Complete Regression of Xenografted Human Carcinomas by Camptothecin Analogue-Carboxymethyl Dextran Conjugate (T-0128)

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ABSTRACT

Clinically available camptothecins (CPTs), such as irinotecan (CPT-11) and topotecan, represent one of the most promising classes of antitumor agents, despite their toxicity. To improve their pharmacological profiles, a new macromolecular prodrug, denoted T-0128, was synthesized. This prodrug is a novel CPT analogue (T-2513)-carboxymethyl (CM) dextran conjugate via a triglycine spacer, with a molecular weight of Mw 130,000. This study was designed to test the concept that the rational design of a CPT-polymer conjugate would increase the efficacy of the parent drug. The in vivo antitumor study against Walker-256 carcinoma demonstrated that T-0128 was 10 times as active as T-2513, supporting this concept. Additionally, comparative efficacy studies of T-0128, T-2513, CPT-11, and topotecan were performed using a panel of human tumor xenografts in nude mice, showing the advantage of T-0128 over these CPTs. The maximal tolerated doses (MTDs) of T-0128, T-2513, and CPT-11 were comparable. Even a single i.v. injection of T-0128 at 6 mg/kg (based on the amount of T-2513 bound to CM dextran) induced complete regression of MX-1 mammary carcinoma. T-0128 at 10 mg/kg weekly for 3 weeks (one-tenth of its MTD) cured LX-1 lung carcinoma. Also, T-0128 below its MTD consistently cured or regressed 3T4 gastric and HT-29 colorectal tumor xenografts that are highly refractory to CPTs. These demonstrate the broad range of therapeutic doses achieved with T-0128. Pharmacokinetic studies were performed to correlate the efficacy results obtained for T-0128 with plasma and tissue drug concentrations using Walker-256 tumor-bearing rats. Results showed that after i.v. administration of T-0128, the conjugate continued to circulate at a high concentration for an extended period, resulting in tumor accumulation. In the tumor, the sustained release of T-2513 occurred. In contrast, T-2513 disappeared rapidly from the body. The significant increases in the amount and exposure time of released T-2513 in the tumor explain well the enhanced efficacy of T-0128. In conclusion, this study indicated that T-0128 improved the potency of T-2513, demonstrating the proof of the above concept.

INTRODUCTION

CPT,2 from an Oriental tree, Camptotheca acuminata, is an inhibitor of topoisomerase I and has exhibited a promising antitumor activity in various experimental tumors. However, CPT is extremely water insoluble, and this feature has severely restricted its clinical application (1). Recently, novel CPT analogues with an improved water solubility, CPT-11 and topotecan, have emerged as one of the most important classes of antitumor agents developed over the last two decades (2, 3). CPT-11 has been available in Japan since 1994, where it is approved for the management of colorectal, cervical, ovarian, uterine, gastric, breast, and lung cancer. In the United States, CPT-11 is presently used for the treatment of advanced colorectal adenocarcinoma. The main clinical adverse effects of the drug are myelosuppression and gastrointestinal toxicity, especially severe diarrhea. These side effects are closely related to its pharmacokinetic property (4, 5).

To alter the pharmacokinetic behavior of a parent anticancer drug, thereby decreasing the toxicity and/or increasing the therapeutic efficacy of the agent, many macromolecules have been used as drug carriers (6, 7). This concept of a macromolecular prodrug is attractive and could well form the basis of a new generation of antitumor agents. Stereoreplicated anhydride neocarzinostatin is the first example of a drug-polymer conjugate approved in Japan as a drug for primary liver cancer. PK1 is another example, which comprises doxorubicin covalently bound to N-(2-hydroxypropyl)methacrylamide copolymer by a peptidyl linker. The Phase I study demonstrated that PK1 decreases important dose-limiting toxicities while maintaining antitumor activity (8). Tumor targeting of these conjugates is assisted by the enhanced permeability and retention effect; tumors usually have a hyperpermeable vasculature, which allows longer circulating macromolecules to pass out through the leaky vessels into the tumor tissue, from where there is no readily available return lymphatic route (9).

In an attempt to overcome the side effects and improve the pharmacological profile of the CPT analogue, T-0128 is designed as a macromolecular prodrug, which consists of CM dextran and T-2513, linked through the triglycine spacer. T-2513 was chosen as a parent CPT analogue for T-0128 from our chemical libraries for the following reasons: (a) an analogue with great potency is favorable; (b) an amino group is needed for the binding to a peptidyl linker, without the opening of the lactone ring, known to be crucial for the activity (10); and (c) a good hydrophobic/hydrophilic balance is required for the synthesis of a conjugate with high water solubility and high drug content. On the other hand, a longer circulation is realized by dextran with appropriate anionic charges. The reason is that macromolecules greater than roughly Mw 70,000 with weak anionic charges are known to circulate in the plasma for a long time because of small hepatic uptake and urinary excretion clearances (11, 12). Therefore, CM groups were introduced to the commercially available dextran with a molecular size of about Mw 110,000. Dextrans have high water solubility and low in vivo toxicity (13). Furthermore, CM groups are available for drug fixation through a peptidyl linker. These favorable properties supported the choice of CM dextran as a drug carrier for T-0128.

