Development of an enzyme-linked immunosorbent assay for camptothecin

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Abstract. The use of camptothecin and its analogues has increased in clinical settings and in agriculture. Therefore, camptothecins and their derivatives, metabolites and degradation products are frequently found in the environment. Therefore, it is important to develop an ELISA for the quantification of camptothecins in human plasma, plants, animal tissues and other matrices. The present study developed a novel competitive indirect ELISA for camptothecin using a monoclonal antibody (MAb). In total, two haptens and various carrier proteins were tested to select the most suitable immunogen for the production of MAbs against camptothecin. Hapten 1 conjugated with keyhole limpet hemocyanin was selected for the preparation of MAb 5A3, and was used to establish a competitive indirect ELISA for camptothecin. A total of three derivatives of camptothecin used in clinical practice were examined. Topotecan showed an IC_{50} value of 0.68 μg/ml with a detection limit of 0.19 μg/ml, belotecan showed an IC_{50} value of 0.87 μg/ml with a detection limit of 0.22 μg/ml and irinotecan showed an IC_{50} value of 2.85 μg/ml with a detection limit of 0.47 μg/ml. The cross-reactivity results suggested that the assay developed in the present study possessed a high sensitivity to camptothecin. Therefore, this immunoassay technique may be suitable for monitoring the levels of camptothecin in compound analysis, clinical applications, and analyses of food and environmental samples.

Introduction

Camptothecin, a pentacyclic quinoline alkaloid, was first isolated by Wall *et al* (1) in 1966 from the bark of *Camptotheca acuminata* Decne, a plant native to Southeast China. Camptothecin has been reported to exhibit antitumor, antifungal, antiviral and insecticidal activities (2,3). As anticancer drugs, camptothecins have attracted increasing interest and attention from both the academic community and the pharmaceutical industry (4). Additionally, camptothecins have been used as pesticides in agriculture due to their insecticidal activity.

Hsiang *et al* (5) reported that camptothecin could selectively block topoisomerase I in complex with DNA. Since the first identification of camptothecin, multiple camptothecin derivatives have been synthesized (6). In total, three water-soluble derivatives of its analogues have gained approval for the treatment of colon, breast, ovarian and small cell lung cancers. The three analogues are irinotecan (NSC no. 616348), sold under the brand name Camptosar® by Pharmacia & Upjohn (7), topotecan (NSC no. 609699), marketed under the name Hycamtin® by SmithKline Beecham (8) and belotecan, sold under the brand name Camtobell® by Chong Kun Dang Pharmaceutical Corporation (9). Various other analogues of camptothecin are under various stages of clinical development (10). However, topotecan, irinotecan and belotecan, drugs used to manage and treat cancer that possess antineoplastic activity, are enzymatically degraded to camptothecin and its metabolites, with toxic side effects (11). Therefore, it is important to detect the degradation products of camptothecins in human plasma.

In agriculture, camptothecin and camptothecin analogues have been reported to have a broad insecticidal activity spectrum, and its action on *Trichoplusia ni* and *Spodoptera exigua* induces alterations in the midgut, loss of the single layer of epithelial cells and disruption of the peritrophic membrane (12). Liu *et al* (13,14) synthesized and tested the insecticidal activity of numerous camptothecin derivatives with a series of modifications at different sites. The use of camptothecins as field pesticides may require the monitoring of camptothecin residues, its metabolites and its degradation products in plants, water and soil.

Immunoassay is a technique used to analyze a particular substance with an antibody or a mixture of antibodies as the main analytical reagent. Immunoassay techniques provide qualitative and quantitative methods for analyzing a substance.

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Since they are simple, rapid and cost-effective, immunoassays have become widely used analysis systems, particularly in clinical settings and in the detection of pesticides (15). Camptothecin and its derivatives have emerged as a promising group of chemotherapeutic agents due to their biological activities in clinical settings and in agriculture (16); however, as the number of drugs based on camptothecin analogues have increased, camptothecins, along with their derivatives, metabolites and degradation products, are increasingly found in humans, plants, animals and the environment. Therefore, to develop an ELISA suitable for the quantification of camptothecins in human plasma, plants and other matrices is required. The aim of the present study was to develop an ELISA selective for camptothecins using monoclonal antibodies (MAbs).

Materials and methods

Reagents and instruments. All reagents and solvents used in the present study were of analytical grade. Camptothecin was provided by Professor Liu Yingqian (Lanzhuo University). N-hydroxysuccinimide (NHS), N,N-dicyclohexylcarbodiimide (DCC), dimethyl formamide (DMF), succinic anhydride, 1-(3-dimethylaminopropyl)-3-ethyl carbon diimide hydrochloride (EDCI), toluene, potassium dichromate, anhydrous pyridine, dimethylaminopyridine (DMAP), isobutylchlorocarbonate, tri-n-butylamine, tetramethylbenzidine (TMB) and DMSO were purchased from Sangon Biotech Co., Ltd. BSA, keyhole limpet hemocyanin (KLH), ovalbumin (OVA), Freund's complete and incomplete adjuvants, and Tween-20 were purchased from Sigma-Aldrich (Merck KGaA). Goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) antibody (cat. no. 31432) was obtained Thermo Fisher Scientific, Inc. Sp2/0 murine myeloma cells was obtained from the American Type Culture Collection. RPMI 1640 medium (cat. no. 11875) was obtained from Thermo Fisher Scientific, Inc. The SBA Clonotyping™ System/HRP kit (cat. no. 5300-05) was obtained from SouthernBiotech.

