AZ10992 is a novel paclitaxel–carboxymethyl (CM) dextran conjugate via a Gly–Gly–Phe–Gly linker with the molecular weight (MW) of 150 kDa. Our previous studies demonstrated that AZ10992 exerts strong antitumor activity against the human tumor xenografts that are highly refractory to paclitaxel, attributable to passive tumor targeting of released paclitaxel. This study examines the effects of carrier MW, anionic charge and drug-contents on the antitumor effects of AZ10992. To study antitumor effects, colon26 carcinoma-bearing BALB/c female mice received repeated (3 injections administered with 7 d intervals) intravenous administration of non-polymer-bound paclitaxel or paclitaxel–CM dextran conjugates. The results indicated that the conjugate comprising dextran T-110 (MW 110 kDa) with the degree of substitution (DS) value for the CM group of 0.50—0.55 per glucose residue and the drug contents of 5.5—6.5% (w/w) would be appropriate for AZ10992 regarding antitumor activity. Maximal tolerated dose (MTD) of AZ10992 was more than twice of non-polymer-bound paclitaxel. Furthermore, normal BALB/c female mice were treated with repeated (3 injections administered with 2 d intervals) intravenous administration of non-polymer-bound paclitaxel or AZ10992 at 50 mg/kg/d (based on the amount of paclitaxel to CM dextran) to study neurotoxicity. AZ10992 did not induce degeneration of myelin or swelling of Schwann cells in sciatic nerves.

Key words  paclitaxel delivery system; antitumor effect; neurotoxicity; polymer–drug conjugate

Paclitaxel shows great promise as an anti-neoplastic agent for a variety of human cancers, including ovarian, breast, and non-small cell lung cancer and acquired immune deficiency syndrome (AIDS)-related Kaposi’s sarcoma.1—6) Docetaxel and paclitaxel together form the drug category of taxanes.14) Such macromolecules are expected to accumulate in tumors effectively after intravenous administration. AZ10992 was thus designed to have the MW of approximately 150 kDa, weak anionic charges, and a peptidyl linker (GlyGlyPheGly) to provide an appropriate release rate of paclitaxel with time-dependent cytocidal activity. Our previous study demonstrated the proof of our design in terms of therapeutic efficacy.15)

However, further studies are required to provide crucial information about safety profile, particularly regarding the neurotoxicity of paclitaxel. Paclitaxel is associated with neurotoxic adverse effects such as peripheral sensory neuropathy. The neurotoxicity is also reported to constitute the significant dose-limiting toxicity of the drug.17,18) Furthermore, no effective or FDA-approved therapies have yet been established to prevent the development or reduce the frequency of paclitaxel-induced neurotoxicity.

The purpose of the present study was three fold: first, to provide a rationale for the selection of CM dextran as a carrier for AZ10992; second, to optimize paclitaxel–CM dextran conjugate in terms of molecular sizes, degrees of carboxymethylation, and loadings of paclitaxel to obtain AZ10992; third, to evaluate reduction of paclitaxel-induced neurotoxicity in mice. To this end, various CM dextrans with...
differing DS values and MWs were synthesized to elucidate relationships between physicochemical properties of CM dextran and biological fates in the body. Moreover, a range of paclitaxel–CM dextran conjugates with differing DS, MW, and drug contents were synthesized to evaluate the effects on antitumor activity in colon26-bearing mice and to evaluate neurotoxicity caused by paclitaxel in mice. The results would provide a clear picture of the carrier, antitumor effects, and neurotoxicity of AZ10992.

MATERIALS AND METHODS

Materials Paclitaxel–CM dextran conjugates via a Gly–Gly–Phe–Gly linker were synthesized and characterized as described previously.16) Paclitaxel (Taxol®) was purchased from Hauser Chemical Research (Boulder, CO, U.S.A.). Dextran (MW approximately 50, 80, and 150 kDa) were purchased from Fluka BioChemika (Buchs, Switzerland), and dextran T-10, T-70 and T-110 (MW approximately 10, 70, and 110 kDa, respectively) were obtained from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of reagent-grade purity or better. Radiolabeled dextrans were synthesized as described previously.19) 3.7 MBq of [3H]-labeled glycine was conjugated with each type of CM dextran in the presence of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in a solution of N,N-dimethylformamide (DMF)–H2O (1:1) to produce the [3H]-labeled CM dextrans. The radiolabeled products were fractionated by a gel filtration method (Sephadex G-25 column). Stability of these [3H]-labeled CM-dextran carriers in rat plasma was evaluated by gel filtration profile analysis. Samples, which were incubated for 1 h, 6 h, and 24 h in rat plasma at 37°C, were loaded onto a TOSOH TSK gel G4000PWXL column and were eluted with 0.9% NaCl solution. Each fraction (0.4 ml) was collected, and the radioactivity was counted. As for the stability of the [3H]-labeled CM dextran in rat plasma, there was no difference between each gel filtration profile of 1 h, 6 h, and 24 h. The [3H]-labeled CM-dextran carriers would be stable at least for 24 h at 37°C in rat plasma. Chemical structure and characteristics of AZ10992 are presented in Fig. 1.