The purpose of the present study is to test the concept that the rational design of a CPT analogue-polymer conjugate would achieve the tumor targeting of the active drug, resulting in improved therapeutic efficacy. To this end, we examined the preclinical profile of the antitumor activity of T-0128 against Walker-256 carcinoma in Wistar rats and a panel of human tumor xenografts in nude mice. The efficacy of T-0128 was compared with those of T-2513, CPT-11, and topotecan. In addition, we investigated the plasma and tissue (tumor and nontumor) pharmacokinetics of both released and polymer-bound T-2513s after the administration of T-0128 to rats bearing Walker-256 carcinoma. These data are also compared with those obtained after

References

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2 The abbreviations used are: CPT, camptothecin; AUC, area under the concentration-time curve; CM, carboxymethyl; HPLC, high-performance liquid chromatography; MRT, mean residence time; MTD, maximal tolerated dose; SN-38, 7-ethyl-10-hydroxy-camptothecin; IS, internal standard.
dosing of T-2513 itself to tumor-bearing rats using the same dosage. Results support potential advantages of T-0128 attributable to the passive tumor targeting.

**MATERIALS AND METHODS**

**Chemicals.** T-0128, T-2513, SN-38, topotecan, and 10-hydroxyethoxy-CPT, a CPT derivative used as an IS in HPLC analysis, were synthesized at Tanabe Seiyaku (Discovery Research Laboratory, Toda, Japan). The synthesis of T-0128 and T-2513 will be published elsewhere (14). All other chemicals were reagent-grade products obtained commercially.

**Characterization of T-0128.** Molecular weight and size distributions of T-0128 were determined by the use of gel permeation chromatography, coupled with the multangle laser light scattering detector at 40°C (15). The chromatographic system consisted of a gel permeation chromatography system 21 (Shodex, Tokyo, Japan), G3000PWXL and G4000PWXL columns 300 × 7.8 mm (Tosoh, Osaka, Japan), a DAWN DSP-F laser photometer (Wyatt Technology, Santa Barbara, CA), and a Wyatt Optilab DSP interferometric refractometer. Data were collected and handled by Astra software (Wyatt Technology). A 0.2 M phosphate buffer (pH 6.9) at a flow rate of 0.8 ml/min was used as a mobile phase. Normalization of the instrument was carried out by Shodex pullulan P-82 standard series. The weight-average molecular weight of T-0128 was $M_r = 130,000$ with narrow dispersity ($M_w/M_n < 1.3$). The content of T-2513 was 4.5–5.5% w/w, determined by measuring absorbance at 360 nm in water. T-0128 contained $<0.05\%$ of the free drug in relation to its total T-2513 content. The degree of substitution of the carboxymethylated groups was determined by measuring the sodium content using an atomic absorption spectrophotometer, when CM dextran was isolated as a sodium salt after the reaction of dextran with chloroacetic acid in alkaline solution. The degree was 0.40 – 0.45 per glucose moiety. The chemical structure and characteristics of T-0128 are presented in Fig. 1.

**Human Tumors.** MX-1 mammary carcinoma and LX-1 lung carcinoma were obtained from Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo, Japan). St-4 gastric carcinoma was kindly provided by Dr. Kubota, Keio University, School of Medicine (Tokyo, Japan). KB oral epidermoid carcinoma and HeLaS3 cervical epitheloid carcinoma were supplied by National Institute of Health Sciences (Tokyo, Japan). Additional tumors (WiDr and HT-29 colon carcinomas, SK-BR-3 breast carcinoma, MKN-1 stomach carcinoma, and SK-LU-1 lung carcinoma) were purchased from American Type Culture Collection (Rockville, MD).

**In Vitro Cytotoxicity.** WiDr, HT-29, SK-BR-3, MKN-1, SK-LU-1, LX-1, KB, and HeLaS3 cells were maintained in the recommended media at 37°C under atmosphere of 5% CO$_2$. Exponentially growing cells were typically seeded in 24-well culture plates (~20000 cells/well) and cultured in Eagle’s MEM supplemented with nonessential amino acids (Life Technologies, Inc., Tokyo, Japan) and 10% heat-inactivated FCS (CosmoBio, Tokyo, Japan) for 48 h. Cells were then treated with drugs in the serum-free medium. After 24 h of exposure to drugs, cells were cultured in the fresh medium with FCS for additional 24 h and then trypsinized and counted by Coulter counter (Coulter Electronics Coulter Z1, Krefeld, Germany). Cell survival was plotted relative to the controls incubated in the medium in the absence of the drug. The mean drug concentration required to inhibit cell growth by 50% (GI$_{50}$) was determined from the dose-response curves.

**Topoisomerase I Inhibitory Activity.** The ability of CPT analogues to inhibit topoisomerase I was quantified in the cleavable complex assays as described elsewhere (16).

