Research Article

Crystalline Camptothecin-20(*S*)-O-Propionate Hydrate: A Novel Anticancer Agent with Strong Activity against 19 Human Tumor Xenografts

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Abstract

To find a more effective and less toxic chemotherapeutic agent, we have successfully prepared crystalline camptothecin-20(*S*)-O-propionate hydrate (CZ48) by reacting camptothecin with propionic anhydride using concentrated sulfuric acid as catalyst. The biological effectiveness of this new anticancer agent was evaluated by using xenografts of human cancers in nude mice as the testing models. The extensive treatment of 21 human tumors with various dose levels of CZ48 has shown that this agent is highly effective against many different human tumors tested with a striking lack of toxicity. Of the 21 human tumor lines tested, 9 regressed, 5 were <10% of the control, 3 were <20%, and 2 were <40%. Two tumors did not respond. The total response rate was 90% (19 of 21). No toxicity was observed in mice. The effective doses required to achieve the positive response varied from 100 to 1,000 mg/kg/d depending on the tumors. The maximum tolerated dose was not reached because of the nontoxic nature of the drug in mice. Thus, this compound has a much wider therapeutic index compared with that of the existing anticancer drugs currently in use. [Cancer Res 2009;69(11):4742–9]

Introduction

Aside from the cancer itself, the biggest enemy that most cancer patients still face, while undergoing treatment with chemotherapy, is the high toxicity (or side effects) associated with the various anticancer drugs. The therapeutic index (TI), defined as a ratio of the maximum tolerated dose (MTD) to the effective dose, of each anticancer drug provides a quantitative assessment of its relative toxic and therapeutic effects. As most chemotherapeutic agents clinically used today have very narrow TI ranges (~1), patients receiving chemotherapy often suffer greatly from many toxic side effects while receiving treatment at therapeutic dose levels. The various anticancer agents are characterized by differences in grades and types of toxicity, influenced by the specifics of their pharmacokinetics and pharmacodynamics. For example, 5-fluorouracil (5-FU) is one of the most commonly used chemotherapeutic agents for the systemic and palliative treatment of patients with cancers arising from the gastrointestinal tract, breast, head, and neck. In the catabolism of 5-FU, dihydropyrimidine dehydrogenase (DPD) serves as the initial and rate-limiting enzyme. A DPD deficiency is increasingly being recognized as an important pharmacogenetic condition involved in the etiology of severe 5-FU–associated toxicity. It has been reported that cancer patients who were genetically heterozygous or homozygous for a mutant allele of the gene encoding DPD suffered from more severe toxicity and even death following the administration of 5-FU (1, 2). Platinum agents, also commonly used in chemotherapy, exhibit other toxicities; a substantial body of literature documents the side effects of platinum compounds. Cisplatin, for instance, has multiple toxicities, including nephrotoxicity, neurotoxicity, ototoxicity, nausea, and vomiting (3). The nephrotoxicity of cisplatin almost led to its abandonment until Cvitkovi and colleagues (4, 5) introduced aggressive hydration, which prevented the development of acute renal failure. Thus, the toxicity of cisplatin actually became a driving force in the history of chemotherapy in the search both for less toxic analogues and for more effective treatment of the side effects of the drug. For other platinum agents such as carboplatin, myelosuppression, which is not usually severe with cisplatin, presents as the dose-limiting toxicity (6), and for oxaliplatin, the dose-limiting toxicity comes from sensory neuropathy (3), a characteristic of all DACH-containing platinum derivatives. Alkylating agents are another class of drugs with an important role in cancer treatment. Each alkylating agent has its own specific associated toxicity and will not be discussed individually here. Instead, listed here are some toxicities common to alkylating agents as a class: hematopoietic toxicity, gastrointestinal toxicity, gonadal toxicity, pulmonary toxicity, alopecia, teratogenicity, carcinogenesis, and immunosuppression. Among these, the usual dose-limiting toxicity for an alkylating agent is its hematopoietic toxicity. Finally, topoisomerase-interactive agents comprise yet another class of chemotherapeutic drugs that have increasingly gained attention from clinical oncologists for their unique mechanisms of action. Topotecan, one of these topoisomerase-interactive agents, is indicated in the second-line treatment of advanced refractory ovarian (7) and small cell lung cancers (8, 9), and it also has been active in the treatment of hematologic malignancies, including myelodysplastic syndromes and multiple myeloma (10). The dose-limiting toxicity of this agent is myelosuppression. Although topotecan has been combined with a variety of other treatments, including radiation, cisplatin, paclitaxel, and doxorubicin, in clinical trials, none of these combinations has achieved any routine use in clinical oncology. This may be due, in part, to the frequent myelosuppressive toxicity of topotecan that has made it difficult to combine in high doses with other bone marrow–suppressive agents (11). Irinotecan, another topoisomerase-interactive agent, is indicated as a single agent or in combination with 5-FU and

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doi:10.1158/0008-5472.CAN-08-4452

