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Synthesis and biological properties of antitumor-active conjugates of ADR with dextran

JAN-AN GUU [1], GING-HO HSIUE [2,*] and TZUOH-MIIN JUANG [1]

[1] *Department of Applied Chemistry, National Chiao Tung University, Hsinchu, 300, Taiwan*
[2] *Department of Chemical Engineering, National Tsing Hua University, Hsinchu, 300, Taiwan*

Abstract—Three kinds of polymeric adriamycin (ADR) conjugates of dextran were synthesized, namely a dextran–Gly-Leu-Gly-ADR (DGLGA) conjugate with a lysosomally degradable tripeptide spacer group, a dextran–Gly-Leu-Gly-ADR-galactosamine (DGLGA-Ga) conjugate with a targeting moiety of galactosamine on DGLGA, and a dextran–C_6H_{10}-ADR (DC$_6$A) conjugate with a hexamethylen spacer group. The content of the ADR moiety in the polymeric-drug conjugate was about 3 mol%. Enzyme hydrolysis of DGLGA and DC$_6$A was carried out by incubation with papain. The total amount of ADR released after 48 h was 43 mol% for DGLGA and less than 1 mol% for DC$_6$A. In an *in vitro* cytotoxicity experiment, the DGLGA-Ga conjugate has higher cytotoxic efficacy than the other conjugates for incubation with Hep-3B cells and consequently, the capability of targeting hepatoma cells of the galactosamine residue was determined. In contrast, for the incubation with SiHa cells of these conjugates, there was no significant cytotoxicity effect. The *in vivo* cytotoxic efficacy of each conjugate (20 mg ADR equiv./kg) against CT-26 mice colon cells implanted subcutaneously in Balb-C mice was studied. The DGLGA conjugate generated the best therapeutic effect with the presence of long-term survival (LTS) at day 50 (2/6).

Key words: Dextran; polymeric pro-drug; antitumor activity; adriamycin; galactosamine.

INTRODUCTION

The anthracycline antibiotic adriamycin (ADR) shows a valuable broad spectrum of antitumour activity in humans [1]. However, its clinical use is often limited by peripheral toxicity [2]. Just like the problems caused by the use of ADR in cancer chemotherapy, low-molecular-weight antitumor drugs generally have serious side-effects, due to a high concentration of toxicity in normal tissue and the short duration of activity. Hence, the development of a potential drug delivery system capable of

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*To whom correspondence should be addressed.

selectively concentrating anticancer agents at the targeting tissue and prolonging the treatment duration of anticancer agents is much needed.

One strategy to improve chemotherapy is to prepare new drug forms by using water-soluble synthetic or natural polymers as carriers of biologically active compounds. Biocompatible water-soluble synthetic and/or natural polymers have been extensively utilized as drug carriers to improve the therapeutic efficacy of anticancer drugs by prolonging the length of their residence in human bodies and increasing the selectivity to the desired sites. Several macromolecular compounds have been suggested as drug carriers, including serum albumin, fibrinogen, immunoglobulin, lectins, polysaccharides, DNA, liposomes, and synthetic polymers [3–5]. In 1974, De Duve *et al.* [6] developed the 'lysosomotropic agent' concept based on drug-carrier conjugates that are selectively taken up into lysosomes. The drugs then demonstrated their pharmacological activity by releasing bioactive agents from the polymeric carriers and penetrating through lysosomal membranes into cytoplasm. Accordingly, Ringsdorf presented a model of the use of a polymer as a targetable drug carrier [7]. The model combines the concept of site-specific drug release with site-specific recognition and predicts some important beneficial properties of polymer–drug conjugates, compared with their low-molecular-weight analogues. Based on this model, it is reasonable to argue that dextran conjugates containing ADR are a promising approach. The drug is covalently bound to the polymer via oligopeptide spacers that are designed specifically for stability in the circulation [8] and terminal cleavage by lysosomal enzymes following pinocytic internalization of the conjugate [9]. Dextran functions as a polymer carrier because it is a water-soluble, biodegradable, and non-antigenetic natural polysaccide [10]. The hydroxy groups of dextran can be replaced by pendant modifiable carboxylic derivatives for the grafting of drug derivatives. It can also be covalently attached to both the parent drug and the targeting unit. Furthermore, between the polymer carrier and the drug, a well-designed oligopeptide spacer could be degraded by enzymes, which could prompt the release of the drug. Kopecek and co-workers synthesized a macromolecular pro-drug, i.e. HPMA copolymers fixing *p*-nitroaniline (NAp) residues as model drugs through various peptidyl spacer groups, and investigated the enzymatic release behavior of NAp from HPMA copolymer by lysosomal proteases *in vitro*. They found that the release rates were significantly accelerated by increasing the length of the oligopeptidyl spacer and also depended on the specific sequence of the oligopeptide. Because the tripeptide Gly-Leu-Gly possesses the essential requisite that it can be degraded by lysosome enzymes, it was used in this study as the spacer far drug binding [11].