**Cell Cycle Analysis.** The effect of T-2513 on cell cycle progression was assayed by flow cytometry using a Cycle Test Plus DNA Reagent kit (Becton Dickinson, Mansfield, MS).
Tumor that is not palpable for a period of time; and cure is defined as an established and control groups, respectively. Complete tumor regression refers to a tumor obtained from each tumor line were implanted s.c. to the right flank of male 8-week-old BALB/c mice (Nippon SLC), and the tumor growth was caused sufficient morbidity as determined by body weight loss. Tumor fragments (3 x 3 x 3 mm) of Walker-256 carcinoma cells (Sasaki Institute, Tokyo, Japan), grown for 7 days in the peritoneal cavity of female Wistar rats (Nippon SLC), were injected s.c. to the inguinal region of 6-week-old female Wistar rats at a dose of 1 x 10^6 cells/rat. After 3 days (day 0), CPT analogues were dissolved in 0.9% NaCl solution and administered once i.v. with five rats/group. Control rats were injected with 0.9% NaCl solution. The rats were killed on day 4, and the tumors were removed and weighted. The efficacy of each drug was assessed as T/C, where T and C were the tumor volume of the treated and control groups, respectively, with a ED50 being calculated from T/C by using the probit method.

Evaluation of Antitumor Activity in Human Tumor Xenograft Models. Tumor fragments (3 x 3 x 3 mm) of MX-1, LX-1, HT-29, WiDr, and St-4, obtained from each tumor line were implanted s.c. to the right flank of male 8-week-old BALB/c nu/nu mice (Nippon SLC), and the tumor growth was followed by measurements of tumor diameters with a sliding caliper two or three times a week. The tumor volume (TV) was calculated according to the formula: TV = L x W^2/2, where L and W are the major and minor dimensions, respectively. Drug treatment with five or six mice/group started on days 8 to 15 after the transplantation, when the tumor volume was 100 – 300 mm^3. Each drug was injected i.v. once, three times with a 4-day interval, or once a week for 3 or 4 weeks. Control mice were treated with 0.9% NaCl solution. The efficacy of the drug was assessed as relative tumor volume to the original. The tumor growth inhibition rate (TIR) was calculated from the equation: 

\[ TIR = 100 \times \frac{1 - TV \text{ of treated group}}{TV \text{ of control group}} \]

where TV is the tumor volume of the treated and control groups, respectively. Complete tumor regression refers to a tumor that is not palpable for a period of time; and cure is defined as an established tumor that is not palpable for >30 days after the final measurement day. These days for MX-1, LX-1, HT-29, WiDr, and St-4 were 30, 21, 32, 37, and 42 days, respectively, after the first drug dosing.

### Table 1: Cytotoxicity of CPTs against a panel of human tumor cell lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>WiDr</th>
<th>HT-29</th>
<th>SK-BR-3</th>
<th>MKN-1</th>
<th>SK-LU-1</th>
<th>LX-1</th>
<th>KB</th>
<th>HeLaS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-2513</td>
<td>32.1</td>
<td>97.6</td>
<td>38.6</td>
<td>111.5</td>
<td>15.6</td>
<td>15.1</td>
<td>34.0</td>
<td>50.9</td>
</tr>
<tr>
<td>Topotecan</td>
<td>16.3</td>
<td>14.0</td>
<td>18.1</td>
<td>52.0</td>
<td>10.7</td>
<td>14.7</td>
<td>15.6</td>
<td>28.5</td>
</tr>
<tr>
<td>SN-38</td>
<td>2.46</td>
<td>5.22</td>
<td>1.79</td>
<td>15.2</td>
<td>1.54</td>
<td>4.36</td>
<td>1.81</td>
<td>8.11</td>
</tr>
<tr>
<td>CPT</td>
<td>5.50</td>
<td>6.02</td>
<td>5.88</td>
<td>15.4</td>
<td>3.73</td>
<td>3.94</td>
<td>5.02</td>
<td>7.84</td>
</tr>
</tbody>
</table>

\* Cells were exposed to the drugs for 24 h, and cell survival was determined after 24-h incubation in the fresh medium as described in “Materials and Methods.”

Data are the mean values of three independent experiments in duplicate.

### Table 2: Therapeutic index of T-0128 and CPT analogues in rats bearing Walker-256 carcinoma

<table>
<thead>
<tr>
<th>Drug</th>
<th>ED50 (mg/kg)</th>
<th>MTD (mg/kg)</th>
<th>Therapeutic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-0128</td>
<td>100</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>T-2513</td>
<td>23</td>
<td>60</td>
<td>2.6</td>
</tr>
<tr>
<td>CPT-11</td>
<td>57</td>
<td>80</td>
<td>1.4</td>
</tr>
<tr>
<td>Topotecan</td>
<td>5.4</td>
<td>25</td>
<td>4.6</td>
</tr>
</tbody>
</table>

\* MTD was determined using healthy SD rats.

\* Therapeutic index was determined by dividing MTD by ED50.

### Toxicity Dose-Finding Studies. Healthy male SD rats (Charles River Japan, Yokohama, Japan) and healthy male BALB/c mice (Nippon SLC, Hamamatsu, Japan) were used for toxicity dose-finding studies. CPT analogues were dissolved in 0.9% NaCl solution and administered i.v. Rats were observed for at least 28 days. The MTD was defined as the maximum dose that caused that drug-related lethality and that produced animal body weight loss of <20% of original weight. The body weight of mice was monitored two or three times weekly for at least 28 days. The loss of >30% of body weight was considered to be lethal. The MTD was defined as the maximum dose that caused sufficient morbidity as determined by body weight loss.