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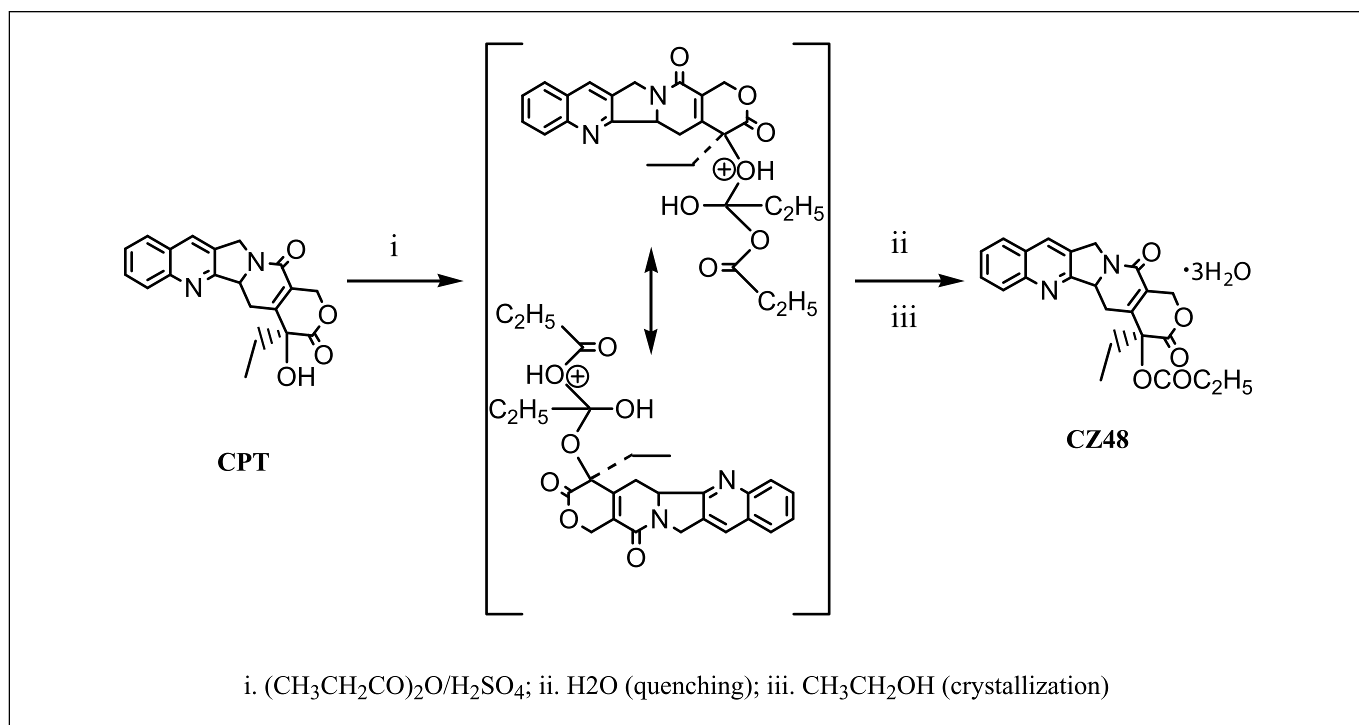

Figure 1. The reaction pathway for synthesizing CZ48.

leucovorin in treating patients with colorectal cancers (12, 13) and has also been found to be active in small cell lung cancer when given in combination with cisplatin. This combination has also been found to be active in non–small cell lung cancer (10). The dose-limiting toxicities of irinotecan are neutropenia and delayed-onset diarrhea; the uses of irinotecan in clinical oncology are thus limited. Other anticancer agents, including recently marketed Erbitux and Avastin, occasionally used by oncologists for specific treatments, are likewise limited due to their associated toxicities. Thus, the biggest challenge is still for cancer researchers and clinical oncologists to find better chemotherapeutic agents with wider TIs.

As a class, the camptothecins have a broad spectrum of anticancer activity. Wall and colleagues (14) isolated and purified the molecule from the Chinese tree *Camptotheca acuminate* in 1966; they subsequently tested the compound against mouse leukemia L1210 system and found that the compound was active. However, the early human clinical trials in 1970s failed to prove the true values of the compound for clinical oncology, instead showed severe, unpredictable toxicities, such as hemorrhagic cystitis. The trials were accordingly halted. The interest in this family of compounds was renewed in the mid-1980s by the finding that the molecular target of camptothecins was the nuclear enzyme topoisomerase I (15). At approximately the same time, new water-soluble derivatives such as topotecan and irinotecan were prepared and biologically evaluated. The subsequent clinical evaluations of these two compounds showed the predictable toxicities and meaningful anticancer activity (16). Topotecan was approved in 1996 as second-line treatment for advanced ovarian cancer, and it later gained the indication for treating patients with refractory small cell lung cancer. In the same year, irinotecan was approved for treating 5-FU–refractory advanced colorectal cancer. The *S*-configured lactone form of camptothecin molecule is thought to be required for antitumor activity. The carboxylate form is only 10% as active as the lactone form as an anticancer agent. The two forms of the molecule exist in equilibrium in aqueous solution. This equilibrium is pH dependent. The lactone is not stable in the body of mammals at the slightly basic physiologic pH (7.4). Even worse, in man, the human serum albumin has a high affinity for the carboxylate form of the molecule (17, 18), binding it and moving the equilibrium between it and the lactone form to the right ($CPT \leftrightarrow CPT^-$). Because of this, when we treated human tumors as xenografts in nude mice with camptothecin or its derivatives such as 9-nitrocamptothecin and 9-aminocamptothecin, we obtained excellent results (19, 20), but when we went into human clinical trials, the complete responses became sporadic (21). This is no surprising because in mice 50% of the CPT was in the active lactone form (22), whereas in man the percentage dropped to 3% to 5%.

In efforts of finding better camptothecin analogues for treatment, we previously synthesized many different camptothecin esters by attaching an ester chain in position C-20 and subsequently evaluated these compounds (23, 24). The treatment of human tumors grown as xenografts in nude mice with our synthetic camptothecin esters was effective and toxicity in mice was minimal (25, 26). Based on these results, we went deeper in the direction and successfully prepared crystalline camptothecin-20(*S*)-O-propionate hydrate (CZ48). We subsequently tested this new agent given by gavage against 21 different human tumors grown as xenografts in mice and found that this compound has a broad spectrum of antitumor activity with a dramatic lack of toxicity in mice at variable dose ranges. The effective doses required for various tumors were established varying from 100 to 1,000 mg/kg/d.

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The corresponding TIs were also calculated and found to be tremendously improved compared with that of most anticancer agents clinically used today by oncologists. We now report our results.

