Paclitaxel Delivery Systems: The Use of Amino Acid Linkers in the Conjugation of Paclitaxel with Carboxymethyldextran to Create Prodrugs

Shu-ichi SUGAHARA,*[,a] Masahiro KAJIKI,[a] Hiroshi KURIYAMA,[b] and To-ru KOBAYASHI[b]

[a] *The Second Research Department of Central Technology Laboratory, AsahiKasei Corporation; and* [b] *Institute for Life Science Research, AsahiKasei Corporation; 2–1 Samejima, Fuji, Shizuoka 416–8501, Japan.*
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Paclitaxel was bound *via* its hydroxyl group to carboxymethyldextran (CMDex, 150 kDa) by means of an amino acid linker; the linker was introduced into the 2′- or 7-hydroxyl group of the paclitaxel through an ester bond. These conjugates—CMDex-2′-paclitaxel and CMDex-7-paclitaxel—were designed to be water-soluble with a paclitaxel content between 6—8% (w/w) with a degree of subsititution (DS) of the CM groups at 0.6 per sugar residue. The release of the paclitaxel from the conjugates was influenced by the hydroxyl group (2′- or 7-) of paclitaxel to which the amino acid linker was introduced, and by what amino acid was used as the linker. In mouse plasma incubated at 37 °C for 72 h, the most paclitaxel was released using CMDex-paclitaxel conjugate with 2′-gly followed by, in descending order, 2′-ala, 2′-leu, 2′-ile, and 7-gly as the amino linkers. Colon 26, a Taxol® resistant cancer, was introduced into mice and the conjugates were intravenously administered by bolus injection for a tumor distribution study, and intermittently intravenously administered for a tumor growth regression study. In both studies the highest amount of paclitaxel release was found in the CMDex-2′-gly-paclitaxel followed by CMDex-2′-ala-paclitaxel, CMDex-2′-leu-paclitaxel and paclitaxel. There was a direct correlation between the amount of paclitaxel released and the observed efficacy. CMDex-2′-ile-paclitaxel and CMDex-7-gly-paclitaxel did not show any anti-tumor activity. These results clearly demonstrate that a CMDex-paclitaxel with an appropriate amino acid linker has significant anti-tumor activity against colon 26, and that these anti-tumor effects appear to correlate with the amounts of paclitaxel released in the tumor.

Key words paclitaxel (Taxol®); carboxymethyldextran; anti-tumor effect; linker; polymeric drug

Paclitaxel (Taxol®), an anti-microtubule agent isolated from the trunk bark of the Pacific Yew tree, *Taxus brevifolia*,[1] shows great promise as an anti-neoplastic agent for a variety of human cancers including breast, ovarian, non small cell lung, head and neck cancers, leukemia, and melanoma.[2—6] Its unique mechanism of action is related to its ability to promote microtubule assembly and inhibit cell replication in the late G2 or M phases of the cell cycle.[7] A major problem associated with the administration of paclitaxel is its low solubility in water as well as in most pharmaceutically acceptable solvents. The Taxol® formulation used clinically contains polyoxyethylated castor oil (Cremophor EL®) and ethanol as excipients. Cremophor EL® has long been considered the source of hypersensitivity reactions observed with paclitaxel infusions.[8] Side effects of Taxol® include nausea and vomiting, diarrhea, mucositis, myelosuppression, cardiotoxicity and neurotoxicity.[9,10] Thus, alternative dosage forms for paclitaxel delivery have been developed to improve the solubility of paclitaxel without the use of Cremophor EL®, for example, liposomes,[11—14] mixed micelles,[15] parenteral emulsions,[16,17] polymeric nanoparticles,[18] cyclodextrin complexes,[19] polyethylene glycol (PEG) esters,[20] polyamino acids,[21,22] and polymer-bound derivatives.[23,24] Although some of the dosage forms can be solubilized to release sufficient quantities of paclitaxel and have shown improved anti-tumor effects in animal models, problems—such as stability—have been observed.[25] The use of macromolecules for the targeted delivery of anticancer agents has generated considerable interest regarding enhancing therapeutic efficacy and reducing systemic side effects, and some satisfactory results have been obtained.[26] We previously reported how to prepare carboxymethyldextran (CMDex) and doxorubicin (DXR) conjugates using a peptide linker, and in our evaluation we showed that CMDex with a suitable anionic nature and MW of more than 150 kDa increased retention of the conjugate in blood circulation and increased accumulation of DXR in tumors.[27] Furthermore, CMDex-peptide-DXR conjugates containing a gly–gly–phe–gly spacer were more efficacious in a Walker-256 carcinoma rat model than a free DXR or a conjugate with no spacer. We chose CMDex as a candidate for a paclitaxel carrier since: CMDex is biocompatible; it contains a large number of carboxyl groups for the drug attachment and provides sufficient carrying capacity of the drug; and the resulting CMDex-drug conjugate has a high probability of being water-soluble.

In this paper, we examine the synthesis and evaluation of CMDex-paclitaxel conjugates bound with an ester bond and using amino acid linkers, namely, gly, ala, leu, and ile. We also look at how polymeric modification of paclitaxel with CMDex significantly improves water solubility and anti-tumor activity. The gly linker was introduced into the 2′-hydroxyl group to form CMDex-2′-gly-paclitaxel and into the 7-hydroxyl group to form CMDex-7-gly-paclitaxel. All other amino acid linkers—ala, leu, and ile—were introduced only into the 2′-hydroxyl group of paclitaxel. These were all designed to be water-soluble. The amounts of paclitaxel released from the conjugates during incubation with a buffer and mouse plasma at 37 °C were measured by HPLC. We compared this to their *in vivo* tumor distribution and *in vivo* anti-tumor effects in a paclitaxel resistant tumor mouse model (colon 26). The tumor and body weights of the mice were monitored after continuous intravenous administration.

MATERIALS AND METHODS

Materials Paclitaxel (Taxol®) was purchased from

* To whom correspondence should be addressed. e-mail: sugawara.sb@om.asahi-kasei.co.jp
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Dabur Pharmaceuticals, Ltd. (New Delhi, India); Dextran T110 was purchased from Pharmacia Biotech (Uppsala, Sweden); Cremophor EL® was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 2-(1-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from NOVA (La Jolla, CA, U.S.A.); and *n*-hexyl *p*-hydroxy benzoate—an paclitaxel internal standard for HPLC assay—was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) All other chemicals were of reagent grade purity or better. Female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan).

Preparation of CMDex-Amino Acid-Paclitaxel The CMDex-amino acid-paclitaxel compounds were prepared as shown in Chart 1. The 2′-amino acid derivatives of paclitaxel **3a**—**d** were prepared by condensing *N*-Carbobenzoxy (*Z*)-L-amino acid **1a**—**d** with paclitaxel in the presence of 1,3-diisopropylcarbodiimide (DIPC) and 4-(dimethylamino) pyridine (DMAP) to obtain 2′-*Z*-amino acid paclitaxel **2a**—**d**, respectively. The *Z* groups of **2a**—**d** were removed by catalytic transfer hydrogenolysis. The 7-gly derivatives of paclitaxel **6** were made by condensing *Z*-chloride with paclitaxel in the presence of *N*,*N*-diisopropylamine to obtain 2′-*Z*-paclitaxel **4**. *Z*-L-gly was condensed with **4** in the presence of DIPC and DMAP to obtain 2′-*Z*-7-*Z*-gly-paclitaxel **5**. 7-gly-paclitaxel **6** was formed from this by removing the *Z* group of 2′-*Z*-7-*Z*-gly-paclitaxel **5** *via* catalytic transfer hydrogenolysis. 7-epi-paclitaxel (7-α-hydroxy configuration) **7**, an epimerization product of paclitaxel, was prepared from paclitaxel by refluxing in toluene in the presence of 1,8-diazabicyclo[5,4,0]-undec-7-ene (DBU).

CMDex **9** could be isolated in its sodium salt form by treating dextran T110 **8** with chloroacetic acid in an alkaline solution.[27] **3a**—**d** and **6** were conjugated with CMDex **9** in the presence of HBTU in a solution of *N*,*N*-dimethylformamide (DMF)–H_2O (1 : 1, v/v) to arrive at conjugates **10a**—**d** and **11**.