### Pharmacokinetic Studies. The tumor-bearing rats received an i.v. bolus injection of either T-2513 or T-0128. Both drugs were administered at doses equivalent to 1 mg of T-2513/kg. A dose of T-0128 is hereafter always expressed as T-2513 equivalent mg/kg of body weight per injection. For T-2513 treatment, 27 rats were divided into nine groups (n = 3), corresponding to 0.017, 0.083, 0.25, 0.5, 1, 2, 4, 6, and 24 h, for terminal blood and tissue sample collection, whereas for T-0128 treatment, 24 rats were divided into eight groups (n = 3), corresponding to 0.083, 1, 3, 6, 12, 24, 48, and 72 h. Tumor, liver, spleen, kidney, heart, lung, duodenum, lymph nodes, and femoral bone marrow were removed and weighed. Immediately before sampling, the rats were anesthetized with ether, and blood was removed from abdominal aorta using a heparinized syringe. Plasma was harvested by centrifugation. Samples were stored at –80°C until analysis.

### Determination of Drug Concentration in Plasma and Tissue. Tissue samples were homogenized on ice using a Polytron mixer (Kinematica, Lucerne, Switzerland) and suspended in PBS (pH 7.0) at a concentration of 25% w/v. By using aliquots of the homogenate and plasma (100 μl), the concentrations of free T-2513 (that released in vivo from T-0128) and polymer-bound T-2513 (that remaining bound to CM dextran) were determined by HPLC analysis. For free T-2513, proteins were precipitated with acetonitrile (300 μl), before adding 180 ng/ml 10-hydroxyethylxyloto-CPT/35 mM formic ammonium buffer (pH 5.0; 100 μl) as an IS. After the addition of 0.1 M HCl (100 μl), the sample was vortexed for 10 s and centrifuged for 3 min at 5000 × g. Prior to analysis, the clear supernatant (100 μl) was diluted with the formic ammonium buffer.

To permit accurate quantitation of polymer-bound T-2513, T-2513 must first be released from the conjugate, because T-0128 is weakly fluorescent. Preliminary experiments revealed that exposure of samples (100 μl) containing T-0128 to 6 M HCl (200 μl; final concentration, 4 μl) at 100°C for 4 h is sufficient to release ~100% of T-2513, and T-2513 is stable during this hydrolysis. Therefore, a hydrolysis time of 4 h was routinely used to permit the quantitation of polymer-bound T-2513. After the hydrolysis, 6 μl NaOH (200 μl) was added to neutralize the acid, followed by a dilution with the formic ammonium buffer. After the addition of 18 ng/ml IS/acetoxin (100 μl), the hydrolysate was filtered through a Millipore KC 0.45 μm filter (Bedford, MA) and analyzed.

### HPLC Chromatography. Reversed-phase HPLC was performed at 40°C on an Inertsil ODS-2 column 150 x 4.6 mm (GL Sciences, Tokyo, Japan) preceded by a Merck LiChrospher 100RP-18 guard cartridge (Darmstadt, Germany). T-2513 and the hydrolysate of polymer-bound T-2513 were eluted with 35 mM formic ammonium buffer (pH 3.0:acetoxin (80:20, v/v) and the buffer-acetonitrile (85:15, v/v), respectively, using a Hitachi L-6200 pump (Tokyo, Japan) at a flow rate of 1.0 ml/min. Detection was carried out using a Shimadzu RF-550 fluorescence detector (Kyoto, Japan) with excitation and...
emission wavelengths of 360 and 420 nm, respectively. Samples (20 μl, prepared as described above) were injected into the HPLC system with a Tosoh AS-8000 automatic sampler. Peak data were recorded by a Shimadzu CR-6A integrator. For every sample, the content of T-2513 was calculated by measuring the relevant peak area and calibrating against the corresponding IS peak area. Polymer-bound T-2513 was determined by subtraction of free T-2513 from total T-2513 of the hydrolysate.

**Data Analysis.** Pharmacokinetic parameters were determined from the average concentration of three rats at each time point using the software WinNonlin (Scientific Consulting, Alexandria, VA). The plasma concentration-time profiles of polymer-bound T-2513, and of T-2513 after dosing of T-2513 itself, were well described by a biexponential equation. The parameters obtained include the area under the concentration-time curve extrapolated to infinity using a fitted model (AUC∞,∞), the mean residence time (MRT), total plasma clearance (CL total), the volume of central compartment (Vc), the volume of distribution at steady state (Vss), the half-life of initial phase [T1/2α], and the half-life of terminal phase [T1/2β]. The AUC∞,∞ values of polymer-bound T-2513 and T-2513 in organs were calculated using the linear trapezoidal rule. The area under the first moment curve (AUMCα) was also calculated similarly. The MRT was determined by dividing AUMCα by AUCα.