**Determination of Paclitaxel Concentration in Plasma**

Amounts of paclitaxel in plasma were determined by HPLC as described previously.16) Paclitaxel concentration in each sample was calculated by comparing the ratio of peak areas of paclitaxel and the internal standard with a corresponding standard curve prepared using appropriate blank samples. The calibration curve used for quantification of paclitaxel was linear over the range of 50—5000 ng/ml in plasma with the correlation coefficient $r^2=0.995$.

**In Vitro Drug Release Test**

Release of paclitaxel from paclitaxel–CM dextran conjugates was examined by adding 20 µl of 0.9% NaCl solution containing conjugates (2 mg/ml) to 200 µl of plasma from BALB/c female mice. Added samples were incubated at 37°C, shaking moderately. The amounts of liberated paclitaxel in the conjugate at the time points 0, 3, 6, 24, 48, and 72 h during incubation were determined by HPLC.

**Procedure for Animal Experiments**

Animals: Animal experiments were performed in compliance with the regulation of the Animal Ethical Committee of Asahi Kasei Pharma Corporation.

Tissue Distribution of [3H]-Labeled Dextran Carriers: Female Wistar rats weighing 110—140 g each were purchased from Japan SLC (Shizuoka, Japan). A Walker256 ascites cell suspension (1×10⁷ cells) was transplanted into the right hind leg muscle of each rat. At 5 d after transplant, radiolabeled carriers were intravenously administered at dose of 10 mg/kg with approximately 1×10⁶ dpm per dose. Radioactivity of [3H]-labeled dextran carriers was determined as described previously.19) The animals were kept in metabolic cages, and urine was collected throughout the study. Blood samples were drawn from the contralateral jugular vein with heparinized syringes at the time points of 0, 5 min, and 30 min and 1 h, 2 h, 4 h and 24 h. Plasma was separated by centrifugation within 10 min of sampling. Immediately after the last sampling of blood, rats were anesthetized with ether and sacrificed. The spleen, kidney, muscle, brain, bone marrow, liver, and tumor were removed and combusted using an ASC-113 automatic sample combustion system (Aloka, Tokyo, Japan). The [3H] was collected as 3H2O, scintillation fluid was added (Aquasol II; Dupont-NEN Research Products, Boston, MA, U.S.A.), and radioactivity was measured in an LSC-3600 liquid scintillation counter (Aloka). Radioactivity levels were expressed as a percentage of injected dose.

Toxicity Dose-Finding Studies: MTD for intravenously administered paclitaxel–CM dextran conjugates was determined in healthy 7-week-old BALB/c female mice (Japan SLC). Survey experiments to define MTD were performed with 3 animals/group. Conjugates were dissolved in a solution containing 0.9% NaCl solution only. Non-polymer-bound paclitaxel was dissolved in a vehicle consisting of Cremophor® EL 50% and ethanol 50%, then diluted with 0.9% NaCl solution to prepare desired concentrations for administration of a single intravenous dose. Drug effects were determined by close observation of body weight and survival. Mouse body weight was monitored 2—3 times/week for more than 28 d. MTD was defined as the maximum dose that caused no drug-related lethality while producing an animal body weight loss of less than 20% of original weight. A fatality within 2 weeks after the first drug treatment was defined as a drug-related death. The body weight loss greater than 30% was considered lethal.