Materials and Methods

Preparation of crystalline CZ48. To a 200 mL round-bottomed flask equipped with a magnetic stirrer and a sand bath were added 20 g camptothecin (0.05747 mole) and 100 mL propionic anhydride (97%; Aldrich Chemical Co.). The mixture was heated by sand bath while stirring. A few drops (8–10) of concentrate sulfuric acid (95–98%; A.C.S. reagent; Aldrich Chemical) were added dropwise when the sand bath temperature reached 80°C. The mixture was then stirred at 110 ± 10°C overnight (∼14 h). After cooling down to room temperature, the reaction mixture was poured onto 1,000 mL ice water portion by portion while stirring. After keeping stirring for ∼45 min, the mixture was filtrated. The residue obtained from filtration was allowed air-drying for 24 h. The dried crude product was transferred into a 500 mL round-bottomed flask equipped with a heating mantle. To this crude product was added 200 mL absolute ethanol (99.5%, 200 proof; Aldrich Chemical). The mixture was allowed to reflux for 2 h and then cooled to room temperature. The pure product (CZ48) was obtained as crystals after crystallization from ethanol. Purity 99.8% [high-performance liquid chromatography (HPLC)], yield 97%, melting point 242°C. A single crystal of CZ48 for X-ray structural analysis was prepared by dissolving 200 mg samples obtained above in 50 mL absolute ethanol.

Crystal structural determination of CZ48. Single-crystal X-ray analysis was done by using a Siemens SMART diffractometer equipped with charge-coupled device area detector. A crystal with dimensions of 0.4 × 0.08 × 0.02 mm was mounted in glass fiber under a stream of cold nitrogen gas at −60°C. Monochromatic Mo $K_{\alpha 1}$ radiation (λ = 0.71073 Å) was used to collect a full hemisphere of data with the narrow-frame method. The data were integrated using the Siemens SAINT program, and the intensities were corrected for Lorentz factor, polarization, air absorption, and absorption due to variation in the path length. Empirical absorption correction was applied and redundant reflections were averaged. Final cell parameters were

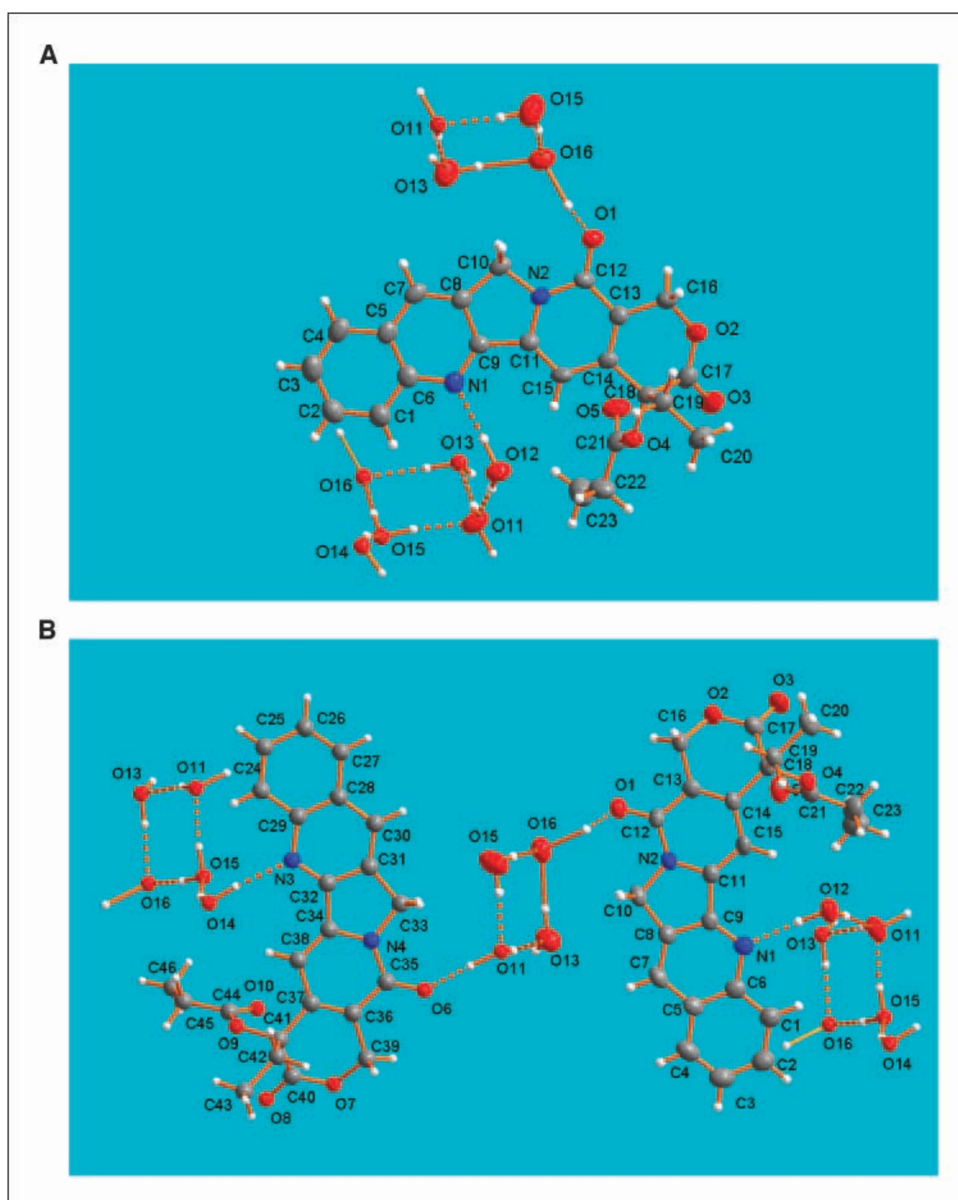

Figure 2. The three-dimensional structure of CZ48. *A,* ORTEP diagram of a single molecule of $C_{23}H_{20}N_2O_5 \cdot 3H_2O$. One molecule of CZ48 is linked to three molecules of water through strong hydrogen bonds. All of the molecules in a crystal unit are linked to each other through a bridge made of H_2O molecules. *B,* ORTEP diagram of a dimer, $[C_{23}H_{20}N_2O_5 \cdot 3H_2O]_2$, showing how two molecules of CZ48 are linked by a water bridge.