**RESULTS**

**In Vitro Cytotoxicity.** T-2513 was compared with CPT analogues, including topotecan, SN-38, and CPT, against a panel of eight human tumor cell lines. Table 1 summarizes the results, indicating that T-2513 has a potent and broad cytotoxicity, although it is less potent than SN-38. In contrast, T-0128 is 1000-fold less potent than T-2513. The IC50s (equivalent to T-2513) of T-0128 against WiDr, SK-BR-3, and HeLaS3 were 17, 39, and 53 μg/ml, respectively.

**Topoisomerase I Inhibitory Activity and Cell Cycle Effects.** T-2513 was found to interact directly with the DNA-topoisomerase I cleavable complex and to produce S-phase arrest and impaired G0 to G1 transition (data not shown). These results demonstrate that T-2513 has a mechanism of action similar to CPT.

**Toxicity Dose-Finding Studies.** To compare the antitumor effects of T-2513 with CPT-11 and topotecan at their optimal doses and to evaluate the improvement in efficacy attributable to the conjugation (T-0128), preliminary studies in healthy rats and BALB/c mice were set up to assess their MTDs. In the case of rats, the MTDs after a single i.v. injection of T-0128, T-2513, and CPT-11 were found to be comparable, i.e., 100 (based on the amount of T-2513 bound to CM dextran), 60, and 80 mg/kg, respectively. In contrast, topotecan at 30 mg/kg caused 25% body weight loss with diarrhea. A similar trend toward the order of the MTDs was observed in the case of mice. The MTDs after a single i.v. injection of T-0128, T-2513, and CPT-11 were apparently comparable, i.e., 100, 80, and 100 mg/kg, respectively. In comparison, the MTD of topotecan was <20 mg/kg. With four times weekly schedule, the MTD of T-0128 was 100 mg/kg. In both animals, the main adverse effects of T-0128 and T-2513 were myelosuppression and gastrointestinal toxicity. Also, our pilot study showed that the MTD of T-0128 in an St-4 tumor xenograft model was 75 mg/kg weekly for 4 weeks or 100 mg/kg weekly for 3 weeks.

**Antitumor Activity against Walker-256 carcinoma.** The ability of T-0128 and T-2513 to affect the growth of rat Walker-256 carcinoma was evaluated. As shown in Fig. 2, T-0128 was able to shrink the tumor to 50% of the control at 2.3 mg/kg (ED50). In contrast, the ED50 of T-2513 was 23 mg/kg; T-0128 was 10-fold as active as T-2513. CPT-11 at 60 mg/kg produced about a 50% reduction in the tumor to 50% of the control at 2.3 mg/kg (ED50).
tumor growth, whereas topotecan at 12 mg/kg produced an 80% reduction. Table 2 compares the therapeutic indexes (MTD/ED50) of the four drugs, clearly indicating the superiority of T-0128 over T-2513, CPT-11, and topotecan. These results mean that the use of macromolecular carrier increases the potency and the range of therapeutic doses of T-2513.

**Antitumor Activity in Human Tumor Xenograft Models.** To confirm the above results, the efficacy of T-0128 was evaluated using MX-1 mammary tumor. Fig. 3 shows the tumor growth and body weight change curves of nude mice given a single i.v. administration of T-0128, as compared with controls or animals given CPT-11 weekly at 60 mg/kg for 4 weeks. A single injection of T-0128 at 4 mg/kg produced a transient inhibition of tumor growth. T-0128 at 6 mg/kg (one-fiftieth of its MTD with three times weekly schedule) achieved complete tumor inhibition in five of the six mice, whereas no obvious decrease in the body weight was detected. CPT-11 at 60 mg/kg with four times weekly schedule produced not only a comparable tumor reduction but also >11% body weight loss. T-2513 and topotecan were less active than CPT-11 (data not shown).

Additionally, the efficacy of T-0128 was assessed in the less sensitive LX-1 lung tumor model. As shown in Fig. 4, T-0128 at 10 mg/kg once a week for 3 weeks (one-tenth of its MTD) regressed the tumor completely and inhibited the body weight loss attributable to cachexia. In contrast, CPT-11 at a dose of 100 mg/kg with the same schedule was only able to slow tumor growth, with virtually no regression of the tumor. T-2513 and topotecan were less potent than CPT-11.

To reinforce the effectiveness of T-0128, further *in vivo* studies were performed using more refractory xenograft models. Table 3 summarizes the antitumor activities of T-0128, compared with CPT-11 or T-2513. T-0128 demonstrated a strong antitumor activity in all tumor models evaluated. It is important to note that T-0128 consistently produced regression of tumor xenografts, which are highly refractory to CPT analogues such as CPT-11 and T-2513, with IR values of >96%. This significant effectiveness of T-0128 is exemplified by the results of St-4 gastric tumor and HT-29 colorectal tumor. Against St-4, the MTDs of CPT analogues only resulted in a delay in tumor growth. However, T-0128 given at four-fifths of its

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**Table 3 Maximum tumor growth inhibition rates of T-0128 and CPT analogues against five human tumor xenografts**

Human tumors were implanted s.c. into nude mice. T-0128 and CPT analogues were administered i.v. according to the schedule. The tumor volumes and body weights were measured two or three times per week. Each group consisted of five or six mice.