The instruments used were the following: UV-visible (vis) spectrometer (DU-640; Beckman Coulter, Inc.), mass spectrometer (HP-5988; Agilent Technologies, Inc.), nuclear magnetic resonance (NMR) spectrometer (Mercury 300 BB; Varian Medical Systems), 96-well polystyrene microplates (MaxiSorp; Thermo Fisher Scientific, Inc.) and Multiskan EX version 1.0 (Thermo Fisher Scientific, Inc.). Electrospray ionization mass spectrometry was conducted as previously described, and ^{1}HNMR and ^{13}CNMR spectra were recorded at 400 MHz and 100 MHz as previously described using tetramethylsilane as the reference (17,18).

In addition, PBS (10 mM, Ph 7.4; NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 2.9 g, KH_2PO_4 0.2 g, H_2O 1 l), carbonate-buffered saline (CBS; 50 mM, pH 9.6; Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, H_2O 1 l) and citrate-PBS (CPBS; 50 mM, pH 5.5; $C_6H_7O_8$ 21 g, Na_2HPO_4 71.6 g, H_2O 1 l) were used.

20(s)-O-succinyl-camptothecin (hapten 1). The structure of camptothecin and the synthetic route of 'hapten 1' (17) are presented in Fig. 1A. Camptothecin (0.352 g, 1.01 mmol) was dissolved in anhydrous pyridine (50 ml). Then, DMAP (0.122 g, 1.00 mmol), EDCI (0.767 g, 4.03 mmol) and succinic anhydride (0.201 g, 2.01 mmol) were added. The mixture was stirred at 70˚C for 3 days. The solution was then cooled to room temperature, diluted with toluene and evaporated under vacuum. Chloroform was added and the organic phase was washed with 0.1 M HCl and saturated NaCl. The solution was then dried using anhydrous $MgSO_4$. The solvent was removed, and the crude product was purified by silica gel chromatography (eluent, $CHCl_3$:CH_3OH, 96:4) and recrystallised from methanol to obtain the pale-yellow solid compound 'hapten 1' (Fig. 1A) with a melting point of 232.1˚C (0.235 g, 51.9%).

The results of the ^{1}H-NMR and ^{13}C NMR [500 MHz; DMSO-d6; δ parts per million (ppm)] were as follows: 0.91 (3H, t, J=7.0 Hz, C19-H), 2.16 (2H, t, J=7.4 Hz, C23-H), 2.50 (2H, t, J=7.4 Hz, C24-H,), 2.69-2.83 (2H, m, C18-H), 5.29 (2H, s, C5-H), 5.48 (2H, s, C17-H), 7.12 (1H, s, C14-H), 7.71 (1H, t, J=7.0 Hz, C10-H), 7.86 (1H, t, J=7.0 Hz, C11-H), 8.12-8.19 (2H, dd, J=8.0 Hz, J=8.5 Hz, C9-H, C12-H), 8.68 (1H, s, C7-H), 12.14 (1H, s, -COOH); and 8.05 (C19), 29.01, 29.16 (C23, C24), 30.97 (C18), 50.73 (C5), 66.87 (C17), 76.45 (C20), 95.61 (C14), 119.45 (C16), 128.20, 128.53, 129.02, 129.58, 130.33, 130.96, 132.08, 145.83, 146.46, 148.47, 152.93, 154.21 (C2, C3, C6-C15), 157.05 (C16a), 167.68 (C21), 171.83, 173.53 (C22, C25) [MS m/z 449 $(M+H)^+$].

7-Hydroxymethyl camptothecin. A solution of 75% H_2SO_4 (75 ml) was added dropwise to a suspension containing 3.00 g (8.6 mmol) of camptothecin (Fig. 1A) mixed with MeOH (90 ml) and H_2O (75 ml). Subsequently, $Fe_SO_4.7H_2O$ (2.4 g, 8.6 mmol) was added. To this ice-cold solution, 30% H_2O_2 (15 ml, 6.6 mmol) was added dropwise for 2 h with constant stirring at room temperature. This solution was stirred at room temperature for 14 h then diluted with H_2O, and the precipitate was collected on a celite pad by vacuum filtration. The pad was eluted with hot DMF and the eluent was evaporated to dryness, and the resulting pale-solid compound '2' was obtained (2.70 g, 82% yield).