Characterization of CMDex-Paclitaxel Conjugates The relative molecular weight of CMDex-paclitaxel was determined by using gel permeation chromatography (GPC). A TSK-gel G4000PW_{XL} (TOSOH, Tokyo, Japan) column was used and its column temperature was maintained at 40 °C with a column oven (CTO-6A; Shimadzu, Kyoto, Japan). The mobile phase, consisting of a 20% (v/v) acetonitrile in 50 mM LiCl, was delivered at a flow rate of 0.8 ml/min with a pump (L-6200; Hitachi, Tokyo, Japan). A 10 μl-aliquot of the samples was injected with an auto injector (SIL-10A; Shimadzu) and column effluent was detected at 230 nm with a UV-VIS detector (L-4200; Hitachi) and refractive index (RI) detector (L-3300; Hitachi). Calibration of the instrument was carried out using a by Shodex pullulan P-82. The area of each eluted peak was integrated with an integrator (C-R6A; Shimadzu).

The degree of substitution (DS) of the carboxymethyl (CM) group of CMDex was determined by the titration method used by Sugahara *et al*.[27] The exact position of car-

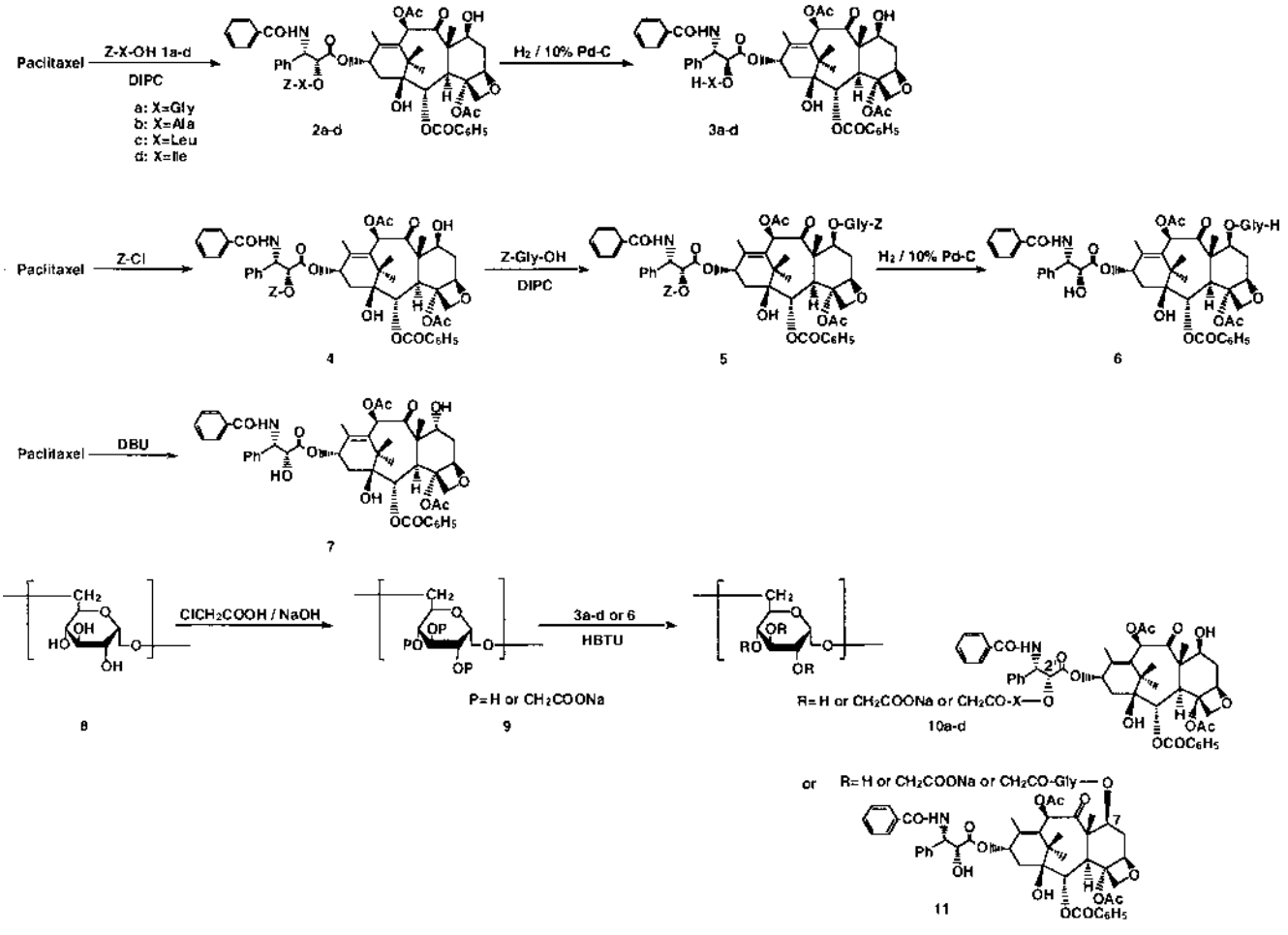

Chart 1

boxymethylation in Dex was determined by the method employed by Daotian and Roger.[28] The monosccharide composition of CMDex was analyzed using an HPLC method where CMDex is first hydrolyzed by trifluoroacetic acid to give constituent monosaccharides. These are subsequently labeled with 1-phenyl-3-methyl-5-pyrazolone (PMP)[29] and separated by reverse-phase HPLC using a column (Waters Puresil, 150×4.6 mm i.d., particle size 5 μm), monitoring UV absorbance at 245 nm. Quantitation was performed using integration values relative to standards. Each peak of the PMP-labeled monosaccharide was identified by mass spectrometry. It has been documented that Pharmacia Biotech's dextran is α(1→6)-linked α-D-glucan with side-chains attached to the 3-position on the backbone units, and that the degree of branching is approximately 5%.[30] The degree of branching of dextran T110 used for the carrier in this study was examined by NMR analysis to determine if it conformed to the same specifications as Pharmacia's dextran.

UV spectra were obtained on a Shimadzu UV-160A spectrophotometer at a wavelength of 227 nm. The content of paclitaxel in the CMDex-paclitaxel conjugate was determined based on a standard curve generated with a known concentration of paclitaxel in a 50% (v/v) methanol solution. The amounts of free paclitaxel in the CMDex-paclitaxel conjugates were determined by HPLC. Solubility of CMDex-paclitaxel conjugates was estimated by dissolving various amounts of these conjugates in 100 μl of saline.

Analysis of Paclitaxel The amount of paclitaxel was determined by HPLC according to Mase *et al*.[31] Samples were pretreated using a solid-phase extraction procedure with Sep-Pak cartridge C18 columns (Waters, Milford, MA, U.S.A.) prior to HPLC analysis. The Sep-Pak columns were treated with 5 ml of acetonitrile and then with 10 ml of distilled water. Test samples were diluted with 5 ml of phosphate buffered saline (PBS, pH 7.4) and loaded in the Sep-Pak columns. Immediately, 1 mg of the internal standard (*n*-hexyl *p*-hydroxyl benzoate, in a 20% (v/v) acetonitrile solution) was applied to the column. The column was washed with 5 ml of distilled water, then with 5 ml of a 20% (v/v) acetonitrile solution, and finally the elutant was collected after the addition of 5 ml of acetonitrile. The solution was dried by evaporation at 40 °C in a water bath with the residue being reconstituted in 500 μl of HPLC mobile phase. These samples were filtered through a 0.5 μm filter (SJLH L04 NS; Millipore, Bedford, MA, U.S.A.) and the filtrates were used for paclitaxel analysis.

For HPLC analysis, a reverse-phase column (Asahipak HIKARISIL C18-4D; 150×4.6 mm i.d.; particle size 5 μm; Asahi Kasei Corporation, Tokyo) was used and the column temperature was maintained at 40 °C with a column oven (CTO-6A, Shimadzu). The mobile phase, consisting of a mixture of acetonitrile and 2 mM phosphoric acid (55 : 45, v/v), was pumped (LC-6AD; Shimadzu) at a flow rate of 1.5 ml/min. A 100 μl-aliquot of the samples was injected with an auto injector (SIL-9A; Shimadzu) and the column effluent was detected at 227 nm with a UV detector (SPD-6A; Shimadzu). The area of each eluted peak was integrated (C-R6A; Shimadzu).