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Table 1. Antitumor activity of CZ48 against human xenografts in nude mice

Tumor line		Dose (mg/kg)	Route	Length (d)	Tumor size changes from day 0 to day last (mm^3)							
					Day 0		Day last		Change		Inhibition (vs control)	
					Control	Test	Control	Test	Control	Test	IR (%)*	GR (%) †
Bladder	BOL	200	Gav	41	400	467	5,001	586	+4,601	+119	97.4	2.6
Breast	BEN	300	Gav	65	461	445	2,352	220	+1,891	−225	>100	NG
	CLO	100	Gav	53	264	300	3,302	11	+3,038	−289	>100	NG
	MUR	300	Gav	45	271	230	2,102	60	+1,831	−170	>100	NG
	WAR	200	IS	104	367	363	3,496	164	+3,129	−199	>100	NG
Colon	HT29	300	Gav	41	730	734	7,005	180	+6,275	−554	>100	NG
	McC	1,000	Gav	25	237	238	7,673	1,117	+7,436	+879	88.2	11.8
	SQU	300	Gav	69	245	162	5,394	201	+5,139	+39	99.2	0.8
	SW48	200	Gav	49	753	763	6,358	799	+5,605	+30	99.5	0.5
DSRCT	MYE	100	Gav	110	570	570	5,101	675	+4,531	+165	96.4	3.6
	ZUC	300	Gav	26	363	361	4,936	434	+4,573	+73	98.4	1.6
Lung	DOY	200	Gav	41	488	498	8,325	276	+7,837	−222	>100	NG
	SPA	300	Gav	35	400	403	5,945	1,228	+5,545	+825	85.1	14.9
Melanoma	BRO	100	Gav	40	353	358	8,089	56	+7,736	=302	>100	NG
	SBC	1,000	Gav	41	222	249	4,142	60	+1,920	−189	>100	NG
Pancreatia	LIE	100	Gav	62	509	526	4,114	314	+3,605	−212	>100	NG
	MIA	1,000	Gav	37	457	463	2,604	1,227	+2,147	+764	64.4	35.6
	PAN	1,000	Gav	68	627	599	2,760	860	+2,133	+261	87.9	12.2
	SU86	1,000	Gav	33	602	602	4,800	2,174	+4,196	+1,572	62.5	37.5

Abbreviations: Gav, gavage; IS, intrastomach; IR, inhibition rate; NG, negative growth; GR, growth rate.
*Inhibition rate was expressed in % and calculated according to the following equation:

$$\text{IR (\%)} = \frac{\text{Net gain of control} - \text{Net gain of test}}{\text{Net gain of control}} \times 100.$$

† Growth rate was also expressed in % and calculated according to the following equation:

$$\text{GR (\%)} = \frac{\text{Net gain of test}}{\text{Net gain of control}} \times 100.$$

refined using 1,971 reflections having $I > 10\sigma$ (I). The tetragonal cell parameters are a = 15.008(2) Å, b = 6.977(1) Å, c = 21.810(3) Å, β = 99.959°, V = 2249.2(5) Å^3, Z = 4, ρ = 1.354 g/cm^3, 2θmax = 56.66°. The structure was solved by direct methods with space group $P2_1$ (No. 4) and refined by full-matrix least-squares calculations on F^2, and the thermal motion of all C, N, O atoms was treated anisotropically. The final R indices [$I > 2\sigma$ (I)], R1 = 0.0454, wR2 = 0.0763, R indices [all data], R1 = 0.1105, wR2 = 0.0933. All calculations were made with using the Siemens SHELXTL programs package.

***In vivo* antitumor activity determination.** All the animal experiments were performed on nude Swiss mice of the NIH, high-fertility strain. They were bred and raised in our laboratory under strict pathogen-free conditions. For antitumor activity determination, a tumor xenograft growing in a nude mouse, ~1 cm^3 in size, was surgically removed under sterile conditions, finely minced with iridectomy scissors, and suspended in cell culture medium at the ratio of 1:10 (v/v). One tenth to one quarter of 1 mL of this suspension, containing ~50 mg of wet weight tumor mince, was s.c. inoculated on the upper half of the dorsal thorax of the mouse. Groups of six animals were used. CZ48 was finely suspended in cottonseed oil and then injected into the stomach cavity of the mouse through the anterior abdominal wall using a 26-gauge needle or administered by gavage. The weekly schedule used for oral administration of CZ48 was once a day for 7 d, or 5 d on and 2 d off. This schedule was used throughout all the animal experiments. Treatment was initiated when the tumor had reached a volume of ~200 mm^3 (i.e., well vascularized, measurable, and growing exponentially). Tumors growing in animals were checked daily and measured with a caliper two times per week. The effective doses were established when a positive response in mouse was reached.

***In vivo* toxicity determination.** Groups of five or six animals having about the same ages and weights were chosen and treated with CZ48 by gavage at doses of 1,000 and 2,000 mg/kg/d, respectively, and continuously or on a schedule of 5 d on, 2 d off. The body weight changes in animals during the treatment were recorded for treated group versus untreated group starting at day 1.