Evaluation of Antitumor Activity against Colon26 Carcinoma: 1×10⁷ cells of colon26 carcinoma (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research,
Tokyo) was administered to the right flank of 6-week-old BALB/c female mice (Japan SLC). Mice were randomized into various treatment groups and numbered. Tumor diameters were measured with a sliding caliper 2—3 times per week. Dose per mouse was adjusted based on body weight as determined at the beginning of treatment. Treatment was started 2 d after tumor implantation, using intravenous administration via the tail vein. The untreated mice and mice treated with a carrier without paclitaxel were used as controls. Tumor volume \( (TV) \) was calculated according to the formula: \( TV = (L \times W^2)/2 \), where \( L \) and \( W \) were the major and minor dimensions of the tumor, respectively. Drug treatment with 5—11 mice per group was applied at q7d through day 16. The 0.9% NaCl solution-treated group served as a control.

We evaluated influences of CM dextran with various MW and anionic charge on tissue distribution and tested for antitumor effects of paclitaxel–CM dextran conjugates with different MW, anionic charges, and drug contents.

Evaluation of Neurotoxicity: Severity of neurotoxicity was mainly assessed histologically. To evaluate the AZ10992’s improvements in efficacy attributable to conjugation, preliminary studies in healthy 8-week-old BALB/c female mice were set up to assess MTD as a prelude to therapeutic experiments in animal models. Dose for each mouse was adjusted based on weight as determined at the beginning of treatment. Treatment was administered intravenously through the tail vein. Drug effects were evaluated by close observation of body weight, behavior, and histopathological examination of sciatic nerves. Mouse behavior and body weight were monitored for at least 30 d.

AZ10992 or non-polymer-bound paclitaxel was given as repeated intravenous administration. Non-polymer-bound paclitaxel in Cremophor® EL-ethanol was administered at a dose of 12.5, 25, or 50 mg/kg/d, and AZ10992 in 0.9% NaCl solution was tested at the same dose of equivalent paclitaxel per kilogram. The 0.9% NaCl solution-treated group served as a control. Sciatic nerves of 3 mice from each of the groups treated with 50 mg/kg/d of non-polymer-bound paclitaxel and AZ10992 as well as the 0.9% NaCl solution-treated control were removed on day 9, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Tissue sections were stained with toluidine blue and examined under light microscopy.

Statistical Analysis: Data are expressed as mean ± S.D. Differences between treatment groups were assessed using one-way analysis of variance. Statistical significance was defined as \( p < 0.05 \) for the rejection of a null hypothesis. When statistical significance was identified, multiple comparisons were determined using the least significant differences technique. Statistical analysis was conducted using StatView software (SAS Institute, Cary, NC, U.S.A.).

RESULTS

**Distribution of CM-Dextran Carriers** DS and MW Effects on Plasma Concentration: To study the relationship between physicochemical property of CM dextrans and their biological fate in the body, we synthesized a range of CM dextrans. Dextran carriers with different DS values of CM groups and MWs were intravenously administered to rats bearing Walker256 at a single intravenous dose of 10 mg carrier/kg (\( 1 \times 10^6 \) dpm/body). Figure 2A shows DS-dependent profiles of CM dextran (CM-D110) synthesized from dextran 110 kDa; Figure 2B shows MW-dependent profiles of CM dextran (CM-D (DS 0.6)) with the DS value of approximately 0.6. The decline seems mono-exponential for all CM dextrans (Figs. 2A, B). The comparison with dextran 110 kDa (DS 0) demonstrated that carboxymethylation and a large MW could endow dextran with long-term circulation. Plasma area under the curve (AUC) profiles up to 24 h after administration peaked around the DS value of 0.2—0.6. As for the MW effects of CM dextrans, the plasma AUC profiles increased with increasing MW, although it appeared to plateau at the MW more than 80 kDa. These results suggest that the selection of DS value and MW would be the key for the longer circulation of CM dextrans in plasma.

DS and MW Effects on Tissue Distribution of CM Dextrans: Using ranges of CM dextrans with DS 0—0.8 (MW 110 kDa) and MW 50—150 kDa (DS 0.6), tissue distributions were studied in Walker256-bearing rats. CM dextran concentrations at 24 h in the kidney, muscle, brain, spleen, liver, bone marrow, and tumor are summarized in Fig. 3 in comparison with dextran 110 kDa (DS 0). Several observations may be made from these studies.