The paclitaxel concentration in each sample was calculated using the ratio of the peak areas of paclitaxel and the internal standard by comparing that ratio with a corresponding standard curve prepared with appropriate blank samples. The calibration curve used for the quantification of paclitaxel was linear over the range of 50—5000 ng/ml in plasma with correlation coefficient of $r^2>0.995$.

***In Vitro* Drug Release Test** The release of paclitaxel from the conjugates was examined by adding 20 μl of a solution containing CMDex-paclitaxel conjugate (**10a**—**d** or **11**, 2 mg/ml) in saline to 200 μl of buffer, plasma and tumor homogenate. Tumor samples were homogenized on ice using a Teflon homogenizer and suspended in PBS (pH 7.4) at a concentration of 50% (w/v). This was incubated at 37 °C, shaking moderately. The amounts of liberated paclitaxel in the conjugate at time points 0.5, 1, 4, 24, 48, and 72 h during incubation were determined by HPLC.

***In Vitro* Stability of Paclitaxel in Mouse Plasma** To examine the stability of the paclitaxel molecule in mouse plasma at 37 °C, the paclitaxel content was measured using HPLC analysis. Twenty μl of solution of paclitaxel (100 μg/ml) in 50% (v/v) methanol was added to 200 μl of mouse plasma, and the solution was incubated at 37 °C, shaking moderately. The amounts of paclitaxel in the plasma solution at time points 4, 24, 48, and 72 h during incubation were determined by HPLC.

Procedure for Animal Experiments. Animals Female BALB/c mice weighing between 16 and 20 g each were purchased from Japan SLC (Shizuoka, Japan). Mice were used as hosts for colon 26, a murine colon tumor model.[12] Subcutaneous tumors on the right flank were induced by the administration of 10^6 cells in a volume of 0.1 ml.

Toxicity of CMDex-Paclitaxel Conjugates The maximum tolerated dose (MTD) for CMDex-paclitaxel conjugates administered i.v. was determined in healthy BALB/c female mice. Survey experiments to define the MTD were performed with three animals per group and doses were escalated in 2-fold increments starting at 25 mg/kg. Drug effects were determined by close observation of body weight and survival. The MTD was defined as the highest nonlethal dose of paclitaxel or of CMDex-paclitaxel conjugates causing a <10% body weight loss within 1 week of cessation of dosing.

Tissue Distribution Study Tumor-bearing BALB/c female mice received an i.v. bolus administration of free paclitaxel or of a CMDex-paclitaxel conjugate; all administrations were at doses equivalent to 50 mg of free paclitaxel. Hereafter, any dose of CMDex-paclitaxel conjugate is expressed as the free paclitaxel equivalent mg/kg of body weight per administration. For each treatment group, 18 mice were divided into six groups ($n=3$), corresponding to the tissue evaluation points of 5 min and 1, 6, 24, 48, and 72 h. Immediately before tissue samples were removed and weighed, mice were anesthetized with ether, and sacrificed by exsanguination through the abdominal aorta using a heparinized syringe. Plasma was obtained by centrifugation and all samples were stored at −80 °C until analyzed.

Tissue samples were homogenized on ice using a Teflon homogenizer and suspended in PBS (pH 7.4) at a concentration of 10% (w/w). Aliquots of the homogenates were used in an HPLC analysis to determine the concentrations of free paclitaxel.

To permit accurate quantitation of polymer-bound paclitaxel, paclitaxel must first be released from the conjugate

Table 1. ^{1}H-NMR Data for Various Paclitaxel Derivatives in Dimethyl-d_6 Sulfoxide[a)]

Protons on:	Paclitaxel	7-Epi-paclitaxel **7**	7-Gly-paclitaxel **6**	2′-Gly-paclitaxel **3a**	2′-Ala-paclitaxel **3b**	2′-Leu-paclitaxel **3c**	2′-Ile-paclitaxel **3d**
C-7	4.12	3.49	5.47	4.10	4.12	4.12	4.12
C-2′	4.60	4.63	4.62	5.46	5.35	5.34	5.37

a) ^{1}H-NMR spectra ware recorded on a JEOL α-500 spectrometer. Chemical shifts are in ppm relative to tetramethylsilane (0.00).

Table 2. Physical Properties and Solubility of CMDex-paclitaxel Conjugates

Compound	Linker	Paclitaxel		Free paclitaxel (%)[a)]	Solubility in saline (as paclitaxel equivalent) (mg/ml)
		w/w%	mol%		
10a	2′-Gly	7.1	1.9	<0.10	>100 (7.1)
10b	2′-Ala	6.3	1.7	<0.10	>100 (6.3)
10c	2′-Leu	6.6	1.8	<0.05	>100 (6.6)
10d	2′-Ile	6.2	1.7	<0.05	>100 (6.2)
11	7-Gly	7.3	2.0	<0.05	>100 (7.3)

a) Free paclitaxel relative to total paclitaxel content.

since it lacks fluorescence. Preliminary experiments revealed that exposure of 200 μl of blood or homogenized tissue samples containing CMDex-2′-gly-paclitaxel to 1500 μl of 2 mM $Na_2HPO_4 \cdot 12H_2O$–MeOH (14 : 1, v/v) at 37 °C for 15 h releases about 80% of the bound paclitaxel. Based on these findings, a hydrolysis time of 15 h was routinely used to permit the quantitation of polymer-bound paclitaxel. Sufficient release of paclitaxel from the CMDex-paclitaxel conjugates with ala, leu, and ile linker was not successful under various hydrolysis conditions, however, because of the stability of the conjugates. After hydrolysis, a solid-phase extraction procedure was employed for sample pretreatment before HPLC analysis, using Sep-Pak cartridge C18 columns in the same manner as when the paclitaxel was analyzed in the corresponding section above.

Evaluation of Anti-tumor Activity against Colon 26 Carcinoma Female colon 26 bearing BALB/c mice were randomized into various treatment groups and numbered. The dose per mouse was adjusted on the basis of its weight as determined at the time of treatment. Treatment began 2 d after tumor inoculation and was administered i.v. through the tail vein. Saline or a carrier without paclitaxel was used as a negative control. Animal weight and tumor volume was measured every 2 d for 32 d after tumor inoculation. Tumor volume was determined by measuring two orthogonal diameters of the tumor and calculated according to the formula: $(L \times W^2)/2$, where L and W are the major and minor dimensions, respectively.

Statistical Analysis The differences between treatment groups were assessed by one-way analysis of variance (ANOVA). Statistical significance was defined as $p<0.05$ to reject a null hypothesis. When there was statistical significance, multiple comparisons were determined using a least significant differences technique. Statistical analysis was conducted with StatView software (SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Preparation and Characterization The hydroxyl group at the 2′-position of paclitaxel is more reactive than the sterically hindered 7-hydroxyl group,[34)] and this difference in reactivity allowed for the introduction of several amino acid esters at the 2′-position. Reaction of paclitaxel with *Z*-amino acid resulted in a larger number of 2′-amino acid ester compounds than those in the 7-position. The ^{1}H-NMR data for the compounds prepared in this study are summarized in Table 1. Identity of these compounds was confirmed by mass spectrometry and proton NMR analysis. Employing an HBTU-mediated coupling reaction between the carboxyl groups of CMDex and the amino groups of the amino acid-paclitaxel provided for a more efficient production of CMDex-paclitaxel: conjugates containing 6.2—7.3% (w/w) paclitaxel were made. Table 2 shows that this is equivalent to approximately 2 mol percent of paclitaxel molecules bound to each conjugate. For conjugate **10a**, a typical UV absorbance spectrum in 50% (v/v) MeOH is shown in Fig. 1 while Fig. 2 is its GPC profile showing its refractive index and absorption at 230 nm. These two figures provide evidence suggesting covalent conjugation after linkage. The UV spectrum of CMDex-paclitaxel obtained in 50% (v/v) MeOH indicated a slight shift (λ_{max}: 230 nm) compared to that of paclitaxel alone under the same conditions (λ_{max}: 227 nm). The possibility of an ester linkage between the free hydroxyl group of paclitaxel and the carboxyl group of CMDex is considered to be small in this case since no cross-linking product

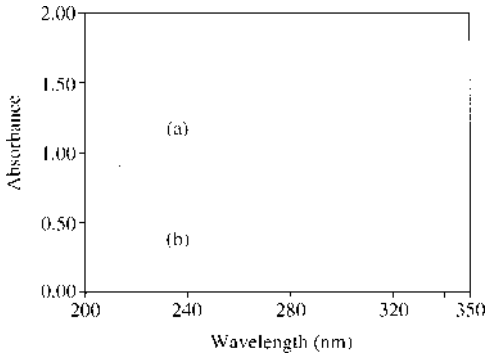

Fig. 1. UV Spectrum of CMDex-Gly-2′-Paclitaxel and Paclitaxel in 50% (v/v) MeOH

a) CMDex-Gly-2′-Paclitaxel (Paclitaxel 3.4×10^{-5} M). b) Paclitaxel (Paclitaxel 2.5×10^{-5} M).

was detected.