In addition, targeted drug deliver may improve the chemotherapy of tumors. Liver-specific targeting can be achieved intravenously by the use of vehicles designed to interact with liver-associated receptors, such as the hepatocyte galactose receptor. Galactosamine, a well-known compound that can be bound with a hepatocyte receptor [12] to achieve active targeting, is introduced into the dextran–ADR conjugate for liver targeting.

The main aim of the present study was the synthesis of dextran–ADR conjugates and to investigate their *in vitro* and *in vivo* properties. The preparation of a dextran–ADR conjugate with a tripeptide spacer (Gly-Leu-Gly) and a hexamethylene spacer is described. Furthermore, galactosamine, a terminal moiety that can be used to target polymer conjugates to liver hepatocytes, was incorporated into one dextran conjugate. The hydrolysis of these conjugates to release ADR in the presence of papain and pH 7.4 buffer solutions was carried out. The cytotoxicity effect of the conjugates against Hep-313 and SiHa human tumor cells and the therapeutic effect on CT-26 tumor cells implanted in mice were also investigated.

MATERIALS AND METHODS

Materials

Dextran was purchased from Pharmacia Fine Chemicals Co., Uppsala, Sweden, and had an average molecular weight of 40 000 (T-40). Adriamycin was from Pharmacia & Upjohn S.p.A. *N*-t-Boc-glycine-*N*-hydroxysuccinimide ester (Boc-Gly-Osu), papain, glutathione (GSH), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from the Sigma Chemical Co., USA. Trifluoroacetic anhydride (TFAA) was provided by Merck, Darmstadt, Germany, while all the other chemicals were of commercial grade and were used without further purification.

Synthesis of Boc-glycyl-L-leucine (Boc-Gly-Leu) (1)

Boc-Gly-OSu (5 g, 18.4 mmol) and leucine (2.74 g, 20.8 mmol) were added to a dimethyl sulfoxide (DMSO; 10 ml) solution and the reaction was conducted by stirring for 3 days at room temperature. The DMSO was then removed and ethyl acetate was added to dissolve the dry residue. The undissolved residue was filtered. The reaction mixture was extracted with 10% citric acid solution (3×20 ml). The organic layer was isolated and the water layer extracted with ethyl acetate (2×20 ml). The ethyl acetate extracts were combined and washed with water and then dried with $MgSO_4$. Ethyl acetate was removed using a rotary evaporator under reduced pressure. The crude product was purified further by recrystallization from ethyl acetate and n-hexane to produce a white powder product. The preparation procedure of this reaction is presented as equation (1) in Scheme 1. Yield 80.6%; m.p. 136–137°C. ^{1}H-NMR [$(CD_3)_2SO$]: δ 0.84 (dd, 6H, CH_3), δ 1.36 (s, 9H, CH_3), δ 1.48 (m, 2H, CH_2), δ 1.59 (m, 1H, CH), δ 3.53 (d, 2H, CH_2), δ 4.21 (m, 1H, CH), δ 6.89 (t, 1H, NH), δ 7.92 (d, 1H, NH).

Synthesis of Boc-glycyl-L-leucyl-4-nitrophenol ester (Boc-Gly-Leu-ONp) (2)

Boc-Gly-Leu (3 g, 10.4 mmol) and *p*-nitrophenol (1.74 g, 12.5 mmol) were dissolved in dimethyl formamide (DMF; 10 ml). The mixture was cooled in an ice

Boc-NH-CH$_2$-COOSu + NH$_2$-CH-COOH (CH$_2$-CH(CH$_3$)$_2$) $\longrightarrow$ Boc-NH-CH$_2$-CONH-CH-COOH (CH$_2$-CH(CH$_3$)$_2$)   (1)

1

1 $\xrightarrow{\text{HONp / DCC}}$ Boc-NH-CH$_2$-CONH-CH-COO—⟨ ⟩—NO$_2$ (CH$_2$-CH(CH$_3$)$_2$)   (2)