The radioactivity of CM dextran in liver were relatively high (7.1% of dose/g) at DS 0, then decreased to lows of 0.4—1.0% of dose/g at DS 0.2—0.6. In bone marrow, the radioactivity showed similar tendencies to those found in liver, starting relatively high (6.1% of dose/g) at DS 0 and decreasing to lows of 0.29—0.80% at DS 0.2—0.6. As expected,

---

**Fig. 2.** Effects of (A) DS Values and (B) MWs on Plasma Concentration—Time Profiles of CM Dextrans after Intravenous Administration to Rats Bearing Walker256 Carcinoma at a Dose of 10 mg/kg with about \( 1 \times 10^6 \) dpm.

Data points are means of three rats; bars, S.D., if not shown, are within the size of the symbols. Solid lines are obtained by fitting data points to a one-component model.
CM dextrans with weak anionic charges (DS 0.2—0.6) avoided accumulation in the liver and bone marrow relative to dextran (DS 0). The accumulation of CM dextran with DS 0.8 in the liver was more than twice of that with DS 0.6. CM dextran concentrations in spleen were relatively high (4.2—6.1% of dose/g) for all DS values and MWs without DS- or MW-dependence. Radioactivity of all CM dextrans in the muscle and brain showed the low levels of 0.01—0.06% with no DS- or MW-dependence. In tumor tissues, the radioactivity increased gradually from 1.4 to 3.7%, as the DS value increased from 0 to 0.8 and showed high radioactivity levels (3.30—4.10% of dose/g) for the MW more than 110 kDa. In addition, tumor accumulation of CM dextrans seemed to increase with DS- and MW-dependence.

Based on these findings, CM dextran with DS of 0.6 and MW of 110 kDa appears to show the characteristically high plasma AUC with high distribution to tumor. This suggests CM dextran may be suitable as a drug carrier for antitumor drugs.

In Vitro Drug Release

In vitro release profiles of paclitaxel from a range of paclitaxel–CM dextran conjugates, up to 72 h in BALB/c female mouse plasma, are shown in Fig. 4. HPLC analysis revealed that different paclitaxel–CM dextran conjugates were converted to different proportions of paclitaxel and 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.

Fig. 3. Effects of (A) DS Values and (B) MWs on Tissue Distribution of CM Dextran for 24 h after Intravenous Administration to Rats Bearing Walker256 Carcinoma at a Dose of 10 mg/kg with About 1×10^6 dpm

Column, mean of 3 rats; bars, S.D.

Fig. 4. Carrier and Drug Content Effects on the Amount of Released Paclitaxel from Paclitaxel–CM Dextran Conjugates in Mouse Plasma for 72 h

Released paclitaxel (■), 7-epi-paclitaxel (●), and total paclitaxel (□). Each point represents the mean±S.D. of three experiments. Effect of a, b, c) DS of CM groups (MW 110 kDa, drug contents 6.3% w/w), d, e, f, g) molecular weights (DS 0.55, drug contents 6.3% w/w) and h, i, j) drug contents (DS 0.55, MW 110 kDa).
Amino acid–paclitaxel or peptide–paclitaxel compounds, such as Gly–paclitaxel, Phe–Gly–paclitaxel, Gly–Phe–Gly–paclitaxel, and Gly–Gly–Phe–Gly–paclitaxel, were not detected during the experiment. These paclitaxel compounds would have been rapidly hydrolyzed to paclitaxel in mouse plasma. The amount of paclitaxel released from conjugates was decreased by increasing drug content (drug contents used: 6.3—13.2%), increasing DS value (DS values used: 0.39—0.80), or decreasing MW (MW used: 40—250 kDa), which was plateaued at MW less than 110 kDa.

**Animal Experiments** Toxicity Dose-Finding Studies: To compare antitumor effects of the paclitaxel–CM dextran conjugate (drug contents 6.5% w/w, DS 0.6, dextran 110 kDa) with non-polymer-bound paclitaxel at optimal doses and to evaluate improvement in efficacy attributable to conjugation, preliminary studies in healthy BALB/c female mice were set up to assess MTD as a prelude to therapeutic experiments in animal tumor models. In the case of single intravenous administration, MTD was found to be more than 100 mg/kg for the paclitaxel–CM dextran conjugate based on amount of paclitaxel to CM dextran and 50 mg/kg for non-polymer bound paclitaxel. In the case of repeated intravenous administration with q2d×3 (3 injections administered with 2 d intervals), q4d×4, q4d×7, and q7d×4, MTD was found to be 50, 70, 75, and 100 mg/kg/d for the paclitaxel–CM dextran conjugates, respectively, and was 50 mg/kg/d for non-polymer-bound paclitaxel with any administration schedule.