Compounds **10a**—**d** and **11** were easily dissolved in saline at more than 10% (w/v) as shown in Table 2 and the resulting solutions remained clear and transparent for a long period of time: more than 6 h. A 100 mg/ml solution of the CMDex-paclitaxel conjugate produces a clear, viscous, yet fluid liquid. On the other hand, when paclitaxel alone was dissolved in a vehicle consisting of Cremophor EL® 50% and ethanol 50% and then diluted with saline to make the desired concentrations, the resulting solutions were slightly hazy and a precipitation formed more quickly: within 30 min.

The exact position of carboxymethylation to the hydroxyl groups of Dex as shown in Table 3, was determined by PMP method, designed by Daotian and Roger.[28] The molar ratio (%) of carboxymethylation to Dex for: mono-carboxymethylation product of 2-hydroxy group with Glc (2-Glc) was 18.5%, with 3-Glc was 13.8%, and with 4-Glc was 18.5%; di-carboxymethylation product of 2,3-, 3,4- and 2,4-Glc, 5.0%; and no tri-carboxymethylation product (2,3,4-Glc) was detected. The exact position of amide formation between the carboxyl groups of CMDex and amino groups of **3a**—**d** and **6** could not be determined. Dextran T110 employed for this study was α (1→6)-linked α-D-glucan with side-chains attached to the 3-position of the backbone units, and the degree of branching was determined to be 3.8% by NMR analysis. The starting DextranT110 had one (1→3)-linked branch point per 25 α (1→6)-linked glucose resides. This degree of branching at the 3-position of Glc accounted for the difference (3.8%) between the formation of monocarboxymethylation of the 3-hydroxy group of the Glc (3-Glc: 13.8%) and the formation of the other monocarboxymethylation products of 2-Glc and 4-Glc (18.5% each).

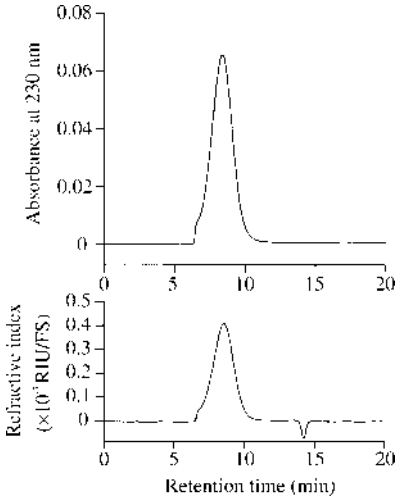

Fig. 2. GPC Profiles of CMDex-Gly-2′-Paclitaxel

Chromatographic conditions: TSK gel G4000PW$_{XL}$ (300×7.8 mm); eluent, 20% (v/v) CH_3CN in 50 mM LiCl; flow rate, 0.8 ml/min; column temperature, 40 °C; sample volume, 10 μl (5 mg/ml).

***In Vitro* Drug Release Test** HPLC analysis was used to examine the release profile of paclitaxel from the CMDex-paclitaxel conjugates in a mouse plasma at 37 °C for 72 h, and, as exemplified in Fig. 3, revealed that different CMDex-paclitaxel conjugates were converted to different proportions of paclitaxel and another compound, which was later identified as 7-epi paclitaxel. 7-epi paclitaxel is an epimerization product of paclitaxel which was previously reported to be formed in a cell culture medium when paclitaxel was incubated with CHO cells and to have similar activity as paclitaxel *in vivo* and *in vitro*.[35] Amino acid-paclitaxel compounds—for example, 2′-gly-paclitaxel—could not be detected during the experiment. The *in vitro* release profiles of paclitaxel from the other CMDex-paclitaxel conjugates are shown in Fig. 4. More than 50% of paclitaxel was liberated from CMDex-2′-gly-paclitaxel in a mouse plasma after a 24 h incubation, while less than 5% of paclitaxel was released from CMDex-2′-ile-paclitaxel or CMDex-7-gly-paclitaxel. After a 72 h incubation period in mouse plasma, the conjugate that released the most paclitaxel was CMDex-2′-gly-paclitaxel followed by CMDex-2′-ala-paclitaxel, CMDex-2′-leu-paclitaxel, CMDex-2′-ile-paclitaxel, and finally CMDex-7-gly-paclitaxel. While the most paclitaxel was released from the CMDex-2′-gly-paclitaxel conjugate, it only was on the order of 70% even after 48 h of incubation. As shown in Fig. 4, the release profiles of paclitaxel from the conjugates in PBS (pH 7.4) or in the colon 26 tumor homogenates were similar in mouse plasma. To clarify why the recovery in mouse plasma was so low, the stability of the paclitaxel under these conditions was examined. The stability of paclitaxel in a mouse plasma at 37 °C, after 48 h incubation, showed that the approximate 30% of starting paclitaxel unaccounted for converted to 7-epi paclitaxel (10%) and to an unknown decomposition product(s) (20%), as shown in

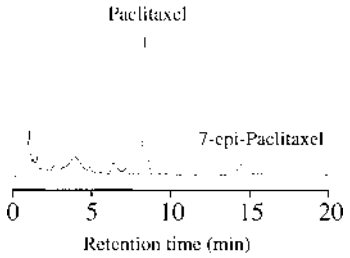

Fig. 3. HPLC Separation of Released Paclitaxel and 7-epi-Paclitaxel from CMDex-2′-Gly-Paclitaxel in Mouse Plasma at 37 °C

For HPLC analysis, Asahipak HIKARISIL C18-4D; 150×4.6 mm i.d., particle size 5 μm; column temperature, 40 °C; eluent, acetonitrile and 2 mM phosphoric acid (55 : 45, v/v); flow rate, 1.5 ml/min; sample volume, 100 μl; detection, 227 nm.

Table 3. Physical Properties of CMDex

	Mono-substituted			Di-substituted	Tri-substituted	Nonsubstituted
	2-CMGlc	3-CMGlc	4-CMGlc	2,3- or 3,4-, or 2,4-CMGlc	2,3,4-CMGlc	Glc
mol% CMGlc or Glc	18.5±1.5	13.8±0.8	18.5±0.8	5.0±0.5	ND[a]	44.1±1.5

a) ND, not detected. Each value represents the mean±S.D. of five experiments.