2

2 $\xrightarrow{\text{NH}_2\text{-CH-COOH}}$ Boc-NH-CH$_2$-CONH-CH-CONH-CH$_2$-COOH (CH$_2$-CH(CH$_3$)$_2$)   (3)

3

Boc-NH-CH$_2$-CONH-CH-CONH-CH$_2$-COOH (CH$_2$-CH(CH$_3$)$_2$) $\xrightarrow{\text{CF}_3\text{COOH}}$ NH$_2$-CH$_2$-CONH-CH-CONH-CH$_2$-COOH (CH$_2$-CH(CH$_3$)$_2$)   (4)

4

4 + Galactosamine $\xrightarrow[\text{30}^\circ\text{C, 4hr}]{\text{DEPC}}$ NH$_2$-CH$_2$-CONH-CH-CONH-CH$_2$-CO-**Gal** (CH$_2$-CH(CH$_3$)$_2$)   (5)

5

Scheme 1. Synthesis of the tripeptide spacer Gly-Leu-Gly.

bath, dicyclohexylcarbodiimide (DCC) (2.79 g, 13.5 mmol) was added with stirring, and the mixture was then stirred at room temperature for 24 h. DMF was removed using a rotary evaporator and ethyl acetate was added to dissolve the dry residue; the precipitate of the mixture was removed by filtration. The residue was extracted with a 10% citric acid solution (3 × 20 ml) and then with saturated sodium bicarbonate (3 × 20 ml). The organic layer was isolated and the water layer extracted with ethyl acetate (2 × 20 ml). The ethyl acetate extracts were combined and washed with water, dried with MgSO$_4$, and evaporated to dryness *in vacuo*. The crude product was purified further by recrystallization from ethyl acetate and n-hexane to produce a white powder product [equation (2) in Scheme 1]. Yield 76%; m.p. 123–125°C. ^{1}H-NMR [(CD$_3$)$_2$SO]: δ 0.96 (t, 6H, CH$_3$), δ 1.40 (s, 9H, CH$_3$), δ 1.67 (m, 2H, CH$_2$), δ 1.75 (m, 1H, CH), δ 3.82 (d, 2H, CH$_2$), δ 4.74 (d, 1H, NH), δ 5.31 (t, 1H, NH), δ 6.88 (m, 1H, NH), δ 7.25 (d, 2H, CH), δ 8.22 (d, 2H, CH).

Synthesis of Boc-glycyl-L-leucyl-glycine (Boc-Gly-Leu-Gly) (3)

Boc-Gly-Leu-ONp (2 g, 4.9 mmol) was dissolved in dry DMSO (5 ml) and then glycine (0.4 g, 5.4 mmol) was added. The mixture was stirred for 3 days at room temperature. DMSO was removed and ethyl acetate was added to dissolve the dry residue; the precipitate was removed by filtration. The reaction mixture was extracted with a 10% citric acid solution (3 × 20 ml). The organic layer was isolated and the water layer extracted with ethyl acetate (2 × 20 ml). The ethyl acetate extracts were combined and washed with water, dried with MgSO$_4$, and evaporated

to dryness *in vacuo*. The crude product was purified by recrystallization from ethyl acetate and n-hexane to produce a white solid product [equation (3) in Scheme 1]. Yield 36%; m.p. 146–147°C. ^{1}H-NMR [(CD$_3$)$_2$SO]: δ 0.83 (t, 6H, CH$_3$), δ 1.36 (s, 9H, CH$_3$), δ 1.43 (m, 2H, CH$_2$), δ 1.60 (m, 1H, CH), δ 3.52 (d, 2H, CH2), δ 3.71 (d, 2H, CH2), δ 4.32 (m, 1H, CH), δ 6.95 (t, 1H, NH), δ 7.84 (d, 1H, NH), δ 8.25 (t, 1H, NH).