The determination of pharmacokinetic parameters of CZ48. Camptothecin-20-O-acetate, an analogue of CZ48, was used as an internal standard for determining all pharmacokinetic parameters of CZ48. A 100 μL plasma from the mouse treated with CZ48 at dose of 2,000 mg/kg was transferred into a 2 mL test tube, and then 100 μL of internal standard working solution (400 ng/mL) were also added to the tube. To the mixture were also added 200 μL of 1% acetic acid solution and 1 mL ethyl ether. After vortex mixing for 10 s, the mixture was incubated at room temperature on a shaker for 10 min and then centrifuged at 10,000 × g for 15 min. The upper layer obtained from the centrifugation was transferred into a clean tube and evaporated to dryness using an

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evaporator at 40°C under a stream of nitrogen. The residue was reconstituted in 200 μL of water/acetonitrile (50/50, v/v) solvent system, and a 20 μL portion of the aliquot was injected into HPLC system for analysis. For the study, a 100 μL blank plasma from untreated mouse was also processed in the same manner as the treated. The important pharmacokinetic parameters of CZ48 and CPT in 48 mice were obtained from the HPLC analysis and this HPLC procedure was previously established in our laboratory (27).

Results

The reaction for synthesizing CZ48 is depicted in Fig. 1. The structure of CZ48 was determined by single-crystal X-ray analysis and shown in Fig. 2. In each crystal unit, one molecule of CZ48 is linked to three molecules of water through strong hydrogen bonds and all of the molecules are linked to each other through a H_2O bridge. CZ48 was tested against 21 different human tumors grown as xenografts in nude mice. The tumors included were one bladder, four breast, four colon, two desmoplastic small round cell tumor, two melanoma, two lung, and five pancreatic lines. The drug suspension was orally administered to the human tumor-bearing mice once a day, 7 days a week, or according to a schedule of 5 days on and 2 days off, for the duration of the treatment period. Table 1 shows that, of these 21 tumor lines tested, 19 showed either regressions or significant growth inhibitions (>50%). The length of treatment was different from tumor to tumor and was decided according to the growth rate of the tumors. Nine tumor lines regressed completely and the other 10 tumor lines showed percentage inhibitions versus controls ranging from 62.5 to 99.5. The continuous daily schedule of CZ48 administration showed a better response rate than the 5/2 (on/off) schedule of administration. The two schedules exhibited no differences with respect to toxicity in mice. The degree of inhibition was shown to be dose dependent. Figure 3*A* shows the direct correlation between the doses and the corresponding inhibitions. Body weight changes for both control and test animals were recorded during treatment as shown in Table 2, and no significant body weight changes were observed for most animals during the treatment. Only the Cln-McC and Cln-SW48 testing groups had 17% and 15% body weight losses, respectively. Animals in the Bl-BOL and P-MIA groups had body weight losses slightly >10%. All other treated groups at all dose levels had body weight losses <10%. Such small body weight changes may even be attributed to causes such as the tumor itself or initial response to the gavage procedure other than the side effects of CZ48. In fact, these slight losses in body weight for most animals were completely reversed when the treatment period was sufficiently long, most likely because the sufficient length of treatment allowed the initial reactions to the gavage procedure to be corrected in the animals. To have an objective judgment of the toxicity of the drug, we performed toxicity studies with healthy mice. We chose three groups of mice, of similar ages and weights. One group was used as control, and the other two groups were treated with CZ48 at two dose levels, 1,000 and 2,000 mg/kg/d, respectively. Figure 3*B* shows the results of the treatment with 2,000 mg/kg/d. Animals in the test groups received the drug in suspension form daily by gavage. Animals in the control group only received the vehicle, also daily by gavage. The results in Fig. 3*B* were from 280 days after treatment initiation. We did not find any body weight losses at all during this long treatment; in fact, the body weights of these treated mice increased slightly.

Pharmacokinetic absorption profile of CZ48 with a single dose of 50, 100, 150, 300, 500, 1,000, and 2,000 mg/kg/d, respectively, was recorded following an oral administration to nude mice. Table 3*A* shows all important pharmacokinetic parameters.

For all responsive tumors, the effective doses required to achieve inhibitions were established and the corresponding TIs were calculated by using 2,000 mg/kg/d as the MTD. The results are shown in Table 3*B*.