Camptothecin 7-carboxylic acid (hapten 2). The structure and synthetic route of 'hapten 2' (18) are presented in Fig. 1A. Starting from a solution containing the compound '2' (1.00 g, 2.6 mmol) dissolved in 10 ml H_2SO_4 (18 mmol), potassium dichromate (1.12 g, 3.82 mmol) was added while stirring in a cooling bath containing ice and salt. The mixture was stirred at room temperature for 2 h and then diluted with H_2O. The precipitate was collected by suction, and the solid substance was purified by recrystallisation (815 mg, 79% yield) to give the 'hapten 2' (Fig. 1A), exhibiting a melting point of 280˚C.

The results of the ^{1}H-NMR and ^{13}C NMR (500 MHz; DMSO-d6; δ ppm) were as follows: 0.85 (3H, t, J =15.0 Hz, C19-H), 1.84 (2H, q, J =15.0 Hz, C18-H), 5.23 (2H, s, C5-H), 5.44 (2H, s, C17-H), 6.58 (1H, brs), 7.19 (1H, s, C14-H), 7.56-8.21 (4H, m, ArH); and 8.03 (C19), 30.91 (C18), 50.70 (C5), 66.82 (C17), 76.39 (C20), 95.56 (C14), 119.41 (C16), 128.16, 128.48, 129.01, 129.53, 130.29, 130.92, 132.04, 145.81, 146.43, 148.44, 152.95 (C2, C3, C6, C8-C15), 157.02 (C16a), 167.54 (C21), 172.83 (C22) [MS m/z 393 $(M+H)^+$].

Immunogen production by conjugation via the active ester method. Haptens were attached to BSA or KLH to use them as immunogens. The haptens were conjugated to produce immunogens using the active ester method, as previously described (19). The synthetic route for the active ester method is presented

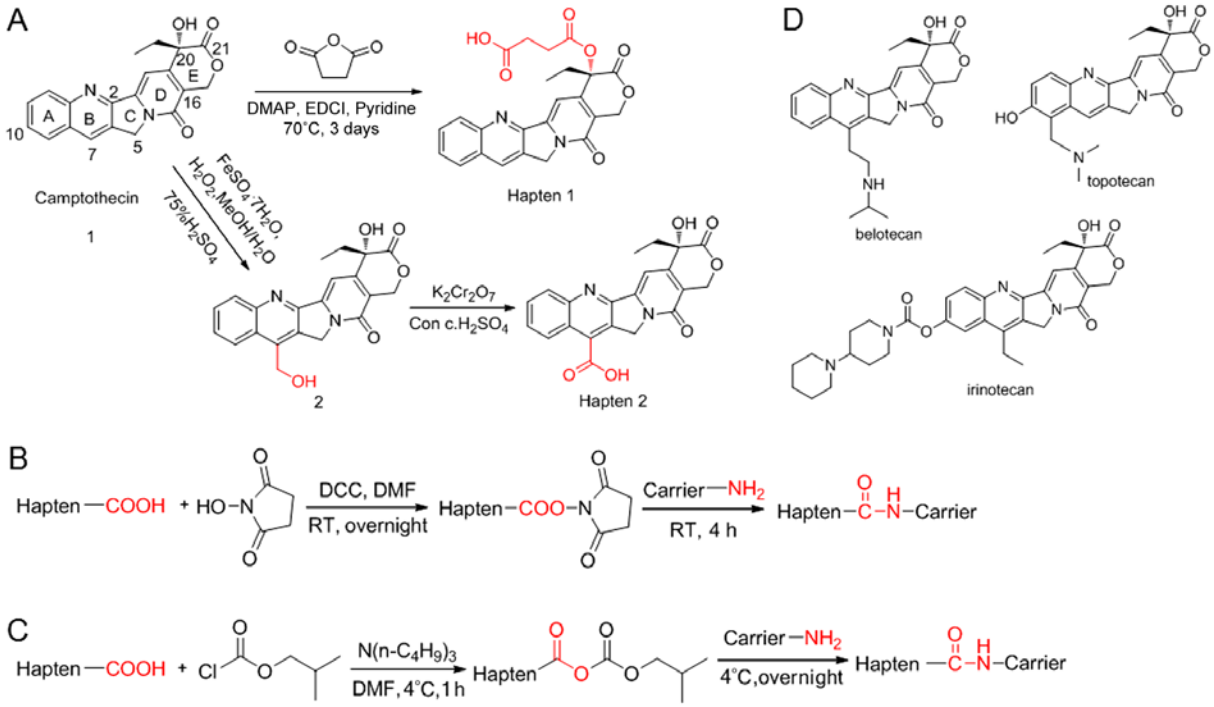

Figure 1. Structures and synthetic routes of haptens and hapten-protein conjugates. (A) Structure of camptothecin and synthetic route of hapten 1 and hapten 2. Red group in hapten 1 indicates the added succinic functional group. Red group in hapten 2 indicates the substituted and oxidized functional group. (B) Synthetic route of the active ester method. Red groups of haptens, carriers and N-hydroxysuccinimide indicate the functional groups involved in the chemical reaction. (C) Synthetic route of the mixed anhydride method. Red groups in haptens, carriers and isobutylchlorocarbonate indicate the functional groups involved in the chemical reaction. (D) Structures of three camptothecins. Con c.H_2SO_4, high concentration of H_2SO_4; DMAP, dimethylaminopyridine; DCC, N,N-dicyclohexylcarbodiimide; DMF, dimethyl formamide; EDCI, 1-(3-dimethylaminopropyl)-3-ethyl carbon diimide hydrochloride; RT, room temperature.