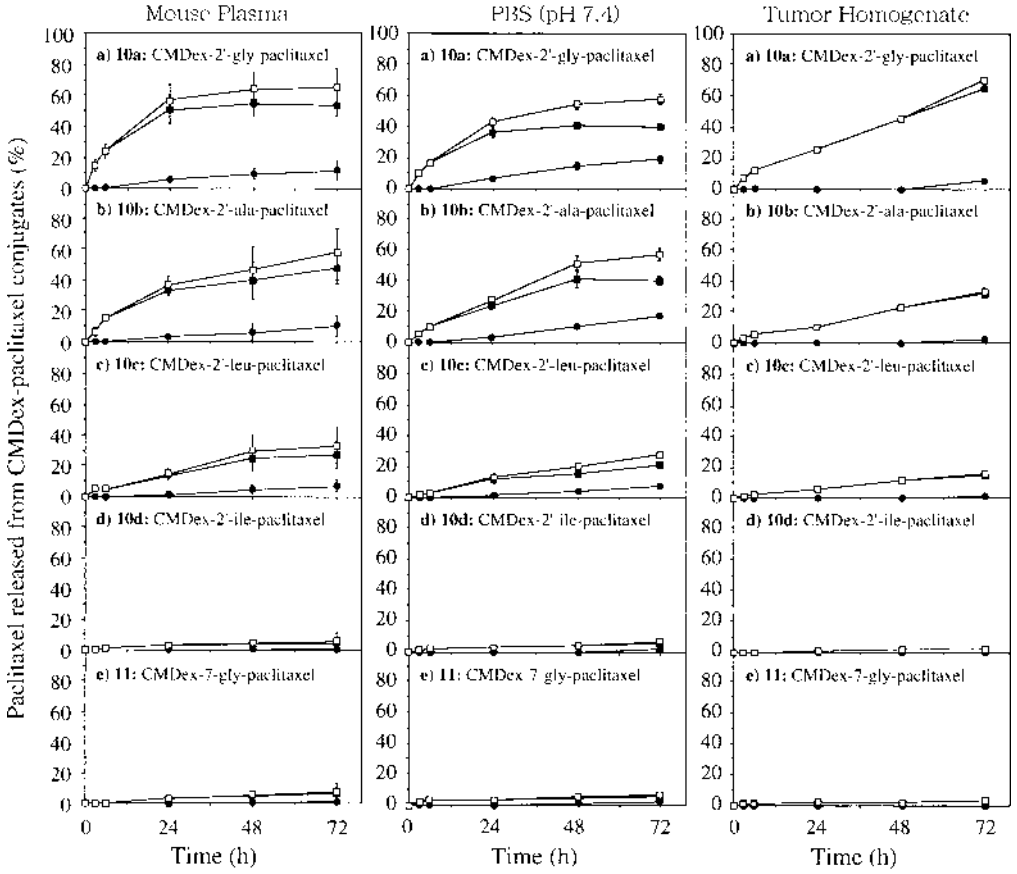

Fig. 4. Effect of Amino Acid Linker on Paclitaxel Release Profile from CMDex-Paclitaxel Conjugates in Mouse Plasma, PBS (pH 7.4), and Tumor Homogenate at 37 °C

Released paclitaxel (■), 7-epi-paclitaxel (●), and total paclitaxel (□). Twenty microliters of solution of CMDex-paclitaxel conjugate (2 mg/ml, respectively) in saline was added to 200 μl of mouse plasma, PBS (pH 7.4), and tumor homogenate , and the solution was incubated at 37 °C with moderate shaking. a) CMDex-2′-gly-paclitaxel; b) CMDex-2′-ala-paclitaxel; c) CMDex-2′-leu-paclitaxel; d) CMDex-2′-ile-paclitaxel; e) CMDex-7-gly-paclitaxel. Each point represents the mean±S.D. of three experiments.

Fig. 5. This implies that the low recovery of the released paclitaxel is due to the instablity of paclitaxel in mouse plasma.

Toxicity of CMDex-Paclitaxel Conjugates *in Vivo* As a prelude to therapeutic experiments in animal tumor models, CMDex-paclitaxel conjugates were tested to determine the MTD in healthy BALB/c female mice. Mice tolerated 50 mg/kg of free paclitaxel in Cremophor EL® and ethanol administered as a single i.v. dose. The CMDex-paclitaxel conjugates, **10a**—**d** and **11**, seemed to tolerate it better, having an MTD exceeding 100 mg of free paclitaxel (eq)/kg.

Tissue Distribution Study Tissue distribution studies were performed to correlate the toxicity and efficacy results obtained for both CMDex-paclitaxel and paclitaxel using plasma and tissue drug concentrations as markers. Colon 26 tumor bearing mice received single i.v. administrations of each drug conjugate at a dose equivalent to the free paclitaxel MTD, 50 mg/kg.

In Fig. 6a the plasma concentration versus time profile of polymer-bound paclitaxel is compared with that of paclitaxel after dosing with free paclitaxel. It is clear from these results that CMDex allows for an extremely long-term circulation of paclitaxel. When CMDex-2′-gly-paclitaxel was administered, polymer-bound paclitaxel continued to circulate at high concentrations for an extended period in the bloodstream, whereas there was a low concentration of released drug in the plasma. In contrast, administrations of free paclitaxel were

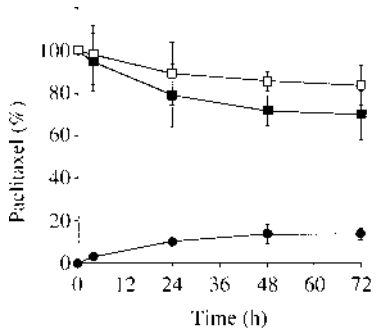

Fig. 5. Stability of Paclitaxel in Mouse Plasma at 37 °C

Released paclitaxel (■), 7-epi-paclitaxel (●), and total paclitaxel (□). Twenty microliters of a paclitaxel-MeOH solution (100 μg/ml, 50% (v/v)) was added to 200 μl of mouse plasma. The solution was incubated at 37 °C while shaking moderately. Each point represents the mean±S.D. of three experiments.

rapidly cleared from the blood.

Ideally, macromolecular prodrugs should be stable and pharmacologically inactive during circulation in the bloodstream but, after reaching the targeted site, they should release the active compound. Figure 6b shows the tumor concentration *versus* time profiles of the polymer-bound paclitaxel and released paclitaxel after dosing with CMDex-2′-gly-paclitaxel. As expected, there was evidence that high levels of released paclitaxel from the conjugate were in tumor tissue. The release of paclitaxel in tumor peaked at around

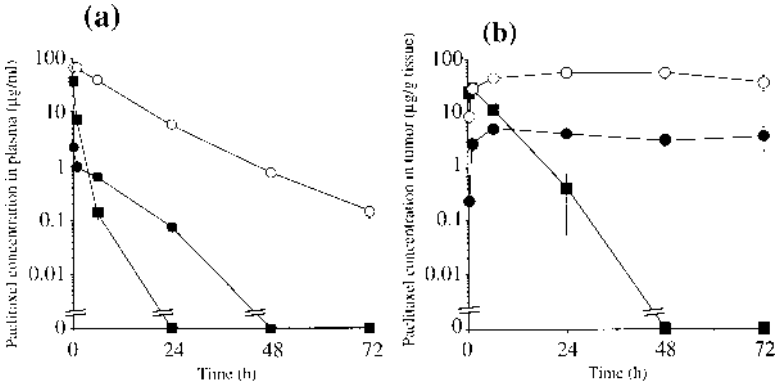

Fig. 6. Paclitaxel Concentration *vs*. Time Profiles of CMDex-2′-Gly-Paclitaxel and Free Paclitaxel: (a) Plasma Concentration *vs*. Time Profiles; (b) Tumor Concentration *vs*. Time Profiles

Polymer-bound paclitaxel (○), paclitaxel released (●) from CMDex-2′-gly-paclitaxel, and free paclitaxel (■) after intravenous administration to mice bearing colon 26 carcinoma at a dose equivalent to 50 mg of paclitaxel. Each point represents the mean±S.D. of three animals.

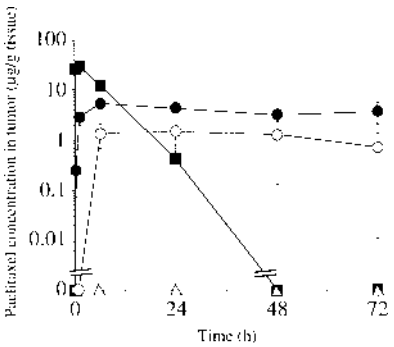

Fig. 7. Paclitaxel Concentration *vs*. Time Profiles of Released Paclitaxel and Free Paclitaxel Released from CMDex-2′-Gly-Paclitaxel (●), CMDex-2′-Leu-Paclitaxel (○), CMDex-2′-Ile-Paclitaxel (△), and Free Paclitaxel (■) after Intravenous Administration to Mice Bearing Colon 26 Carcinoma at a Dose Equivalent to 50 mg of Paclitaxel

Each point represents the mean±S.D. of three animals.