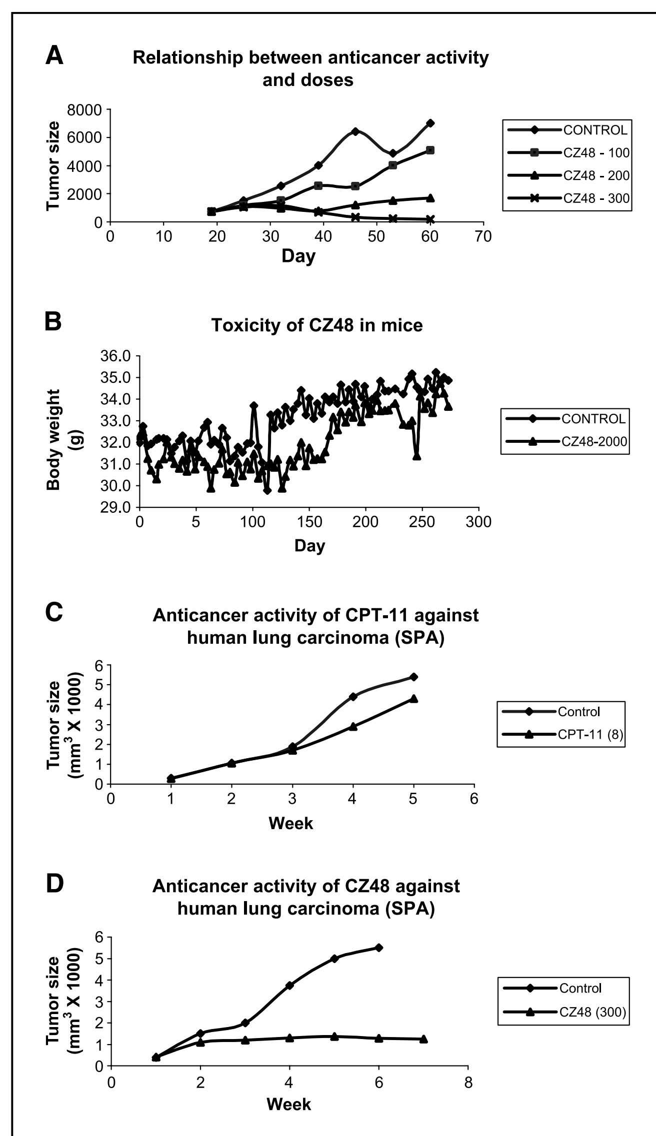

Figure 3. *A,* correlations between dose levels and the corresponding inhibitions. Three groups of tumor-bearing mice were treated with 100, 200, and 300 mg/kg/d of CZ48, respectively. The mean of the tumor sizes of each group measured at each time point was plotted versus treatment time. *B,* body weight changes in healthy mice during 280 d of the treatment. The test group was treated with 2,000 mg/kg/d CZ48 (suspended in cottonseed oil) daily for the duration of the treatment period by gavage. The control group was treated by the cottonseed oil only, also daily for the duration of treatment period by gavage. *C,* antitumor activity of commercial CPT-11 against human SPA lung carcinoma with oral administration of 8 mg/kg/d. This dose was safe to animals treated under our experimental conditions. Under this dose level, CPT-11 did not show significant inhibitory effects. *D,* antitumor activity of CZ48 against human SPA lung carcinoma with oral administration of 300 mg/kg/d. Under this dose level, CZ48 was effective and showed great inhibitions.

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Table 2. Body weight changes in mice during the treatment

Mouse groups	Dose (mg/kg)	Route	Length (d)	Body weight changes from day 0 to day last					
				Control (g)			Test (g)		
				Day 0	Day last	Change	Day 0	Day last	Change
Bl-BOL	200	Gav	41	30.2	29.5	−0.8	27.2	24.1	−3.1
Br-BEN	300	Gav	65	33.4	36	2.6	33.5	33.1	−0.4
Br-CLO	100	Gav	53	29.5	32.3	2.8	29.6	28.6	−1
Br-MUR	300	Gav	45	28.4	29	0.6	28.5	27.3	−1.2
Br-WAR	200	IS	104	27.2	29	1.8	26.3	24.1	−2.7
Cln-HT29	300	Gav	41	27.9	32.9	5	27.8	25.5	−2.3
Cln-McC	1,000	Gav	25	32.6	31.3	−1.3	32.9	27.2	−5.7
Cln-SQU	300	Gav	69	33.2	35.5	2.3	33.9	33.2	−0.7
Cln-SW48	200	Gav	49	32.6	34.2	1.6	32.7	27.9	−4.8
D-MYE	100	Gav	110	33.4	31.7	−1.7	33.4	34	0.6
D-ZUC	300	Gav	26	32.1	34.1	2	33.4	31.8	−1.6
L-DOY	200	Gav	41	31.6	34.3	2.7	33.3	32.5	−0.8
L-SPA	300	Gav	35	31.3	32.3	1	30.9	29.3	−1.6
M-BRO	100	Gav	40	32.2	34.6	2.4	31.5	35.7	4.2
M-SBC	1,000	Gav	41	32.6	31.6	−1	31	29.4	−1.6
P-LIE	100	Gav	62	32.1	33.4	1.3	32.6	32.5	−0.1
P-MIA	1,000	Gav	37	33.2	34.1	0.9	33.5	29.7	−3.8
P-PAN	1,000	Gav	68	34.2	34.9	0.7	33.7	31.5	−2.2
P-SU86	1,000	Gav	33	33.8	34.9	1.1	33.5	31.4	−2.1

Discussion

Compared with most conventional anticancer agents clinically used today by oncologists, CZ48 is more effective and has a much higher response rate. Nineteen of 21 human tumor lines treated with CZ48 in our laboratory (90%) achieved either regressions or growth inhibitions. We also treated seven human tumor lines grown in mice as xenografts with nine conventional anticancer agents, such as Adriamycin, Alkeran, BiCNU (carmustine), cyclophosphamide, 5-FU, methotrexate, methyl CNU, vincristine, and vinblastine. The seven lines were BRO melanoma, CLO breast, FOS melanoma, HT29 colon, MUR breast, SQU colon, and WAR breast. Each tumor line was treated, respectively, with these nine agents. Totally, 56 treating experiments were conducted. The dose for each treatment with one of these agents was calculated according to the commercial recommendation. Of the 56 treatments, only 5 were found to be effective; CLO breast was responsive to the treatments, respectively, with Alkeran, cyclophosphamide, vincristine, and vinblastine, and WAR breast with 5-FU; all other 51 were essentially ineffective. Thus, all these nine agents combined gave a 9% (5 of 56) response rate, one tenth of CZ48's. Irinotecan (CPT-11) has probably been a camptothecin analogue mostly used by oncologists for certain treatments. To compare CZ48 with irinotecan, the *in vitro* IC_{50} data of CZ48 and irinotecan were, respectively, obtained by measuring the inhibitory effects of these two drugs against HT29, McCN, DOY, and BRO human cancer cell lines. CZ48 is more potent than irinotecan across all four tested cell lines. Table 3*C* summarizes the results. The *in vivo* anticancer activities of CZ48 and irinotecan against human lung carcinoma (SPA) were also compared with each other by using same schedules and same oral administrations. Irinotecan was toxic in mice if the dose was higher than 12 mg/kg/d under our experimental conditions, and thus, we chose 8 mg/kg/d as the dose for irinotecan treatment; this dose was safe to mice. CZ48 was safe to mice ranging from 1 to 2,000 mg/kg/d. We chose 300 mg/kg/d as the dose for CZ48 treatment. This dose previously showed effectiveness against SPA lung carcinoma. The results (Fig. 3*C* and *D*) showed that irinotecan was not effective at this chosen dose level and CZ48 expressed great anticancer activity at this nontoxic dose of 300 mg/kg/d. In addition to its shown effectiveness, CZ48 was found to be nontoxic in mice. Healthy animals treated with CZ48 at 2,000 mg/kg/d for >9 months slightly gained body weights. This indicates that CZ48 is completely nontoxic in mice even. Under our experimental conditions, we were not able to reach MTD in mice. The dose of 2,000 mg/kg/d was the highest one we were able to reach by gavage and the highest dose we had ever been able to reach in our laboratory. The required effective doses varied depending on the types of tumors. Two tumor lines showed great inhibitory effects by administration of 100 mg/kg/d, and others required as high as 1,000 mg/kg/d to achieve the same. Using 2,000 mg/kg/d as MTD, the TIs for CZ48 were calculated according to the definition given in the introduction section ranging from 2 to 20 (Table 3*B*). This TI range is much wider than that (~ 1) of most anticancer agents currently used in clinical oncology; none of which can be continuously used for long periods of time at the effective dose. Speculatively, the lack of toxicity of CZ48 in mice is because of the inactive nature of the drug itself and the high stability of the lactone moiety of the molecule in blood. The drug is probably activated in cancerous tissues by an enzyme (or enzymes) called esterase (or esterases).