<table>
<thead>
<tr>
<th>Tumor lines</th>
<th>Schedule</th>
<th>Dose (mg/kg)</th>
<th>IRmax (%)</th>
<th>BWLmax (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mammary cancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MX-1</td>
<td>q4d × 1</td>
<td>6</td>
<td>99.8 (23–30): 5/6 cure</td>
<td>3.5 (7)</td>
</tr>
<tr>
<td>MX-1</td>
<td>q7d × 3</td>
<td>3</td>
<td>97.9 (23–25): 5/6 cure</td>
<td>2.6 (12)</td>
</tr>
<tr>
<td><strong>Lung cancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LX-1</td>
<td>q4d × 3</td>
<td>10</td>
<td>98.8 (18): 6/6 cure</td>
<td>14.6 (8)</td>
</tr>
<tr>
<td>LX-1</td>
<td>q7d × 3</td>
<td>10</td>
<td>98.8 (18): 6/6 cure</td>
<td>8.8 (7)</td>
</tr>
<tr>
<td><strong>Colorectal cancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT-29</td>
<td>q4d × 1</td>
<td>80</td>
<td>92.9 (22, 25)</td>
<td>10.1 (8)</td>
</tr>
<tr>
<td>HT-29</td>
<td>q7d × 2</td>
<td>20</td>
<td>97.8 (32)</td>
<td>2.7 (6)</td>
</tr>
<tr>
<td><strong>Gastric cancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>St-4</td>
<td>q4d × 1</td>
<td>100</td>
<td>82.7 (25)</td>
<td>10.4 (10)</td>
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<tr>
<td>St-4</td>
<td>q7d × 3</td>
<td>80</td>
<td>95.7 (42)</td>
<td>29.2 (17)</td>
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<td><strong>Control</strong></td>
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<tr>
<td>Control</td>
<td></td>
<td></td>
<td>80</td>
<td>54.5 (20)</td>
</tr>
</tbody>
</table>

*a The dose of T-0128 is expressed as a dose equivalent to T-2513.

*b IRmax (%), maximum tumor growth inhibition rate (1 − T/C) × 100, where T and C are the tumor volume of the treated and control groups, respectively; number in parentheses, days after drug dosing when IRmax was observed; cure is defined as an established tumor that is not palpable for >30 days after the final measurement (day 30 for MX-1, day 21 for LX-1, and day 42 for St-4 after the administration). ND, not done.

*c BWLmax (%), maximum body weight loss; number in parentheses, days after drug dosing when BWLmax was observed.

*d q4d × 1, single injection; q7d × 4, four injections administered with a 7-day interval; q4d × 3, three injections administered with a 4-day interval; q7d × 3, three injections administered with a 7-day interval.
MTD (at 60 mg/kg weekly for 4 weeks) cured the mice. As shown in Fig. 5, a single injection of T-0128 at 60 mg/kg obtained an IR of >90% against HT-29 tumor. Three injections of T-0128 at the same total dose (at 20 mg/kg weekly for 3 weeks) regenerated the tumor more effectively. The optimal schedule for administering T-0128 was not determined here. Therefore, the dose required to achieve cures may be further reduced with schedule optimization.

**Plasma Pharmacokinetics.** Pharmacokinetic studies were performed to correlate the toxicity and efficacy results obtained for both T-0128 and T-2513 with plasma and tissue drug concentrations. Walker-256 tumor-bearing rats received single i.v. injections of each drug at a dose equivalent to 1 mg of T-2513/kg. Data points, means of three rats; the bars (SD) are within the size of the symbols.

**Tissue Distribution.** Ideally, macromolecular produgs should be stable and pharmacologically inactive during circulation in the bloodstream. However, after reaching the targeted site, they should release the active compound. Fig. 7 shows the tumor concentration-time profiles of polymer-bound T-2513 and released T-2513 after dosing of T-0128. These profiles in several tissues are summarized in Fig. 8. As was expected, there was evidence that the liberation of T-2513 from T-0128 occurred in various tissues. High levels of released T-2513 were particularly observed in the tumor, spleen, liver, and lymph nodes (data not shown for lymph nodes). The concentrations in the tumor, liver, spleen, and lymph nodes peaked ~12 h and subsequently declined very slowly, suggesting the cellular uptake and retention of T-0128. In contrast, the profiles in the kidney, heart, lung, and duodenum (data not shown for duodenum), not corrected by remaining blood in the tissue, showed a decline similar to that of the plasma. For comparison, the profiles of T-2513 after the administration of T-2513 itself are also shown in Figs. 7 and 8. T-2513 was cleared rapidly from the body. At 24 h after dosing, no retention of T-2513 in the tumor was observed, as in all of the tissues measured. To compare the tissue accumulations of released T-2513 with those of T-2513 after dosing T-2513 itself, the AUC values in each tissue are summarized in Table 5. T-0128 increased the relative presence of T-2513 particularly in the tumor, liver, spleen, and lymph nodes. The accumulations were attained by factors ranging from 52 to 86, as determined by AUC evaluation, whereas the AUC values in the kidney and the heart were unaffected.