in Fig. 1B. Hapten 1 (22.4 mg, 0.05 mmol) was dissolved in DMF (0.5 ml). Then, NHS (6.9 mg, 0.06 mmol) was added, followed by addition of DCC (12.36 mg, 0.06 mmol) dissolved in 0.2 ml DMF. After stirring overnight at room temperature in the dark, the mixture was centrifuged at 10,000 x g at room temperature for 10 min. Then, the supernatant was collected for the following step. BSA (33 mg, 0.5 μmol) was dissolved in 5 ml 10% CBS-PBS. The supernatant was added to the protein solution dropwise and stirred for 4 h at room temperature. The method used to conjugate KLH to hapten 1 was the same that used for BSA, with the exception that KLH was 75 mg (0.25 μmol). Conjugates were dialyzed in PBS at 4˚C for 2 days to allow separation of the unreacted haptens. Purified conjugate solutions were then freeze-dried. UV-vis spectral data were used to confirm the structures of the final conjugates as previously described (18). Same method were used for the synthesis of the active ester of hapten 2 conjugated to BSA. The hapten densities of the conjugates, defined as the number of hapten molecules per molecule of protein, were directly estimated by the molar absorbance ε (20), with the following formula: Hapten density=($^{\varepsilon}$conjugates-$^{\varepsilon}$protein)/$^{\varepsilon}$hapten.

Coating antigen production by conjugation via the mixed anhydride method. Haptens were attached to OVA as coating antigens. The method of conjugation used for coating antigens was the mixed anhydride method, as previously described (21). The synthetic route for the mixed anhydride method is presented in Fig. 1C. Hapten 1 (22.4 mg, 0.05 mmol) and 23 μl tri-n-butylamine (9.26 mg, 0.05 mmol) were dissolved in DMF (0.5 ml) and cooled to 4˚C. To the resulting solution, 7 μl isobutylchlorocarbonate (0.05 mmol, 6.829 mg) was added, and the mixed anhydride was stirred at 4˚C for 1 h. Then, the mixed anhydride was collected for the following steps. OVA (30 mg, 0.5 μmol) was dissolved in 5 ml PBS. The OVA solution was added to the protein solution dropwise, and stirred gently for 30 min at room temperature and then overnight at 4˚C. Conjugates were dialyzed in PBS at 4˚C for 2 days to allow the separation of unreacted haptens. The purified conjugate solutions were then freeze-dried. The same method was used for the synthesis of the mixed anhydride of hapten 2 conjugated to OVA. Confirmation of the structures of the final conjugates was performed by UV-vis spectroscopy as aforementioned in the description of the active ester method.

MAb preparation. Female BABL/c mice, (n=9; 6-8 weeks; 15-18 g) were supplied by the Xi'an Jiaotong University Medical Laboratory Animal Center. Mice were were housed at a temperature of 23˚C with 50% humidity under a standard 12:12-h light/dark cycle, with access to specific pathogen-free-grade food and water *ad libitum*. Mice were subcutaneously immunized with a mixture of an immunogen (100 μg/mouse) diluted in PBS and Freund's complete adjuvant (v/v 1:1). At 2 weeks following the initial injection, booster injections of an equal quantity of immunogen were given, with Freund's incomplete adjuvant instead of Freund's complete adjuvant. Then, blood (0.2 ml/mouse) was collected from the tail vein, centrifuged at 1,000 x g at 4˚C for 3 min, and the antisera were collected and tested to determine the anti-hapten antibody titer by a non-competitive indirect ELISA coated with the corresponding antigen. Immunized mouse

