialysis tubing. The total antisera obtained from rabbits who had been immunized by [KLH]-glycine-SN38 was not satisfactory compared to immunization by [KLH]-4-amino-4-oxobotanyol-glycine-SN38 immunogen (Fig. 4c). The low potential immunogenicity of the [KLH]-glycine-SN38 was also confirmed when our first try for mice immunization via [KLH]-glycine-SN38 immunogen was

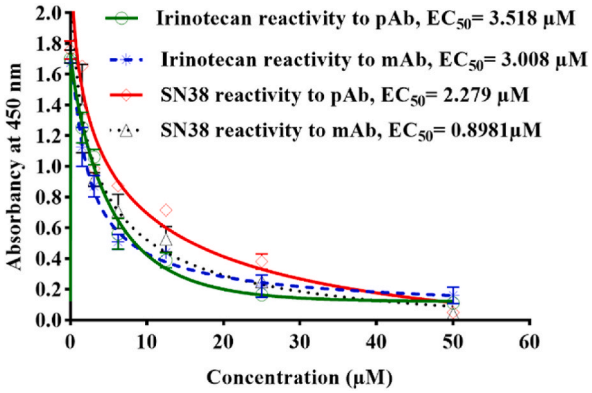


Fig. 8. The binding reactivity of SN38 and Irinotecan to the anti-SN38 pAb and mAb was compared using competitive ELISA: The analysis resulted in an inverse curve that is relative to the concentration of SN38 or Irinotecan. Data were analyzed using GraphPad Prism 8.0 software, employing non-linear regression with an asymmetric sigmoidal 5 PL equation and fitted using the least-squares method.

Table 1
Cross-reactivity of anti-SN38 pAb and mAb.

Compound	EC_{50} (μM)		% Cross-reaction	
	SN38	Irinotecan	SN38	Irinotecan
anti-SN38 pAb	2.279 ± 0.04	3.518 ± 0.09	100	64.79 ± 0.43
anti-SN38 mAb	0.8981 ± 0.23	3.008 ± 0.05	100	29.77 ± 7.17

Values were expressed as mean ± standard error of the mean (SEM) from three experiment.

failed. These observations attest to the SN38 modification with the 5-atom spacer ([KLH]-4-amino-4-oxobotanyol-glycine) could improve immune responses compared to linked via a shorter linker ([KLH]-glycine). Similar views are expressed in ex-reports that report on the possible effects of drug loading, stability, and immunogenicity of linkers [56]. It seems that the latter conjugation method also provides more stability for the SN38 lactone ring hydrolysis at a physiological pH. Purified pAb and mAb respectively showed reactivity at the well avidity (Kd 8.32 nM) and affinity (Kd 22.47 nM) values.

The ELISA for SN38 as a free form could detect as little as at the LOD and LOQ, which is in excellent agreement with the value determined for the anti-SN38 pAb of 51 nM [45,47]. However, there have yet to be comprehensive reports on the production of SN38-specific mAbs in the literature. The mAb sensitivity (LOQ = 25 nM) would be expected to be adequate for the quantitation of SN38 in plasma samples without any requirement for pre-concentration of SN38 in the samples prior before their analysis as the reported level of SN38 in plasma was 0.66 μM*h/L at the beginning of the infusion of irinotecan (180 mg/m^2 IV) up to the 24 h [58].

The use of generated antibodies to detect SN38 in SN38-conjugated therapeutic platforms has been the main subject of the study, as the SN38-conjugated therapeutic platform like nanoparticles, liposomes, micelles and ADCs, represent a highly successful paradigm for direct delivery of SN38 [5], especially for targeted delivery and treatment of tumors. The generated antibodies were used to develop an ELISA for an SN38-based ADC detection [31] at test midpoint EC_{50} of 2.5 μg/mL. To our knowledge, no previous study demonstrating specific antibody against SN38 capable of detecting it as a payload in an ADC.

The SN38-based ADCs with at least two products approved to market and several candidates have been navigating through clinical trials [5,59], illustrating their well-established and clinical acceptance. Availability, affordability, and FDA approval of the SN38 are advantages of this moderately-cytotoxic small molecule to use in ADC designs [31].

Despite such progress regarding SN38-based ADCs, their pharmacokinetic studies (PK) and ADME analysis, like other ADCs, remained one of the main challenges [60,61]. Three main parameters must be evaluated for ADME and PK studies of the ADCs: total antibody, drug conjugated to the antibody, and unconjugated drug. The drug conjugated to the antibody is the same active form of ADC and plays a crucial role in the dose-response analysis of ADCs [62]. The drug conjugated to the antibody is calculated using the average number of drugs conjugated to each antibody (DAR). It is currently calculated using HPLC and LC-MS techniques, which are expensive and labor-intensive for clinical diagnostic laboratories [36]. Based on ICH S9 guideline, the DAR affects both efficacy and safety of an ADC [63] and also concerning personalized medicine [39]. In this respect, our generated antibodies offer a favored tool for DAR estimation through ligand binding assay (LBA). The LBA is applicable for quantifying any SN38-based ADCs, and similar therapeutic platforms conjugated with SN38, facilitating their successful development and clinical translation. However, to ensure accurate DAR estimation via LBA, it is essential to supply at least 3–5 SN38-conjugated antibodies with known DAR as standards for each specific project. Although providing these standards with specified DAR requires costly methods, such as LCMS, it is a one-time expensive, and these standards can be utilized for numerous evaluations after that. Other potential applications of the anti-SN38 antibodies may include reducing SN38-induced systemic toxicity through an inverse targeting strategy. This strategy is an approach utilizes i.p administration of an antineoplastic drug with simultaneous i.v. administration of anti-drug antibodies. The anti-drug antibody enhances targeted drug therapy by obstructing drug distribution to sites associated with drug side effect and toxicity [64–66]. The inverse targeting strategy is a novel strategy that can also benefit from it to overcome drug resistance posed by anti-drug antibody production [67].

Quantification of SN38 in free and conjugated forms is essential for SN38-based drug developments from bench top to the clinics, particularly in personalized medicine. Personalized medicine involves administering drugs based on patients' genetics to reduce side effects and increase overall survival rates. There is a vast inter-individual inconsistency in pharmacokinetic values and pharmacodynamics of SN38-based drugs related to patients' genetic background. Especially, a polymorphism in the UGT1A1 gene (UGT1A1*28) has been reported to be associated with impaired detoxification of SN38 (the active form of all camptothecin-based drugs) to SN-38 glucuronide (SN-38G) leading to augmented toxicities [39]. TDM of the SN38 is recommended for discontinuing or lowering the unnecessary dose of all camptothecin-based drugs, wherein serum drug concentrations are used to guide adequate drug dosing on an individual.

Anti-SN38 mAb antibodies helped detect SN38 in Western blotting analysis (Fig. 5c); however, it is unclear whether the antibodies are applied to other analyses, such as flow cytometric and immunohistochemistry. Future studies need to assess the usability of the antibodies in flow cytometric and immunohistochemistry analyses of the SN38 in tissue or fluidic samples.

Additional information

Additional information is available in supplementary data file.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Tahereh Zarnoosheh Farahani: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Mohammad-Reza Nejadmoghaddam:** Writing – review & editing, Writing – original draft, Validation, Data curation, Conceptualization. **Soyar Sari:** Writing – review & editing, Writing – original draft, Supervision, Investigation. **Ramin Ghahremanzadeh:** Writing – review & editing, Visualization, Validation, Supervision, Methodology. **Arash Minai-Tehrani:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33232.

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