**DISCUSSION**

T-0128 comprises a CPT analogue T-2513 covalently bound to CM dextran by a triglycyl linker. Through a selection process that involved the evaluation of a variety of our CPT analogues, suitable peptidyl linkers, and polysaccharide carriers, T-0128 was chosen for a CPT tumor-targeting system. This process will be published elsewhere. The object of this study is to test the concept that the rational design of a CPT-polymer conjugate would achieve the tumor targeting of the active drug, resulting in improved therapeutic efficacy. To this end, we compared the antitumor activities of T-0128 with those of T-2513, CPT-11, and topotecan and evaluated the ability of T-0128 to target the active drug to tumors. The results reported here demonstrate the proof of this concept.

T-2513 had a broad cytotoxicity against a range of human tumor cell lines, whereas T-0128 was inactive in vivo. However, when used in vivo, the efficacy of T-0128 was 10-fold superior to that of T-2513 against Walker-256 carcinoma in Wistar rats. Although the MTDs of T-0128 and T-2513 were comparable, the conjugate gained a significant advantage in the tumor-bearing rats in terms of therapeutic index (MTD/ED50). Furthermore, T-0128 demonstrated a strong antitumor activity against a panel of human tumor xenografts that were mostly

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**Table 4** Pharmacokinetic parameters of polymer-bound T-2513 after i.v. administration of T-0128 and of T-2513 after dosing of T-2513 to rats bearing Walker-256 carcinoma at a dose equivalent to 1 mg of T-2513/kg.

<table>
<thead>
<tr>
<th>Drug</th>
<th>T1/2 (α) (h)</th>
<th>T1/2 (β) (h)</th>
<th>Vc (ml/kg)</th>
<th>Vss (ml/kg)</th>
<th>MRT (h)</th>
<th>CLtotal (ml/kg/h)</th>
<th>AUC0-∞ (µg/h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-0128</td>
<td>4.04 (5.3)</td>
<td>17.0 (18.6)</td>
<td>61.5 (6.2)</td>
<td>72.4 (6.2)</td>
<td>7.31 (3.3)</td>
<td>9.91 (4.5)</td>
<td>101 (4.5)</td>
</tr>
<tr>
<td>T-2513</td>
<td>0.017 (23.4)</td>
<td>0.88 (25.1)</td>
<td>206 (54.4)</td>
<td>1558 (65.1)</td>
<td>0.22 (37.8)</td>
<td>6987 (31.9)</td>
<td>0.14 (31.8)</td>
</tr>
</tbody>
</table>

---

Fig. 6. Plasma concentration-time profiles of polymer-bound T-2513 after i.v. administration of T-0128 (●) and T-2513 after dosing of T-2513 (△) to rats bearing Walker-256 carcinoma at a dose equivalent to 1 mg of T-2513/kg. Data points, means of three rats; the bars (SD) are within the size of the symbols. Solid lines are obtained by fitting data points to a two-compartment model.
refractory to CPT-11, T-2513, and topotecan. Overall, T-0128 was more active and had a much broader range of therapeutic doses than T-2513 in vivo.

The improvements in potency of a drug through the drug-polymer conjugation, especially in the case of doxorubicin (17), have been reported elsewhere. An example is PK1, a doxorubicin-N-(2-hydroxypropyl)methacrylamide copolymer conjugate. Another example, BR96-doxorubicin immunoconjugate, was shown to exhibit a strong antitumor activity in a panel of human tumor xenografts (18).

It is true that the use of drugs with an established clinical profile, such as doxorubicin, may offer a safety advantage over more potent but less defined agents. However, we believe that the rational design of a drug-polymer conjugate, including the optimization of a drug, will achieve the maximal therapeutic efficacy. This is because the chemical structures of conventional chemotherapeutics are mostly restricted for the linkage to a polymeric carrier. On the basis of this belief, we synthesized and evaluated T-0128. The results described here proved our concept and encouraged us to develop this conjugate.

The ultimate effectiveness of T-0128 in the tumors is likely a result of various factors. On the basis of the pharmacokinetic data in this study and information available on the pharmacological and physicochemical properties of CPT analogues, several hypotheses might apply.

(a) Tumor targeting of T-0128 attributable to the enhanced permeability and retention effect should account for the efficacy. It is interesting to compare the amounts of free T-2513 found in Walker-256 carcinoma after the administration of T-0128 and T-2513. Comparing the two profiles of free T-2513 levels in tumors, it is clear that the AUC value for T-0128 is ~50 times greater than that for T-2513 (Table 5). This remarkable elevation in AUC is sufficient to explain the increased efficacy of T-0128. Our preliminary study also confirms the tumor accumulation of T-0128 and the release of T-2513 in a panel of human tumor xenografts.

(b) A slow prolonged release of T-2513 is likely to be responsible for the improvement of therapeutic efficacy (19). Successful CPT-based chemotherapy, which is only effective during the comparatively short S-phase of the cell cycle, will require continuous presence of the drug (20). In fact, the time period of exposure to T-2513 is a great determinant of cytotoxicity. T-2513 is more active with >24-h incubation in vitro (data not shown). Therefore, the potential therapeutic benefit of T-0128 lies in a very long tumor exposure time of T-2513, reflected by the significant extension in MRT (Table 5).