Anti-tumor Activity against Colon26 Carcinoma: A paclitaxel-resistant colon26 tumor model was used to evaluate anti-tumor activity of paclitaxel–CM dextran conjugates. To determine the effect of these drugs on colon26 tumor growth, non-polymer-bound paclitaxel or paclitaxel–CM dextran conjugates were given as repeated intravenous administration of q7d×3 after subcutaneous tumor implantation.

Figure 5 shows relative tumor volume and body weight change of mice on day 20 that were given repeated intravenous administration of paclitaxel–CM dextran conjugates as compared with the controls or the animals given non-polymer-bound paclitaxel. Non-polymer-bound paclitaxel in Cremophor® EL and ethanol was administered at its MTD of 50 mg/kg/d but showed no effect on tumor growth compared to the 0.9% NaCl solution-treated controls. However, this treatment did cause an obvious decrease in body weight. Paclitaxel–CM dextran conjugate at 100 mg/kg/d (q7d×3) caused significant tumor regression and at the same time inhibited body weight loss as shown in Table 1. As a result, conjugates with dextran 110 kDa, the DS value of CM group of 0.6 per sugar residue, and the paclitaxel contents of 6.3%

### Table 1. Tumor Growth Inhibition Rates of Paclitaxel–CM Dextran Conjugates and Non-polymer-Bound Paclitaxel in Mice Bearing Colon26 Carcinoma Cells

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Physicochemical property of conjugates</th>
<th>Dose (mg/kg/d)</th>
<th>Mean tumor volume (mm³) at day 20</th>
<th>Tumor growth inhibition rates</th>
<th>Mean body weight change day 20 vs. day 1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution</td>
<td>—</td>
<td>—</td>
<td>1358±183</td>
<td>0</td>
<td>91.6</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>—</td>
<td>50</td>
<td>939±80</td>
<td>30.9</td>
<td>96.9</td>
</tr>
<tr>
<td>Conjugate a</td>
<td>DS 0.39</td>
<td>100</td>
<td>128±33 (a)</td>
<td>90.5</td>
<td>105.6</td>
</tr>
<tr>
<td>Conjugate b</td>
<td>DS 0.55</td>
<td>100</td>
<td>29±11 (a)</td>
<td>97.9</td>
<td>106.6</td>
</tr>
<tr>
<td>Conjugate c</td>
<td>DS 0.80</td>
<td>100</td>
<td>240±104 (a)</td>
<td>82.3</td>
<td>107.2</td>
</tr>
<tr>
<td>Conjugate d</td>
<td>MW 40 kDa</td>
<td>100</td>
<td>164±41 (a)</td>
<td>87.9</td>
<td>111.2</td>
</tr>
<tr>
<td>Conjugate e</td>
<td>MW 70 kDa</td>
<td>100</td>
<td>89±42 (a)</td>
<td>93.5</td>
<td>106.2</td>
</tr>
<tr>
<td>Conjugate f</td>
<td>MW 110 kDa</td>
<td>100</td>
<td>29±11 (a)</td>
<td>97.9</td>
<td>106.6</td>
</tr>
<tr>
<td>Conjugate g</td>
<td>MW 250 kDa</td>
<td>100</td>
<td>90±23 (a)</td>
<td>93.4</td>
<td>104.3</td>
</tr>
<tr>
<td>Conjugate h</td>
<td>Content 6.3%</td>
<td>100</td>
<td>29±11 (a)</td>
<td>97.9</td>
<td>106.6</td>
</tr>
<tr>
<td>Conjugate i</td>
<td>Content 9.0%</td>
<td>100</td>
<td>69±25 (a)</td>
<td>94.9</td>
<td>106.8</td>
</tr>
<tr>
<td>Conjugate j</td>
<td>Content 13.2%</td>
<td>100</td>
<td>151±46 (a)</td>
<td>88.9</td>
<td>99.8</td>
</tr>
</tbody>
</table>