The peak concentrations (i.e., C_{max}) in blood for all doses were reached within 2 hours. C_{max} (ng/mL) increased from 77.9 to 190.9 when the dose (mg/kg/d) increased from 50 to 2,000. The area

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Table 3. Pharmacokinetic parameters, TIs, and IC_{50} data of CZ48

A. Absorption parameters of CZ48 after single dose*

Dose (mg/kg/d)	50	100	150	300	500	1,000	2,000
Pharmacokinetic parameters							
T_{max} (h)	0.5	1.0	0.5	1.0	1.0	2.0	1.0
C_{max} (ng/mL)	77.9	97.9	91.6	104.5	141.0	143.5	190.9
$AUC_{0\text{-}t}$ (ng·h·mL^{-1})	798.9	657.9	989.4	941.0	1,205.4	1,672.4	1,687.0
$AUC_{0\text{-}\infty}$ (ng·h·mL^{-1})	786.7	715.6	1,293.1	1,088.1	1,564.9	2,076.6	3,283.9
$t_{1/2}$ (h)	18.9	15.4	14.2	7.6	11.8	9.8	24.9
K_e (1/h)	0.037	0.000	0.000	0.091	0.059	0.071	0.028

B. Effective doses and TIs of CZ48 against 19 human tumor lines†

Lines	Bl-BOL	Br-BEN	Br-CLO	Br-MUR	Br-WAR	Co-HT29	Co-McC	Co-SQU	Co-SW48
ED	200	300	100	300	200	300	1,000	300	200
TI	10	6.7	20	6.7	10	6.7	2	6.7	10
DSRCT-MYE	DSRCT-ZUC	Lung-DOY	Lung-SPA	Mel-BRO	Mel-SBC	Pan-LIE	Pan-MIA	Pan-PAN	Pan-Su86
100	300	200	300	100	1,000	100	1,000	1,000	1,000
20	6.7	10	6.7	20	2	20	2	2	2

C. IC_{50} comparisons between CPT-11 and CZ48

Tumors	HT29 colon	McC colon	DOY lung	BRO melanoma
CPT-11	1,098.7 ± 53.9	697.3 ± 158	582.8 ± 28.6	459 ± 29.6
CZ48	436.5 ± 164.3	472.5 ± 16.2	369.9 ± 127.5	250.2 ± 38

Abbreviation: ED, effective dose.
*Experimental conditions for pharmacokinetic absorption studies: species, four female mice; feeding conditions, fasting; vehicle/formulation, suspension in cottonseed oil; method of administration, gavage; sample, plasma; analyte, CZ48; assay, HPLC.
†TIs were calculated according to the definition given in Introduction using 2,000 mg/kg/d as MTD (i.e., TI = 2,000/effective dose).

under the curve (AUC; ng·h·mL^{-1}) also increased from 789.9 to 1,687.0 for $AUC_{0\text{-}t}$ and from 786.7 to 3,283.9 for $AUC_{0\text{-}8}$ when the dose increased from 50 to 2,000. The ranges of elimination half-lives ($t_{1/2}$) of CZ48 were from 8 to 25 hours for these seven doses. CZ48 stayed in blood longer when the dose was lower. The elimination $t_{1/2}$ was ~19 hours for dose 50 mg/kg/d, 15 hours for 100 mg/kg/d, and 14 hours for 150 mg/kg/d. The $t_{1/2}$s became much shorter when the dose reached ≥300 mg/kg/d. The $t_{1/2}$ became much longer (24.9 hours) again when the dose was 2,000 mg/kg/d. This 24.9-hour value probably did not reflect the real elimination rate of the drug because the stomach of the mouse was full of drug when 2,000 mg/kg/d was administered, and thus, the mouse needed the longer time to digest the drug and/or to get the drug excreted.

Multiple daily doses (i.e., two or three times per day) may be even better for cancer treatment, as the mean elimination $t_{1/2}$ of CZ48 for six single doses (50–1,000 mg/kg/d) is ~13 hours and the mean elimination $t_{1/2}$ for three single higher doses (300, 500, and 1,000 mg/kg/d) is <10 hours (9.7 hours).

Thus, the new anticancer agent CZ48 has shown a broad spectrum of anticancer activity against xenografts of human tumors in nude mice with a striking lack of toxicity. An Investigational New Drug application to the American Food and Drug Administration was approved to proceed. Human clinical trials with this new agent are ongoing and the results from these human studies will be separately reported.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 11/24/08; revised 3/26/09; accepted 4/6/09; published OnlineFirst 5/19/09.
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We thank CHRISTUS Stehlin Foundation for Cancer Research and the friends of the Stehlin Foundation for Cancer Research for financial supports.