(c) Part of the increased efficacy of T-0128 might be explained based on the pH-mediated structural change of the parent drug. Under acidic conditions, CPT analogues are known to exist predominantly in the ring-closed lactone form, whereas at neutral or alkaline pH, they are converted into the ring-open carboxylate form, which is a much less potent inhibitor of topoisomerase I (21). Therefore, the cellular uptake of

![Passive Tumor Targeting for a CPT Analogue](image-url)

Table 5  **AUC and MRT values in tissues after the administration of either T-0128 or T-2513 in rats bearing Walker-256 carcinoma**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>T-2513 AUC (µg·h/g)</th>
<th>T-2513 MRT (h)</th>
<th>Free T-2513 AUC (µg·h/g)</th>
<th>Free T-2513 MRT (h)</th>
<th>Polymer-bound T-2513 AUC (µg·h/g)</th>
<th>Polymer-bound T-2513 MRT (h)</th>
<th>T-2513 AUC ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% released&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>2.74</td>
<td>1.05</td>
<td>3.23</td>
<td>37.3</td>
<td>43.2</td>
<td>26.1</td>
<td>1.2</td>
<td>7.0</td>
</tr>
<tr>
<td>Liver</td>
<td>0.65</td>
<td>1.23</td>
<td>38.1</td>
<td>31.2</td>
<td>183.7</td>
<td>26.8</td>
<td>59</td>
<td>17.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.80</td>
<td>1.10</td>
<td>68.4</td>
<td>35.7</td>
<td>289.4</td>
<td>33.0</td>
<td>86</td>
<td>19.1</td>
</tr>
<tr>
<td>Heart</td>
<td>1.44</td>
<td>1.34</td>
<td>1.64</td>
<td>33.4</td>
<td>17.2</td>
<td>19.0</td>
<td>1.1</td>
<td>8.7</td>
</tr>
<tr>
<td>Lung</td>
<td>0.30</td>
<td>1.34</td>
<td>1.42</td>
<td>23.9</td>
<td>28.1</td>
<td>18.4</td>
<td>1.1</td>
<td>4.8</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>NM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>99.4</td>
<td>35.4</td>
<td>352.1</td>
<td>32.6</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22.0</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0.56</td>
<td>1.80</td>
<td>8.04</td>
<td>36.8</td>
<td>78.5</td>
<td>29.7</td>
<td>14</td>
<td>9.3</td>
</tr>
<tr>
<td>Duodenum</td>
<td>NM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.23</td>
<td>31.4</td>
<td>13.5</td>
<td>20.9</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.2</td>
</tr>
<tr>
<td>Tumor</td>
<td>0.73</td>
<td>1.68</td>
<td>38.1</td>
<td>31.0</td>
<td>230.2</td>
<td>52</td>
<td>52</td>
<td>14.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> T-2513 AUC ratio is determined by dividing the AUC values of free T-2513 after the administration of T-0128 by those after the administration of T-2513.

<sup>b</sup> % released is determined by dividing the AUC values of free T-2513 by those of total (free plus polymer-bound) T-2513 after the administration of T-0128.

<sup>c</sup> NM, not measured.

<sup>d</sup> ND, not determined.
T-0128, probably followed by exposure to acidic medium in lysosomes, might make the lactone form of released T-2513 predominant, close to and inside the target cells, promoting antitumor activity (22).

Having considered the mechanisms for the efficacy of T-0128, we should now focus on the toxicity of T-0128. The principal dose-limiting toxicities of both T-0128 and T-2513 are myelosuppression and diarrhea. The reasons for their comparable MTDs and toxicities (Table 2) are not yet clear. However, pharmacokinetic studies in tumor-bearing rats may give a clue. One possible explanation is hepatic and splenic accumulations after dosing of T-0128 (Fig. 8 and Table 5). These accumulations may sacrifice the advantage of the conjugate of reducing peak drug concentrations, resulting in the close MTD. In particular, hepatic accumulation of released T-2513 may produce a continuous drug exposure of the intestine attributable to the pharmacokinetic property of CPT analogues (23). Therefore, the use of a carrier with less hepatic clearance might increase the range of therapeutic doses of T-0128.

In summary, T-0128 consistently produced regression of tumor xenografts that are highly refractory to CPT analogues. Altered pharmacokinetic properties of T-0128 in terms of plasma half-life, tumor targeting, and kinetics of T-2513 release may account for the effectiveness. Results demonstrate the broad range of therapeutic doses that were achieved with T-0128, suggesting that T-0128 is a promising agent. In particular, hepatic accumulation of released T-2513 may produce a continuous drug exposure of the intestine attributable to the pharmacokinetic property of CPT analogues (23). Therefore, the use of a carrier with less hepatic clearance might increase the range of therapeutic doses of T-0128.

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Complete Regression of Xenografted Human Carcinomas by Camptothecin Analogue-Carboxymethyl Dextran Conjugate (T-0128)

Satoshi Okuno, Mitsunori Harada, Toshiro Yano, et al.


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