Paclitaxel–CM dextran conjugates, non-polymer-bound paclitaxel and 0.9% NaCl solution were administered intravenously on days 2, 9 and 16. Tumor volumes and body weights were measured 2—3 times/week. Non-polymer-bound paclitaxel in Cremophor® EL-ethanol was administered at a dose of 50 mg/kg/d and paclitaxel–CM dextran conjugates in 0.9% NaCl solution were tested at 100 mg of equivalent paclitaxel per kilogram per day. The 0.9% NaCl solution-treated group served as a control. a, b, c, d, e, f, g, h are relative tumor volume versus control on day 20. a, d, f, h are relative body weight on day 20 versus day 1. Paclitaxel–CM dextran conjugates or non-polymer-bound paclitaxel were given as repeated intravenous administrations on days 2, 9 and 16 (q7d×3) after subcutaneous tumor inoculation. Non-polymer-bound paclitaxel in Cremophor® EL-ethanol was administered at a dose of 50 mg/kg/d and paclitaxel–CM dextran conjugates in 0.9% NaCl solution were tested at 100 mg of equivalent paclitaxel per kilogram per day. The untreated group served as controls. Each column represents mean±S.D. for 5 mice, except the untreated group of 11 mice.

---

**Footnotes:**
- © Significant difference in comparison with the 0.9% NaCl solution-treated group (p<0.01).
(w/w) showed strongest antitumor activity and no body weight loss in this model.

These results indicate the superiority of paclitaxel–CM dextran conjugates over non-polymer-bound paclitaxel, demonstrating that the proper use of macromolecular carriers would increase the potency and range for therapeutic doses of paclitaxel.

Evaluation of Neurotoxicity: Treatments with paclitaxel in humans have been reported to display cumulative predominant sensory distal neuropathy, which was characterized clinically by a mixture of paresthesias and dysesthesias of the extremities. Pathologically, axonal swelling, vesicular degeneration and demyelination have been reported. We therefore evaluated the effects of paclitaxel–CM dextran conjugate and non-polymer-bound paclitaxel at their MTD using histopathological method in healthy BALB/c female mice.

Figure 6 shows the body weight change curves of the mice given repeated q2d×3 (on days 0, 2, and 4) intravenous administration of paclitaxel–CM dextran conjugate compared with the 0.9% NaCl solution-treated controls or the animals given non-polymer-bound paclitaxel. 50 mg/kg/d doses of paclitaxel–CM dextran conjugate and non-polymer-bound paclitaxel, which is their MTD, with q2d×3 caused obvious decreases in body weight (maximum body weight decreasing was −11.5% and −9.3% for paclitaxel–CM dextran treated and non-polymer-bound paclitaxel treated group, respectively). However, after the last dose, body weight of the both groups began to increase and recovered to the levels of the control group within 5 d.

In the case of repeated intravenous administration with q2d×3 of non-polymer-bound paclitaxel 50 mg/kg/d, neurological signs such as loss of stretch reflex in the hind limbs, abnormal walking, and paralysis of hind limbs were observed from day 5 through day 18, then gradually disappeared. In contrast, the mice that were treated with the same dose of paclitaxel–CM dextran conjugate (based on the amount of paclitaxel to CM dextran) showed no obvious neurological signs.

To confirm the above results, the influence of paclitaxel–CM dextran conjugate against nerves was evaluated using histopathological method. Sciatic nerves, which were removed on day 9, were fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissue sections were stained using toluidine blue and examined under light microscopy. (Fig. 7) Non-polymer-bound paclitaxel at 50 mg/kg/d induced degeneration of myelin and swelling of Schwann cells in sciatic nerves as shown in Fig. 7. In contrast, the same dose of AZ10992 did not induce neurological changes in sciatic nerves. These results indicate the superiority of paclitaxel–CM dextran conjugate (AZ10992) over non-polymer-bound paclitaxel and demonstrate the improvement in neurotoxicity attributable to macromolecular conjugation with a macromolecule.

DISCUSSION

The present study was designed to obtain a clear picture of the carrier, antitumor effects, and neurotoxicity of AZ10992. Our data would provide a rationale for the selection of CM dextran as a carrier for AZ10992 in terms of plasma half-life, tumor targeting, and anti-tumor effects. In this study, the main consideration in selecting an appro-
priede carrier for AZ10992 was the ability to enhance tumor accumulation of paclitaxel since paclitaxel is rapidly cleared from the body. To evaluate this ability, we synthesized a range of radiolabeled CM dextrans and examined their pharmacokinetics. Appropriate negative charge and MW would be required for effective tumor targeting. DS within the range of 0.2—0.6 and MW more than 80 kDa appeared to be suitable. For AZ10992, paclitaxel bound to the carrier through a GlyGlyPheGly linker may also affect the pharmacokinetic behavior of the carrier. However, such a label effect, if any, is expected to be minimal because the paclitaxel content in AZ10992 is sufficiently low (0.014—0.016 mol of paclitaxel/mol of glucose residue and 5.5—6.5% w/w). Therefore, the data obtained from the present study on radiolabeled CM dextrans would be applicable to the consideration of AZ10992.