6 h, and thereafter declined very slowly. Concentration levels were maintained in the tumor site for more than 72 h, thereby suggesting cellular uptake and retention of paclitaxel. A similar profile of polymer-bound paclitaxel in the tumor was obtained. In contrast, with an administration using free paclitaxel, concentration levels in the blood cleared rapidly—within 48 h.

Figure 7 shows the tumor concentration *versus* time profiles of released paclitaxel after the administration of various CMDex-paclitaxel conjugates and free paclitaxel at doses equivalent to 50 mg of paclitaxel. As for CMDex-paclitaxel conjugates with 2′-gly and 2′-leu linker, the concentration in the tumor peaked from 6 to 24 h and subsequently declined very slowly maintaining nearly a constant level for more than 72 h, again suggesting the cellular uptake and retention of paclitaxel. In contrast, there was virtually no released paclitaxel from CMDex-2′-ile-paclitaxel in the tumor during the experiment.

Anti-tumor Activity *in Vivo* Because resistance of tumors to drugs is a frequent—and often lethal—occurrence in humans suffering from cancer, a paclitaxel-resistant strain, the colon 26 tumor model,[12] was used to evaluate the anti-tumor activity of CMDex-paclitaxel conjugates. Anti-tumor activity was evaluated using MTD dose and treatment given once a week for four weeks. To determine the effect of these drugs on colon 26 tumor growth, free paclitaxel or CMDex-paclitaxel conjugates were given as intermittent i.v. administrations on days 2, 9, 16, and 23 after s.c. tumor inoculation. Figures 8 and 9 show the tumor growth and body weight change curves of mice given a single i.v. administration of CMDex-paclitaxel, as compared with controls or animals given free paclitaxel weekly for 4 weeks.

Free paclitaxel in Cremophor EL® and ethanol was administered at its MTD of 50 mg/kg but showed no effect on tumor growth compared to the saline treated controls. It did, however, cause an obvious decrease in the body weight. CMDex-paclitaxel conjugates in saline were tested at 100 mg of paclitaxel (equivalent)/kg. CMDex-gly-2′-paclitaxel caused significant tumor growth delay compared to the paclitaxel or saline treated controls whereas no obvious decrease in the body weight was detected. CMDex-ala-2′-paclitaxel and CMDex-leu-2′-paclitaxel caused significant tumor growth delay as compared with the paclitaxel or saline treated controls, but also caused a decrease in the body weight. CMDex-ile-2′-paclitaxel and CMDex-gly-7-paclitaxel showed no effect on tumor growth delay nor did they show any obvious decrease in body weight. These tumor growth and body weight results are illustrated in Figs. 8 and 9.

The variation in tumor volume (mean±S.E. of 5 animals/group), on day 32 after tumor inoculation and once a week treatment for four weeks is shown in Table 4. The saline treated group of mice showed a progressive increase in tumor growth with the mean tumor volume increasing to 4092±268 mm^3 by day 32. Administration of the CMDex carrier or Cremophor EL® and ethanol resulted in a similar tumor volume growth as that observed in the saline treated group of mice (data not shown). Treatment of free paclitaxel resulted in no effect on tumor growth with the mean tumor volume close to that of the saline treated control (3351±326 mm^3 on day 32). The mice treated with CMDex-gly-2′-paclitaxel, CMDex-ala-2′-paclitaxel, and CMDex-leu-2′-paclitaxel conjugates showed significant tumor growth inhibition (mean tumor volume 1048±319 mm^3, 969±172 mm^3, and 2165±174 mm^3 on day 32, respectively) compared to either the saline or free paclitaxel treated groups.

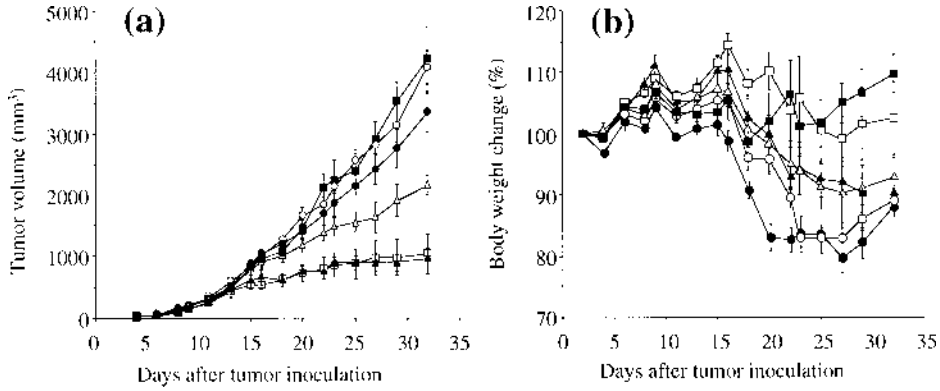

Fig. 8. *In Vivo* Efficacy of CMDex-Paclitaxel Conjugates and Free Paclitaxel in Mice Bearing Colon 26 Carcinoma: (a) Tumor Growth; (b) Body Weight Change

Paclitaxel or CMDex-paclitaxel conjugates were given as intermittent intravenous injections on days 2, 9, 16, and 23 after subcutaneous tumor inoculation. Free paclitaxel in Cremophor\ EL-ethanol (●) was administered at a dose of 50 mg/kg. CMDex-paclitaxel conjugates in saline were tested at 100 mg of equivalent paclitaxel per kilogram. The untreated group (○) served as controls. Each point represents the mean±SE for five mice, except the untreated group, which was for nine mice. The linkers 2′-gly (□), 2′-ala (▲), 2′-leu (△), and 2′-ile (■) were introduced into the CMDex-paclitaxel conjugates.

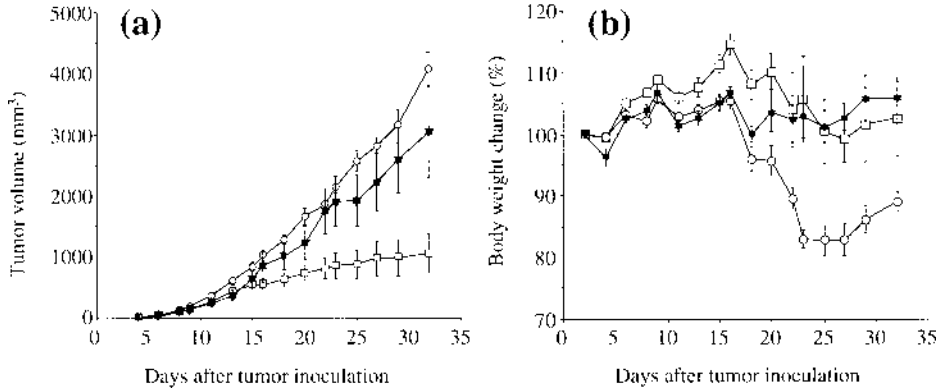

Fig. 9. *In Vivo* Efficacy of CMDex-Paclitaxel Conjugates and Free Paclitaxel in Mice Bearing Colon 26 Carcinoma: (a) Tumor Growth; (b) Body Weight Change

Paclitaxel or CMDex-paclitaxel conjugates were given as intermittent intravenous injections on days 2, 9, 16, and 23 after subcutaneous tumor inoculation. CMDex-paclitaxel conjugates in saline were tested at 100 mg of equivalent paclitaxel per kilogram. The untreated group (○) served as controls. Each point represents the mean±SE of five mice, except the untreated group, which was of nine mice. The linkers 2′-gly (□) and 7-gly (✱) were introduced into the CMDex-paclitaxel conjugates.