Synthesis of dextran 4-nitrophenyl carbonate [*dextran*–*COO(C$_6$H$_4$)NO$_2$*]

The activation of dextran was carried out according to a similar method described by Vandoorne *et al.* [13]. Dextran (5 g) was dissolved in 20 ml of DMSO/pyridine 1/1 to (v/v) mixture. The solution was cooled to -10°C and then 4-dimethylaminopyridine (DMAP) (0.2 g, 1.6 mmol) and 4-nitrophenyl chloroformate (2.3 g, 11.4 mmol) were added. The reaction mixture was stirred at a low temperature (-10°C) for 30 min and then slowly dropped into ethanol (300 ml) with stirring to obtain a white precipitate. The precipitate was washed with a large excess of ethanol (3×100 ml) and evaporated to dryness *in vacuo* to produce a white dextran–COO(C$_6$H$_4$)NO$_2$ powder. The degree of actvation of dextran was determined by the hydrolysis of activated dextran in NaOH solution. Activated dextran (100 mg) was dissolved into 10 ml of 0.1 N sodium hydroxide solution and the absorption of the carbonate residue was monitored by UV–visible spectroscopy at 402 nm for the *p*-nitroaniline group. The content of carbonate was determined using Beer's law with $\varepsilon = 18\,400$ [13].

*Synthesis of the dextran–Gly-L-Leu-Gly conjugate (**6**)*

Boc-Gly-Leu-Gly (1 g, 2.9 mmol) (**3**), prepared previously as shown in Scheme 1, was placed into a 25 ml round-bottomed flask. Trifluoroacetic acid (2 ml) was added and the reaction was conducted under stirring at room temperature for 45 min. The mixture was evaporated *in vacuo* to produce the oily glycyl-L-leucyl-glycine (Gly-Leu-Gly). The oily residue of Gly-Leu-Gly (0.2 g, 0.8 mmol) was dissolved in a DMSO solution in which dextran–COO(C$_6$H$_4$)NO$_2$ (4.3 g) was dissolved and then triethylamine (0.2 ml) was added. The mixture was stirred at room temperature for 3 days and then slowly dropped into ethanol (300 ml) with stirring to obtain a white precipitate. The precipitate was washed with a large excess of ethanol (3×100 ml) and evaporated to dryness *in vacuo* to produce a white dextran–Gly-Leu-Gly conjugate powder (see Scheme 2).

Synthesis of the dextran–C$_5$H$_{10}$COOH conjugate

N-t-Boc-6-aminohexanoic acid (Boc-C$_5$H$_{10}$COOH) (1 g, 4.3 mmol) was placed into a 25 ml round-bottomed flask. Trifluoroacetic acid (2 ml) was added and the reaction was conducted by stirring at room temperature for 45 min. After removal of excess trifluoroacetic acid and by-products by evaporation, the oily

$$\text{(6)}$$
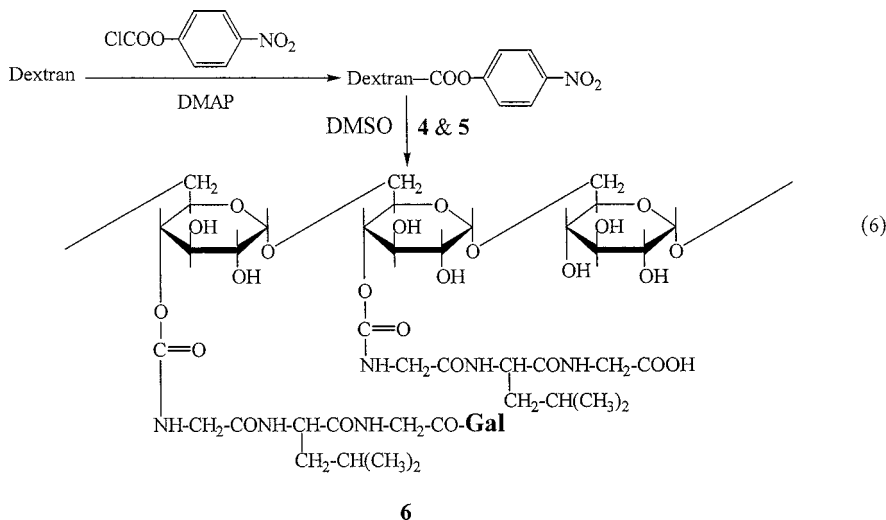
Scheme 2. Synthesis of the dextran–Gly-Leu-Gly-galactosamine derivative.

$NH_2C_5H_{10}COOH$ residue (0.2 g, 1.52 mmol) was dissolved in a DMSO solution in which dextran–$COO(C_6H_4)NO_2$ (4.3 g) was dissolved and then triethylamine (0.2 ml) was added. The mixture was stirred at room temperature for 3 days, then slowly dropped into ethanol (300 ml) and stirred to achieve a white precipitate. The precipitate was washed with a large excess of ethanol (3 × 100 ml) and evaporated to dryness *in vacuo* to produce a white dextran–$C_5H_{10}COOH$ conjugate.