Electric charges markedly alter the pharmacokinetics and in vivo distribution of macromolecules. When the two profiles of CM dextran (110 kDa) with DS 0.6 and 0.8 in tissues are compared, both of them showed high tumor accumulation, whereas the distribution of CM dextran with DS 0.6 in the liver was almost the half of that with DS 0.8 (Fig. 3). Appropriate anionization reduces the hepatic accumulation of dextrans attributable to nonspecific adsorptive endocytosis, resulting in prolonged circulation. However, strongly negatively charged macromolecules like dextran sulfate or succinylated albumin are rapidly taken up by nonparenchymal hepatocytes as well as by macrophages via scavenger receptor-mediated endocytosis. CM dextran (110 kDa, DS 0.6) exhibited the most extended half-life in the bloodstream (Fig. 2). Conversely, CM dextran (110 kDa, DS 0.8) was rapidly cleared from the circulation, which would be the result of being taken up efficiently by macrophages and accumulated significantly in the liver. Therefore, CM dextran (110 kDa, DS 0.6) seemed to be more suitable for drug delivery than that with DS 0.8. The in vivo antitumor study of the conjugate using CM dextrans (DS 0.6) synthesized from dextran 110 kDa against colon26 carcinoma confirmed a potent antitumor effect, supporting the rationale for the design of AZ10992.

Furthermore, MW also plays a crucial role in the pharmacokinetics of macromolecules. Dextrans (not carboxymethylated, DS 0) with a MW less than 40 kDa are predominantly excluded from the kidney, whereas higher MW dextrans are concentrated in the liver and spleen. In this study, CM dextran with a low MW of 50 kDa exhibited rapid clearance from the circulation, while CM dextrans with higher MW of 80—150 kDa showed an extended half-life in the bloodstream (Fig. 2). Conversely, polymeric carriers should be eliminated from the body within a reasonable time, although accumulation in the tumor is desirable. This means that one of the characteristics of an ideal carrier is susceptibility to biodegradation and/or excretion, implying that carriers with an excessively large MW are unsuitable. Collectively, these results provide a rationale for the selection of CM dextran (DS 0.6) from dextran 110 kDa as a carrier for AZ10992.

Having considered the carrier effects of electric charges and MW, focus was placed on the effects of drug contents. Paclitaxel—CM dextran conjugates from dextran 110 kDa with DS 0.6 and various contents of paclitaxel were synthesized. The amount of paclitaxel released from conjugates in mouse plasma were affected by electric charges (DS value of CM group per glucose residue), MW, and paclitaxel contents (Fig. 4). The amount of released paclitaxel was large for the conjugates comprising DS 0.39—0.55, MW 40—110 kDa, and paclitaxel content 6.3% (w/w). Antitumor activities and effects on body weight change were evaluated by the q7d × 3 dose to the colon26 carcinoma-bearing BALB/c female mice (Fig. 5). Conjugates comprising dextran 110 kDa with DS 0.6 and paclitaxel contents of 6.3% (w/w) showed strongest antitumor activity and no body weight loss in this model. Thus, it would be appropriate for paclitaxel—CM dextran conjugate (AZ10992).

Tissue distribution of the conjugates would be greatly affected by binding hydrophobic drugs such as paclitaxel. In particular, paclitaxel was reported to show high distribution in the liver after intravenous administration. Therefore, the paclitaxel bound conjugates might show higher accumulation in the liver and lower in the tumor than those of carriers as paclitaxel content increases. Liver distribution of each carrier—linker—drug conjugate as well as tumor distribution would be responsible for its potent antitumor activity. CM dextran carrier's advantage of being highly efficient at low accumulation in the liver would be decreased by binding paclitaxel; therefore, antitumor activities of the conjugates were decreased as paclitaxel content increased.