Table 4. Effect of Amino Acid Linker on Antitumor Effect of CMDex-paclitaxel Conjugates after Intermittent Intravenous Administration in Colon26-bearing Mice

Treatment group	Mean tumor volume (mm^3)[a)]	Tumor range (mm^3)	Mean body weight day 32 *vs.* day 2 (%)[a)]
	On day 32		
Saline	4092±268	3369—5292	89.4±1.8
Paclitaxel	3351±326	2791—4623	82.6±1.9
10a	1048±319[b,c)]	108—1913	104.2±6.1[b,c)]
10b	969±172[b,c)]	550—1250	92.8±2.0
10c	2165±174[b,c)]	1479—2304	94.9±3.9
10d	4221±534	2746—5292	106.5±5.5[b,c)]
11	3047±742	405—5000	102.4±3.7[b,c)]

a) Each value represents the mean±S.D. of five animals, except for the saline-treated group which is of nine mice. *b*) Significant difference in comparison with the saline-treated controls ($p<0.01$). *c*) Significant difference in comparison with the paclitaxel-treated group ($p<0.01$).

DISCUSSION

There has been a great deal of interest in the use of polymers as carriers for anti-tumor drugs. The basis for this school of thought is that binding anti-cancer drugs to high MW prodrug carriers can lead to: alternations in biological distribution; longer retention time within the body; reduction in systemic toxicity; and improvements in therapeutic efficacy.[36)] As long as a polymer and anti-tumor drug conjugate exists in the body—whether it is circulating or is in the target site—it has the potential to release the drug. Of course, the rate of drug release will depend on the nature of the polymer-drug linkage and that linkage could theoretically be chosen for either pH or enzymatic degradation in order to better me-

diate prolonged drug release. Furthermore, the stability of the drug-conjugate linkage and its potential for controlled degradation is an important determinant in the efficacy and toxicity of any prodrug. A general rule is that if the conjugate is designed as a circulatory depot, the drug should be liberated according to a prescribed schedule without immediate dissociation upon administration. Similarly, if the conjugate is meant to reach a particular extracellular or intracellular target, the linkage must be sufficiently stable to maintain its chemical integrity until its reaches its final destination.

Although many types of linkages can be employed in a prodrug strategy, enzymatic hydrolysis of ester or amide functionality have been most often used. Paclitaxel has a unique structure in that there are three hydroxyl groups and, of these three, two are reactive giving it its anti-cancer activity. By converting the 2′- or 7-hydroxyl group to an ester, a water-soluble prodrug can be formed. In earlier attempts to make 2′-glycyl paclitaxel and other amino acid ester derivatives of paclitaxel difficulty was reported in obtaining pure 2′-esters.[32,33] These derivatives were characterized as unstable compounds that readily reverted to paclitaxel in the presence of formic acid or trifluoroacetic acid at the deprotection steps. The instability of the 2′-glycyl ester salt is probably due to a simple inductive effect of the protonated amino group assisting in the attack of external nucleophiles on the 2′-acyl group.[33] We have circumvented this inherent instability by using *Z*-amino acid and catalytic transfer hydrogenolysis. Amino acids are ideal as linkers since, as bi-functional molecules, they provide a reactive carboxyl group with which to attach paclitaxel and an amino group that can be easily modified by CMDex. It also appears that an alteration of the amino acid linker group within the conjugate, or an alteration of steric hindrance[37] of the linker group resulting from α-substitution can lead to different rates of both enzymatic and nonenzymatic breakdown. In this way, the rate of dissociation can be taken to extremes: exclusively rapid circulatory hydrolysis, or slow intracellular breakdown. Both extremes are less than optimal, since rapid circulatory hydrolysis can result in toxicity that causes profound damage to normal cells, while, on the other hand, most intracellular breakdown depends on unpredictable endocytotic transport and slow enzymatic degradation.[38] Of interest is the possibility of reaching some middle ground by way of the introduction of an amino acid linker between the CMDex and paclitaxel which would cause moderate and predictable circulatory dissociation, but would still allow for substantial CMDex-induced tumor accumulation *via* passive targeting. Any accumulated conjugate could then presumably be released by either extracellular or intracellular means.

The fact that amino acids have simple esters which can be used as linker groups in the coupling with CMDex and that selective introduction of amino acid to the 2′- or 7-hydroxyl group of paclitaxel appears to be sufficient for adjusting the dissociation of the conjugate, led us to choose the type of prodrug system described here. The changes in kinetics *in vitro* also appear to correlate with both the safety and efficacy of the drug. For example, looking at the differences among the 2′-gly, 2′-ala, 2′-leu, 2′-ile and 7-gly derivatives, the 2′-gly derivative showed a relatively fast dissociation half-life among the conjugates tested in mouse plasma, PBS (pH 7.4), and tumor homogenate. However, *in vivo* testing of this conjugate demonstrated good tumor distribution, tumor retention, and significant anti-tumor activity in the solid tumor model compared to both the control and paclitaxel treatment groups in mice. This enhanced tumor distribution may be the basis for the extreme differences in safety and efficacy effects seen between the conjugated and unconjugated forms of paclitaxel in the colon 26 model. In fact, our earlier study has shown that CMDex-modified drugs cause an increased intra-tumor accumulation, together with an increased therapeutic index.[27] The 2′-ala and 2′-leu analogs demonstrated slow dissociation in mouse plasma. Somewhat similar to 2′-gly, they resulted in significant anti-tumor activity in the colon 26 tumor model as compared with both control and paclitaxel treatment groups in mice. However, unlike 2′-gly derivative, the 2′-ala and 2′-leu analogs caused body weight loss similar to both the control and paclitaxel treated mice. This suggests that the rate of normal tissue distribution and/or cellular uptake is not appropriate, leading to little distinction between the safety of these conjugates and the unconjugated forms of paxlitaxel. The use of 2′-ile or 7-gly derivatives produced a highly stable conjugate with very low toxicity, but demonstrated the smallest anti-tumor activity observed among all the analogs.

Significant differences in kinetics (*in vitro* plasma, PBS (pH 7.4) and tumor homogenate) were observed for the various CMDex-amino acid-paclitaxel conjugates illustrated above. These observed variations in kinetics are probably due to a combination of steric factors and the hydrophobicity for a particular amino acid which impacts bond hydrolysis. These analogs could be hydrolyzed with enzymatically-mediated pathways. It is possible that specific amino acids may result in favorable hydrolysis of the ester bond between the amino acid and paclitaxel *via* pH or esterase release. In contrast, others may initially encourage amide bond breakage between CMDex and amino acid resulting in an amino acid paclitaxel conjugate, which would still have its bioavailability.[39] The ester bond would subsequently be cleaved to activate paclitaxel. As Fig. 4 illustrates, the conjugate's differences in rates of dissociation need to be considered whenever examining its usefulness.

Several mechanisms may explain the differences in efficacy observed among the conjugates: alterations in pharmacokinetics, tumor and tissue distribution, or rate of cellular uptake, to mention a few. Polymer conjugation has been shown to alter the pharmacokinetics and biodistribution of the bound drug as compared with the free form.[27] Pharmacokinetics data of CMDex-DXR suggest that the CMDex transport form releases paclitaxel in a relatively slow and sustained manner.[27] This release, *in vivo*, is governed by the circulatory retention of the high MW polymeric drug and its gradual dissociation. The rate of the conjugate's tissue uptake and/or circulatory dissociation must therefore be faster than its rate of circulatory elimination to allow for optimal activity of the bound drug. Hence, the rate of *in vivo* conjugate breakdown affects the drug's biodistribution and may ultimately impact both safety and efficacy. For example, the rather slow breakdown of 2′-ala and 2′-leu following an i.v. administration causes toxicity of free paclitaxel, an effect that was not observed with the 2′-gly derivative. In contrast, the stable 2′-ile and 7-gly derivatives, which display the slowest hydrolysis within the series, appear ineffective in this study.

It is well established that large macromolecules circulating for extended periods show substantial tumor accumulation.[40] In fact, CMDex molecules of 150 kDa or greater MW demonstrate significantly higher accumulation in tumors than in normal tissue with the exception of the spleen.[27] The underlying physiological mechanism appears to be a combination of increased tumor vascular permeability with insufficient lymphatic drainage, resulting in what is termed the 'enhanced permeability and retention (EPR) effect', thought to be a universal solid tumor phenomenon for macromolecular drugs.[41]

It is quite possible that the use of different amino acids within the CMDex-paclitaxel conjugate could affect the amount and form of the paclitaxel reaching the tumor site. Ideally, it would be desirable for release of the drug to occur only in the vicinity of tumor cells, thereby sparing normal cells from concomitant destruction. In the case of intact conjugate accumulation, there exist two possible pathways for this conjugate's breakdown in the tumor: simple ester hydrolysis, or amide cleavage. The presence of functional peptidase within the extracellular space of tumors[42] certainly makes the second pathway feasible. The production of amino acid conjugated species has been implicated in the enhanced cellular uptake of daunorubicin.[43] A recent paper has reported on the cellular uptake of amino acid ester prodrugs by a peptide transporter,[44] making entrance into the cell theoretically possible through either active or passive transport.