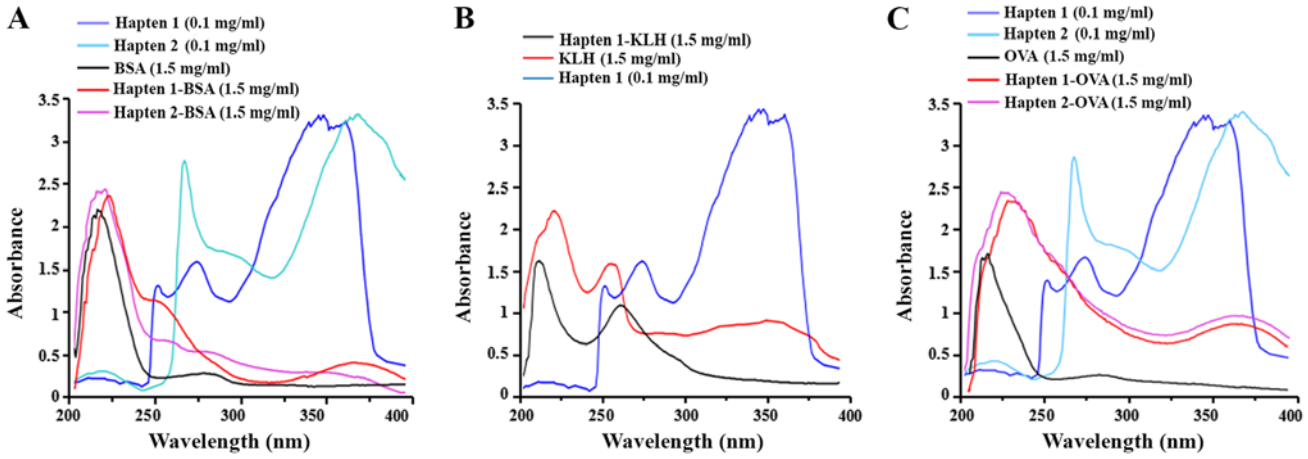

Figure 2. UV absorbance spectrum of haptens, carrier proteins and hapten-protein conjugates solutions in PBS. Concentration of all analytes was 100 μg/ml. Haptens, carrier proteins and hapten-protein conjugates presented in different panels were measured under the same conditions. (A) Hapten-BSA conjugates. Hapten 1 profile is shown in blue. Hapten 1-BSA profile is presented in black. BSA profile is presented in red. (B) Hapten-KLH conjugates. Hapten 1-KLH profile is presented in black. KLH profile is presented in red. (C) Hapten 1-OVA conjugates. Hapten 1-OVA profile is presented in black. OVA profile is presented in red. OVA, ovalbumin; KLH, keyhole limpet hemocyanin.

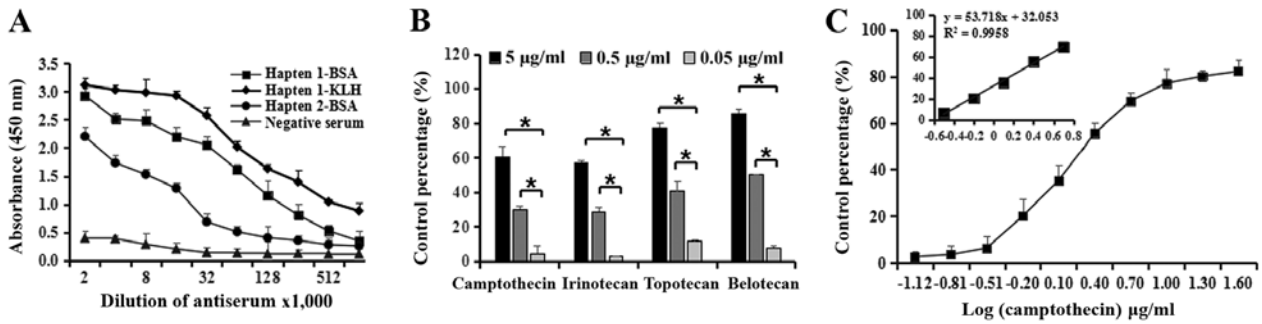

Figure 3. Indirect ELISA results of antisera and four camptothecins. (A) Non-immunized serum collected 1 week before immunization from every mouse was used as control serum. (B) Inhibition rate for four camptothecins at various concentrations by indirect ELISA. MAb 5A3 was diluted 1:128,000, with a final concentration of 0.014 μg/ml. (C) Inhibition curve and linear regression analysis of camptothecin by indirect ELISA. MAb 5A3 was diluted 1:128,000, with a final concentration of 0.014 μg/ml. Data are presented as the mean ± standard deviation (n=3). *P<0.05. R^2, angular coefficient; KLH, keyhole limpet hemocyanin; MAb, monoclonal antibody.

splenocytes were obtained by extruding the mouse spleen and filtering with 200-mesh stainless steel mesh. Then, splenocytes were mixed with Sp2/0 murine myeloma cells (ratio of mice splenocytes to myeloma cells, 5-10:1) and centrifuged at 200 x g at 4˚C for 10 min. Then, 50% polyethylene glycol 1500, hypoxanthine-aminopterin-thymidine medium (RPMI 1640 supplemented with 100 μM hypoxanthine, 16 μM thymidine and 0.4 μM aminopterine) and hypoxanthine-thymidine medium (RPMI 1640 supplemented with 100 μM hypoxanthine and 16 μM thymidine) were used for the selection of targeted hybridoma cells. The cells were cultivated at 37˚C and 5% CO_2 for 7 days. The supernatants of targeted hybridoma cell cultures were collected, and antibodies were detected using non-competitive indirect ELISAs for hybridoma screening. Selected hybridomas were cloned by limiting dilution (22), and stable antibody-producing clones were expanded. Competitive indirect ELISA was then employed to identify the antibodies that reacted with camptothecin. The selected clones were cryopreserved in liquid nitrogen.