Furthermore, the neurotoxicity of AZ10992 was evaluated by repeated intravenous administration at its MTD with BALB/c female mice, and drug effects were evaluated by close observation of body weight and behavior as well as histopathological examination of sciatic nerves. Regarding the neurotoxicity profiles, AZ10992 treatment (q2d × 3 at 50 mg/kg/d) to mice showed the body weight decrease (Fig. 6) but produced less toxic effects on peripheral nerves than non-polymer-bound paclitaxel (Fig. 7). This reduced neurotoxicity of AZ10992 was demonstrated by the histopathological method and was probably attributable to the lower distribution of paclitaxel into normal neural tissue following AZ10992 administration.

Many dosage forms of paclitaxel have been developed to date, such as polyglutamate-conjugated paclitaxel (CT-2103), polymeric micelle-formulated paclitaxel (Genexol-PM), paclitaxel-incorporating micellar nanoparticle formulation (NK105), and albumin-stabilized nanoparticle formulation of paclitaxel (Abraxane). The advantage commonly shared with these dosage forms is that they are intravenously injectable without the mixture of Cremophore EL and ethanol, which has the potential of provoking serious allergic reactions. This paclitaxel—CM dextran system is thus expected to possess clinical advantages similar to that of the above paclitaxel dosage forms. So what is the difference between AZ10992 and the other paclitaxel dosage forms?

Abraxane and Genexol-PM were reported to have plasma and tumor AUCs nearly comparable or slightly lower than those of free paclitaxel. Furthermore, plasma and tumor AUC for CT-2103, NK105, and AZ10992 are higher than that of free paclitaxel in respective studies employing proper tumors and proper rodent models. However, whether enhanced accumulation of an anticancer drug into tumor is sufficient in leading the drug to exert antitumor activity in vivo remains debatable. Ideally, these macromolecular produgs should be stable and pharmacologically inactive during circulation in
the bloodstream but must release the active compound at an appropriate rate after reaching the tumor via the EPR effect. For sufficient distribution of drug to cancer cells distant from tumor vessels, the formulation should not be too stable in the tumor interstitium. Dosage forms displaying excess stability in tissue components such as liposomes were reported not to allow the free drug therein to be released.28) Such dosage forms therefore have been speculated to be not so effective against cancers such as scirrhous cancer of the stomach or pancreatic cancer, in which the tumor vessel network is irregular and loose because of an abundant collagen-rich matrix.29) We previously reported the necessity of controlled paclitaxel release to achieve antitumor effects in a colon26 carcinoma-bearing mouse model.29) In that report, we discussed that the appropriate drug release rates from conjugates are essential to achieve antitumor effects. AZ10992 would be predicted to have more controlled drug release via the peptide linker as compared with the other paclitaxel dosage forms.

Furthermore, we examined long-term toxicity (28 d) by repeated intravenous q4d×7 administration of AZ10992 (3 mg/kg/d based on amount of paclitaxel to CM dextran), non-polymer-bound paclitaxel, and carrier (carboxymethylated dextran of dose equivalent to AZ10992) in male SD rats. As for histopathological findings in the liver of AZ10992-treated groups at week 4 (day 28), we found: 1) extramedullary hematopoiesis that might be caused by paclitaxel (the same findings were found between AZ10992- and non-polymer-bound paclitaxel-treated groups); and 2) foamy cells that might be caused by the carrier (the same findings were found with the AZ10992- and carrier-treated groups). Both findings might result in a slight lesion. Foamy cells might be caused by high molecular weight carriers retained by or not eliminated from the liver. Regarding bone marrow toxicity, no difference was seen between paclitaxel and AZ10992 when 3 mg/kg of free-paclitaxel equivalent dose (q4d×7) was administered (data not shown).

AZ10992 has a MW of 150 kDa, which seems too large for renal excretion, and CM dextran carrier may be undegradable. However, just like other polymer compounds such as polysaccharides, CM dextran has a molecular weight distribution, so its molecular weight of 150 kDa only represents the average molecular weight. CM dextran also contains from a low-MW-fraction to a high-MW-fraction of CM dextran. The low-MW-fraction CM dextran would be eliminated smoothly, and part of the high-MW fraction would be retained in tissues such as liver.

The pharmacokinetics of paclitaxel was greatly improved by AZ10992 with vastly longer retention in the bloodstream and an appropriate release rate of paclitaxel into tumor tissue, resulting in preferential tumor-targeting. On the basis of these pharmacokinetic improvements, AZ10992 would exhibit enhanced antitumor effects with reduced neurotoxicity at its MTD, compared with non-polymer-bound paclitaxel. In conclusion, AZ10992 represents a promising agent for cancer treatment.

REFERENCES