References

1. Van Kuilenburg ABP, Haasjes J, Richel DJ, et al. Clinical implications of dihydropyrimidinedehydrogenase (DPD) deficiency in patients with severe 5-fluorouracil-associated toxicity: identification of new mutations in the DPD gene. Clin Cancer Res 2000;6:4705–12.
2. Van Kuilenburg ABP, Muller EW, Haasjes J, et al. Lethal outcome of a complete dihydropyrimidine dehydrogenase (DPD) deficiency after administration of 5-fluorouracil: frequency of the common IVS14+1G>A mutation causing DPD deficiency. Clin Cancer Res 2001;7:1149–53.
3. DeVita VT, Hellman S, Rosenberg SA. Cancer, principles and practice of oncology. 7th ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 335–459.

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4. Cvitkovic E, Spaulding J, Bethune V, et al. Improvement of *cis*-dichlorodiammineplatinum (NSC 119875): therapeutic index in an animal model. Cancer 1977;39: 1357–61.
5. Hayes D, Cvitkovic E, Golbey R, et al. High dose *cis*-platinum diamine dichloride: amelioration of renal toxicity by mannitol diuresis. Cancer 1977;39:1372–81.
6. Evens B, Raju K, Calvert A, et al. Phase II study of JM8, a new platinum analogue, in advanced ovarian carcinoma. Cancer Treat Rep 1983;67:997–1000.
7. Huinink W, Gore M, Carmichael J, et al. Topotecan versus paclitaxel for the treatment of recurrent epithelial ovarian cancer. J Clin Oncol 1997;15:2183–93.
8. Schiller JH, Adak S, Cella D, et al. Topotecan versus observation after cisplatin plus etoposide in extensive-stage small-cell lung cancer: E7593—a phase III trial of the Eastern Cooperative Oncology Group. J Clin Oncol 2001;19:2114–22.
9. Von Pawel J, Schiller JH, Shepherd FA, et al. Topotecan versus cyclophosphamide, doxorubicin, and vincristine for the treatment of recurrent small-cell lung cancer. J Clin Oncol 1999;17:658–67.
10. Pizzolato JF, Saltz LB. The camptothecins. Lancet 2003;361:2235–42.
11. Miller AA, Lilenbaum RC, Lynch TJ, et al. Treatment-related fatal sepsis from topotecan/cisplatin and topotecan/paclitaxel. J Clin Oncol 1996;14:1964–5.
12. Saltz LB, Cox JV, Blanke C, et al. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. N Engl J Med 2002;343:905–14.
13. Douillard JY, Cunningham D, Roth AD, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicenter randomized trial. Lancet 2000;355:1041–7.
14. Wall ME, Wani MC, Cook CE, Palmer KH, McPhail AT, Sim GA. Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. J Am Chem Soc 1966;88:3888–90.
15. Hsiang YH, Liu LF. Identification of mammalian DNA topoisomerase I as an intracellular target of anticancer drug camptothecin. Cancer Res 1988;48:1722–6.
16. Takimoto CH, Arbuck SG. Topoisomerase I targeting agents: the camptothecins. In: Chabner BA, Longo DL, editors. Cancer therapy and biotherapy: principles and practice. 3rd ed. Philadelphia: Lippincott Williams & Wilkins 2001. p. 579.
17. Burke TG. Chemistry of the camptothecins in the bloodstream: drug stabilization and optimization of activity in the camptothecins—from discovery to patients. Ann N Y Acad Sci 1993;803:29–31.
18. Mi z, Burke TG. Marked interspecies variations concerning interactions of camptothecin with serum albumin. Biochemistry 1994;33:1240–5.
19. Giovanella BC, Stehlin JS, Wall ME, et al. Topoisomerase I-targeted chemotherapy of human colon cancer xenografts. Science 1989;246:1046–8.
20. Giovanella BC, Hinz HR, Kozielski AJ, et al. Complete growth inhibition of human cancer xenografts in nude mice by treatment with 20-(*S*)-camptothecin. Cancer Res 1991;51:3052–5.
21. Stehlin JS, Giovanella BC, Natelson EA, et al. A study of 9-nitrocamptothecin (RSF-2000) in patients with advanced pancreatic cancer. Int J Oncol 1999;14:821–32.
22. Giovanella BC, Harris N, Mendoza J, Cao Z, Liehr J, Stehlin JS. Dependence of antitumor activity of camptothecins on maintaining their lactone function. In: The camptothecins: unfolding their anticancer potential. Ann N Y Acad Sci 2000;922:27–35.
23. Cao Z, Harris N, Kozielski A, Vardeman D, Stehlin J, Giovanella B. Alkyl esters of camptothecin and 9-nitrocamptothecin: synthesis, *in vitro* pharmacokinetics, toxicity, and antitumor activity. J Med Chem 1998; 41:31–7.
24. Cao Z, Mendoza J, DeJesus A, Giovanella B. Synthesis and antitumor activity of alkenyl camptothecin esters. Acta Pharmacol Sin 2005;26:235–41.
25. Cao Z, Pantazis P, Mendoza J et al. Structure-activity relationship of alkyl camptothecin esters. Ann N Y Acad Sci 2000;922:122–35.
26. Cao Z, Pantazis P, Mendoza J, at al. Structure-activity relationship of alkyl 9-nitrocamptothecin esters. Acta Pharmacol Sin 2003;24:109–19.
27. Lui X, Wang Y, Vardeman D, Cao Z, Giovanella B. Development and validation of a reverse-phase HPLC with fluorescence detector method for simultaneous determination of CZ48 and its active metabolite camptothecin in mouse plasma. J Chromatogr B Analyt Technol Biomed Life Sci 2008;867:84–9.

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