MAb purification. Saturated ammonium sulfate precipitation was performed for the purification of MAb 5A3. Mice were intraperitoneally injected with hybridoma cells 5A3, then ascites were removed from mice 1 week later, and centrifuged at 2,000 x g at 4˚C for 5 min. The supernatant was mixed with PBS, then equal volumes of saturated ammonium sulfate solution was added and stirred evenly; the mixture was then refrigerated at 4˚C overnight. The mixture was centrifuged at 4˚C at 10,000 x g for 20 min. The precipitate was dissolved in PBS, and the procedure described above was repeated twice. Finally, the precipitate was dissolved in 5 ml PBS and dialyzed with a large volume of PBS at 4˚C, and then frozen at -20˚C. The subtypes of purified antibodies were identified using an SBA Clonotyping System/HRP kit according to the manufacturer's protocols.

ELISA. For the non-competitive indirect ELISA, 96-well plates were coated with 100 μl/well of coating antigens (2 μg/ml in CBS) and incubated overnight at 4˚C. The plates were washed three times with PBST solution (10 mM PBS containing 0.05% Tween 20; pH 7.4) and then blocked with 200 μl/well 2% BSA (mg/ml) for 2 h at 37˚C. After three washes with PBST, the 96-well plates were incubated with 100 μl/well of serum, supernatant (containing 0.4 mg/ml antibody) or

Table I. Ratios of hapten/protein conjugates.

Hapten	Conjugate	Binding ratio to carrier protein	Wavelength, nm
Hapten 1	BSA	48	347
	KLH	65	
	OVA	30	
Hapten 2	BSA	43	276
	OVA	28	

OVA, ovalbumin; KLH, keyhole limpet hemocyanin.

purified antibody solution (2.8 mg/ml) serially diluted in PBS for 1 h at 37˚C. The plates were washed three times with PBST. Then, 100 μl/well of goat anti-mouse IgG-HRP diluted 1:4,000 with 1% BSA (mg/ml) was added. After incubation for 1 h at 37˚C and three washes with PBST, 100 μl/well TMB solution (400 μl 0.6% TMB-DMSO and 100 μl 1% H_2O_2 diluted in 25 ml CPBS) was added, and plates were incubated for 10 min at 37˚C. To stop the reaction, 50 μl H_2SO_4 (2 M) was added per well, and the absorbance was measured at 450 nm.

For checkerboard assays (22), antibodies (2.8 mg/ml) and coating antigens (2 mg/ml) were titrated using sequential concentrations. All procedures were the same as the non-competitive indirect ELISA, and were conducted under the same conditions. These assays were used to obtain an approximate estimate of the appropriate coating antigen and antibody concentrations for further competitive assays.

In the competitive indirect ELISA, the coating antigen (2 μg/ml) and antibodies (0.014 μg/ml) were used. All procedures were the same as those used for the non-competitive indirect ELISA, and were performed under similar conditions with the exception of the addition of antigen homologues to competitively bind the antibodies. To each well, 50 μl camptothecins dissolved in 10% methanol-PBS and 50 μl antibody (0.014 μg/ml) diluted in PBS were added. All samples were tested on three different days, and the final titer value was calculated as the average of three separate experiments. The cross-reactivity was calculated with the following formula: Cross-reactivity=(IC_{50} of camptothecin/IC_{50} of other compounds) x100%.

Linear regression analysis. Competition curves were obtained by plotting the inhibition rate against the logarithm of the analyte concentration. The five values showing a linear trend in the sigmoidal curves were selected as the numerical basis of the linear regression analysis and the linear regression equation. Using the linear regression equation, the concentration corresponding to the inhibition rate within this range was calculated. The IC_{50} and IC_{10} were determined with the following formula based on the optical density (OD) of samples: Inhibition rate=[($OD_{positive}$-OD_{sample})/$OD_{positive}$] x100%. Hapten 1 was used as a positive control.

Statistical analysis. Data were collected and plotted using Microsoft Excel v16.0 (Microsoft Corporation) and SPSS 22.0 software (IBM Corp.). Data are presented as the mean ± standard deviation. Data were analyzed with ANOVA followed by Tukey's test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Hapten synthesis. Hapten 1, a 20-O-linked succinate-based camptothecin ester derivative, and hapten 2, a water-soluble 7-substituted camptothecin analogue, were synthesized (Fig. 1A). Camptothecin was reacted with succinic anhydride to synthesize hapten 1 as previously described (23). By contrast, hapten 2 was synthesized from camptothecin by substitution and oxidation, as previously described (24). The structures of these products were confirmed by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry. Haptens with substituents at different sites were designed to investigate the effect of various hapten analogues on the immunisation sensitivity. In addition, the structures of the spacer arms of these two haptens varied, potentially affecting the sensitivities of the two haptens.