Our current study has extended CMDex prodrug strategies to include the use of spacer groups in paclitaxel modification and was undertaken to determine the effect of various amino acid spacers on the activity of CMDex-paclitaxel conjugates. It was found that the use of specific amino acid spacers affected the breakdown (dissociation) of CMDex-paclitaxel, its *in vivo* efficacy, and its toxicity. Conjugating 150 kDa of CMDex to 2′-gly paclitaxel results in a homogeneous water-soluble prodrug with less toxicity and enhanced anti-tumor activity compared with the resultant native drug. Work is now expanding on the effect of substituting other linkage for gly in the CMDex-paclitaxel delivery system, as well as examining different CMDex-spacer-paclitaxel transport forms in a variety of *in vivo* applications.

REFERENCES

1) Wani M. C., Taylor H. L., Wall M. E., Coggon P., McPhail A. T., *J. Am. Chem. Soc.*, **93**, 2325—2327 (1971).
2) Long H. J., *Mayo Clin. Proc.*, **69**, 341—345 (1994).
3) Cahan M. A., Walter K. A., Colvin O. M., Brem H., *Cancer Chemother. Pharmacol.*, **33**, 441—444 (1994).
4) Wiernik P. H., Einzig A. I., *Monogr. Natl. Cancer Inst.*, **15**, 185—187 (1993).
5) Arbuck S. G., Christian M. C., Fisherman J. S., Cazenave L. A., Sarosy G., Suffness M., Adams J., Canetta R., Cole K. E., Friedman M. A., *Monogr. Natl. Cancer Inst.*, **15**, 11—24 (1993).
6) Foa R., Norton L., Seidman A. D., *Int. J. Clin. Lab. Res.*, **24**, 6—14 (1994).
7) Lopes N. M., Adams E. G., Pitts T. W., Bhuyan B. K., *Cancer Chemother. Phrmacol.*, **32**, 235—242 (1993).
8) Weiss R. B., Donehower R. V., Wiernik P. H., Ohnuma T., Gralla R. J., Trump D. L., Baker J. R., Van Echo D. A., VonHoff D. D., Leyland Jones B., *J. Clin. Oncol.*, **8**, 1263—1268 (1990).
9) Enzig A., Wiernik P. H., Sasloff J., Garl S., Runowicz C., O'Hanlon K. A., Goldberg G., *J. Clin. Oncol.*, **10**, 1748—1753 (1992).
10) Rogers B. B., *Oncol. Nurs. Forum*, **20**, 1483—1489 (1993).
11) Riondel J., Jacrot M., Fessi H., Puisieux F., Potier P., *In Vivo*, **6**, 23—27 (1992).
12) Sharma A., Mayhew E., Straubinger R. M., *Cancer Res.*, **53**, 5877—5881 (1993).
13) Sharma A., Straubinger R. M., *Pharm. Res.*, **11**, 889—895 (1994).
14) Straubinger R. M., Sharma A., Murray M., Mayhew E., *J. Natl. Cancer Inst. Monogr.*, **15**, 69—78 (1993).
15) Alkan-Onyuksel H., Ramakrishnan S., Chai H.-B., Pezzuto J. M., *Pharm. Res.*, **11**, 206—212 (1994).
16) Tarr B. D., Sambandan T. G., Yalkowsky S. H., *Pharm. Res.*, **4**, 162—165 (1987).
17) Wheeler J. J., Wong K. F., Ansell S. M., Masin D., Bally M. B., *J. Pharm. Sci.*, **83**, 1558—1564 (1994).
18) Sharma D., Chelvi T. P., Kaur J., Chakravorty K., De T. K., Maitra A., Ralhan R., *Oncol. Res.*, **8**, 281—286 (1996).
19) Sharma U. S., Balasubramanian S. V., Straubinger R. M., *J. Pharm. Sci.*, **84**, 1223—1230 (1995).
20) Greenwald R. B., Pendri A., Bolikal D., Gilbert C. W., *Bioorg. Med. Chem. Lett.*, **4**, 2465—2470 (1994).
21) Li C., Yu D.-F., Newman R. A., Cabral F., Stephens L. C., Hunter N., Milas L., Wallace S., *Cancer Res.*, **58**, 2404—2409 (1998).
22) Chun L., Price J. E., Milas L., Hunter N. R., Shi K., Dong-Fang Y., Chusilp C., Wallace S., *Clin. Cancer Res.*, **5**, 891—897 (1999).
23) Monelli N., Angelucci F., Pesenti E., Suarato A., Biasoli G., US Patent, 5,362,831 (1994).
24) Pesentie E., Franzetti C., Biasoli G., Ciomei M., Paston W., Marsiglio A., Stegnjaich S., Mongelli N., Grandi M., *Proc. Am. Assoc. Cancer Res.*, **1995**, 1824 (1995).
25) Greenwald R. B., Gilbert C. W., Pendri A., Conover C. D., Xia J., Martinez A., *J. Med. Chem.*, **39**, 424—431 (1996).
26) Vasey P. A., Duncan R., Kaye S. B., Cassidy F., *Eur. J. Cancer*, **31A**, S193 (1995).
27) Sugahara S., Okuno S., Yano T., Hamana H., Inoue K., *Biol. Pharm. Bull.*, **24**, 535—543 (2001).
28) Daotian F., Roger A. O., *Anal. Biochem.*, **227**, 377—384 (1995).
29) Honda S., Akao E., Suzuki S., Okuda M., Kakei K., Nakamura J., *Anal. Biochem.*, **180**, 351—357 (1989).
30) Larm O., Lindberg B., Svensson S., *Carbohyd. Res.*, **20**, 39—48 (1971).
31) Mase H., Hiraoka M., Suzuki A., Nakanomyo H., *Yakugaku Zasshi*, **114**, 351—355 (1994).
32) Mathew A. E., Mejillano M. R., Nath J. P., Himes R. H., Stella V. J., *J. Med. Chem.*, **35**, 145—151 (1992).
33) Ringel I., Horwitz S. B., *J. Pharmacol. Exp. Ther.*, **242**, 692—698 (1987).
34) Takakura Y., Hashida M., *Crit. Rev. Oncol. Hematol.*, **18**, 207—231 (1995).
35) Deutsch H. M, Glinski J. A, Hernandez M., Haugwitz R. D., Narayan V. L., Suffness M., Zalkow L. H., *J. Med. Chem.*, **32**, 788—792 (1989).
36) Zhao Z., Kingston D. G. I., Crosswell A. R., *J. Natur. Prod.*, **54**, 1607—1611 (1991).
37) Hansch C., Leo A., "Exploring QSAR," ed. by Heller S. R., ACS Professional Reference Book, American Chemical Society, 1995, pp. 69—96.
38) Soyez H., Schacht E., Vanderkerken S., *Adv. Drug Deliv. Rev.*, **21**, 81—106 (1996).
39) Mathew A. E., Mejillano M. R., Nath J. P., Himes R. H., Stella V. J., *J. Med. Chem.*, **35**, 145—151 (1992).
40) Maeda H., Seymour L., Miyamoto Y., *Bioconjugate Chem.*, **3**, 351—362 (1992).
41) Maeda H., *Adv. Drug Deliv. Rev*, **6**, 181—202 (1991).
42) Hess R., *Cancer Res.*, **20**, 940—943 (1960).
43) Baurain R., Masquelier M., Deprez-De C. D., Trouet A., *J. Med. Chem.*, **23**, 1171—1174 (1980).
44) Han H., Oh D., Amidon G. L., *Pharm. Res.*, **15**, 1382—1386 (1998).