Identification of artificial antigens and coupling ratio. The immunizing conjugates were prepared using different activation methods. The mixed anhydride method was used to prepare the coating antigens, whereas the active ester method was conducted to obtain the immunogens. Analysis of the UV-vis spectra is an effective method for the verification of the conjugation and estimation of the hapten/protein ratio. UV-vis spectra were obtained by scanning the samples using wavelengths of 200-400 nm (25). The haptens, carrier proteins and conjugates were all analyzed by UV-vis spectrometry (Fig. 2). The profiles of the three curves were distinct, and the values differed between haptens, carrier proteins and conjugates. The absorbance patterns of the conjugates were different from the corresponding carriers, suggesting that the hapten was successfully conjugated to the carrier protein. Hapten/protein ratios were estimated by spectroscopy at the same wavelength (18). A specific wavelength for each hapten was selected to detect the absorbance values of haptens, carrier protein and conjugate at selected concentrations, and the values of the molar absorbance coefficients were subsequently calculated. The concentration of all analytes tested was 100 μg/ml, and the results are presented in Table I. The present results suggested that the hapten/protein ratios varied with different carrier proteins and coupling methods.

Selection of the immunogens. To determine the ideal hapten for eliciting the production of antibodies against camptothecin, 6 mice were divided into two groups, and injected with hapten 1-BSA or hapten 2-BSA. Following the administration of booster injections (22), antisera from these mice were collected and tested for the presence of antibodies that recognized the corresponding immunizing hapten by non-competitive indirect ELISAs using hapten 1-OVA and hapten 2-OVA. The results of the titration experiments are presented in Table II. The present results suggested the presence of a marked difference in titer values between hapten 1-BSA and hapten 2-BSA, with a higher antibody titer generated from the immunogen hapten 1-BSA compared with the hapten 2-BSA. The antiserum titer curves of hapten 1-BSA and hapten 2-BSA determined by indirect ELISA

Table II. Summary of titers of antisera.

Antiserum	Sample	Boost				
		1st	2nd	3rd	4th	5th
Hapten 1-BSA						
	Mouse 1A	1:3,200	1:16,000	1:256,000	1:256,000	1:512,000
	Mouse 2A	1:1,600	1:8,000	1:128,000	1:256,000	1:256,000
	Mouse 3A	1:800	1:4,000	1:32,000	1:32,000	1:64,000
Hapten 2-BSA	Mouse 1B	1:400	1:800	1:3,200	1:3,200	1:6,400
	Mouse 2B	1:200	1:600	1:1,600	1:3,200	1:3,200
	Mouse 3B	1:800	1:1,600	1:3,200	1:6,400	1:12,000
Hapten 1-KLH	Mouse 1C	1:6,400	1:128,000	1:256,000	1:512,000	1:512,000
	Mouse 2C	1:6,400	1:256,000	1:512,000	1:1,024,000	1:1,024,000
	Mouse 3C	1:6,400	1:128,000	1:512,000	1:512,000	1:1,024,000

Titer of antisera was defined as the antiserum dilution that induced a 2.1-fold increase in the absorbance compared with the control serum. In total, 2 μg/ml hapten-OVA was used as coating antigen. OVA, ovalbumin; KLH, keyhole limpet hemocyanin.

Table III. Cross-reactivities and limit of determination of camptothecins.

Compound	IC_{50}, μg/ml	IC_{10}, μg/ml	Cross-reactivity, %	R^2 of the linear equation
Camptothecin	2.19±0.08	0.39±0.02	100.00	0.9958
Irinotecan	2.85±0.10	0.47±0.01	76.63	0.9943
Topotecan	0.68±0.01	0.19±0.01	321.27	0.9884
Belotecan	0.87±0.03	0.22±0.01	250.84	0.9958

Results are presented as the mean ± standard deviation from three experiments. R^2, angular coefficient.

are presented in Fig. 3A. The present results suggested that hapten 1 may be more suitable for the generation of antibodies against camptothecin. Different carrier proteins were conjugated with hapten 1 to examine the effects of the carrier protein on the immunogenicity of hapten. Mice were immunized with hapten 1-KLH and hapten 1-BSA. Mouse antisera samples were screened against hapten 1-OVA using a non-competitive indirect ELISA. The results of the titration experiments are presented in Table II. The titration of hapten 1-KLH was observed to be higher than hapten 1-BSA, and the antiserum titer curves of hapten 1-KLH and hapten 1-BSA determined by indirect ELISA exhibited distinct profiles (Fig. 3A). The titer of antibodies generated from the immunogen hapten 1-KLH was higher compared with hapten 1-BSA. Therefore, the present results suggested that KLH was a superior carrier protein for MAb preparation. Therefore, hapten 1-KLH was selected for the generation of antibodies against camptothecin.

Establishment of ELISA using MAb 5A3. The MAb against camptothecin, MAb 5A3, was purified from ascites in sensitized BALB/c mice. The identified subtype of MAb 5A3 against camptothecin was IgG1 with κ-light chains, as determined by using an SBA Clonotyping System/HRP kit. An ELISA was used to analyze the titer and the limit of determination value of MAb 5A3. The inhibition rate for camptothecin was determined using checkerboard assays, and the concentration of hapten 1-OVA and MAb 5A3 was determined as aforementioned. Then, a standard curve for camptothecin was obtained by plotting the inhibition rate against the concentration of camptothecin, and the linear relation graph for camptothecin was calculated (Fig. 3C). The present results suggested an I_{50} value of 2.1898 μg/ml with a detection limit of 0.3886 μg/ml (I_{10}) for MAb 5A3. According to the linear relation diagram of the sigmoidal curve, the working range for MAb 5A3 ranged between 0.5965-7.8085 μg/ml, which were defined as I_{20} and I_{80}, respectively. The present results suggested that the ELISA established using MAb 5A3 was stable and sensitive for the detection of camptothecins. Therefore, MAb 5A3 was selected for subsequent experiments.

Cross-reactivity. To evaluate the specificity of MAb 5A3, irinotecan, topotecan and belotecan were used to test the cross-reactivity of the developed assay. The inhibition rate for the four camptothecins is presented in Fig. 3B. The present results suggested that the inhibition rate of topotecan and belotecan was increased compared with camptothecin and irinotecan. The IC_{50}, IC_{10}, cross-reactivity and the angular coefficient of the linear equations for each molecule are

presented in Table III. The highest cross-reactivity was identified for topotecan (321.27%), followed by belotecan (250.84%) and irinotecan (76.63%). The present results suggested that the developed assay was more sensitive for topotecan and belotecan than irinotecan. The structures of irinotecan, topotecan and belotecan are presented in Fig. 1D. The structures of topotecan and belotecan are similar to the structure of camptothecin, whereas irinotecan present marked differences compared with camptothecin; however, these three compounds exhibited high cross-reactivity with camptothecin (Fig. 1A).

Discussion

For a specific immunoassay, a critical step is the selection of the haptens (22). Importantly, the selected hapten should preserve the structure of the target compound (26). Hapten design is important for the development of effective immunoassays for small molecular compounds (27). In the present study, various haptens for camptothecin were designed. As pentacyclic alkaloids maintain a planar structure, numerous analogues of camptothecin were synthesized by semi-synthetic approaches based on A-, B-, C-, D- and E-ring modifications (28,29). Due to high toxicity, relative instability and rapid inactivation of camptothecins by lactone ring hydrolysis at a physiological pH, the present study designed haptens based on B- and E-ring modifications in order to investigate the effects of hapten analogues on the sensitivity of immunization.

The prediction of the effects of heterogeneous haptens on the immune response is challenging, due to differences in the immune response among individual animals (30). Additionally, the ability to discriminate the immune responses induced by haptens containing distinct groups in different sites is limited (31). However, the present results suggested conserved trends among the antibodies induced by the injection of hapten 1. Hapten 1 was synthesized using succinic anhydride. Notably, the biological life span of the lactone form of 20-O-alkyl camptothecin ester in human and mouse plasma is significantly higher than succinate-based camptothecin ester (32). Therefore, the succinate-based camptothecin ester may be more suitable for designing haptens, as succinate is not only relatively stable under physiological conditions, but it has also been extensively used as a spacer in the preparation of conjugates or pro-drugs (33). Hapten 1 contains a more stable lactone form and demonstrated decreased cytotoxicity of the 20-hydroxyl group, which was masked by esterification with succinic anhydride. In addition, the lactone E-ring, which includes an α-hydroxy lactone ring with (S)-configuration, is characterized by the presence of an (R)-enantiomer and (S)-enantiomer (34). Therefore, hapten 1 may possess more exposed camptothecin-specific functional groups compared with hapten 2.

The carrier protein is another important factor that affects the immune effects of immunogens (35). The differences in antisera affinity between hapten 1-KLH and hapten 1-BSA may be due to distinct immunogenicities and hapten-carrying capabilities of the carrier proteins. KLH, derived from molluscs, is less conserved in mammalian species than BSA, and therefore produces antibodies that are less likely to cross-react with typical target samples (34). Due to its high molecular weight and complexity, KLH elicited a stronger immune response than other carrier proteins (32). KLH is a large protein that possesses hundreds of primary amines and carboxyl groups, whereas BSA contains a total of 59 lysine-amine groups that are prone to be conjugated with other factors (35). Collectively, KLH is a more suitable carrier protein for haptens.

The present study aimed to develop an ELISA for the detection of camptothecins, and a MAb 5A3 against the designed camptothecin hapten 1, 20(s)-O-succinyl-camptothecin, was identified. Additionally, the characteristics of MAb 5A3 were examined with a competitive indirect ELISA. MAb 5A3 showed high specificity to camptothecin and its three derivatives. Although the developed assay does not meet the criteria for the detection of all camptothecins, the present results suggested that it was able to efficiently detect various common camptothecins. Therefore, the present study suggested the feasibility of the detection of certain camptothecins using immunochemical methods. Collectively, the developed assay could be used for a variety of applications such as compound analysis, clinical applications, and analyses of food and environmental samples.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CZ, LY and YL conceived and designed the experiments. LY and XN performed the experiments. LY drafted the manuscript. HW and XH analyzed the data. JH interpreted the data and critically revised the manuscript for important intellectual content. YL supervised all research and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocols for animal studies were approved by The Committee on the Ethics of Animal Experiments of the Shaanxi Provincial People's Hospital (approval no. 01-0420).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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