Synthesis of the dextran–Gly-Leu-Gly-ADR (DGLGA) conjugate (7)

For the synthesis of the dextran–Gly-Leu-Gly-ADR conjugate, dextran–Gly-Leu-Gly (88 mg, 0.54 mmol), adriamycin (10 mg, 0.016 mmol), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (363 mg, 1.62 mmol) were mixed and stirred in 15 ml of purified water, and the pH of tie mixture was adjusted to 8 using HCl (1% w/w). The solution was under reaction by stirring for 48 h at room temperature. Purification of the mixture was carried out by dialysis with an 8000–10 000 molecular weight dialysis membrane. The purified solution was freeze-dried to obtain the red-colored DGLGA conjugate. The content of ADR in DGLGA was determined by UV–visible spectroscopy. The maximum absorption wavelength of ADR and DGLGA has been detected to be about 254 nm. A calibration curve was made by detecting different concentration of ADR solution at 254 nm. The absorption of DGLGA was measured at 254 nm and then the ADR content of DGLGA was found by comparison with the calibration curve of ADR. In this study, the ADR content of DGLGA was about 3 mol%. Moreover, the synthesis dextran–C_6H_{10}-ADR (DC$_6$A)(**7**) followed the same procedure of the synthesis of DGLGA.

Release of ADR from the dextran–ADR conjugate

The enzymatic hydrolysis of DGLGA was carried out by incubation with papain. DGLGA was dissolved in a buffer solution containing 0.10 M citric acid, 0.10 M disodium hydrogen phosphate, and 1 mM EDTA and the mixture was adjusted to pH 5.50 with NaOH. After DGLGA was dissolved, glutathione (23 mg) and papain (12 mg) were added. The mixture was incubated in a shaking bath at 37°C. A 1 ml sample of the mixture was removed every hour in the first 24 h and every 12 h thereafter. The samples were analyzed by HPLC with a Phenomenex LUNA 5u C18 (2) column. The eluant was prepared by mixing PBS (pH 7.4) and acetonitrile (80/20, v/v) and the analysis was run with a flow rate of 1 ml/min. The eluate was monitored at 254 nm with a TSP SPECTRA 100 VARIABLE UV/visible CE detector. The enzymatic hydrolysis experiment of DC_6A was conducted using the same method.

Cytotoxicity of DGLGA against Hep-3B cells

A semi-automated tetrazolium-based colorimetric (NTT) assay [14] was employed to examine the cytotoxicity effect of DGLGA and DC_6A. Exponentially growing cells were cultured in 96-well plates and treated with various concentrations (5, 2.5, 0.5, 0.05, 0.005 μM) of DGLGA, DC_6A, and ADR for 3 days at 37°C. After exposure of the cells to the dextran–ADR conjugates, the cells were washed twice with a serum-free medium and once with water. 0.5 ml of a medium solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well and the MTT assay was performed 4 h later. The entire assay was repeated twice and growth inhibition was calculated using the following equation:

$$\text{growth inhibition (\%)} = \frac{[T] - [B]}{[C] - [B]} \times 100\%.$$

In this equation, $[T]$ is the amount of cells in the treatment group after incubation for 72 h; $[C]$ is the number of cells in the control group after incubation for 72 h; and $[B]$ is the initial number of cells.

In vivo *cytotoxicity of DGLGA against CT-26 cells in mice*

The experiments of conjugates against Balb-C mice (males, 7–8 weeks old, 20–30 g) were inoculated subcutaneously at day 0 with 10^6 viable CT-26 cells. ADR or polymeric ADR conjugates were administered intraperitoneally as a single dose of 20 mg ADR-equiv./kg at day 4. Five groups of mice were tested, with six mice in each group. The weight and tumor size of the animals were measured daily. The survival time and number of long-term survivors (LTS) at day 50 were monitored.

## RESULTS AND DISCUSSION

Preparation of the dextran–ADR conjugate

Dextran is a water-soluble, biodegradable, and non-antigenetic natural polysaccaride; thus, it has many essential properties to be a polymer carrier in a conjugation pro-drug system. However, the hydroxy groups of dextran cannot connect with spacer and drug directly. Hence, pre-activation of dextran is required, to replace the hydroxy group with pendant modifiable carboxylic derivatives before being coupled with the spacer and drug. A number of methods can be used to transform the polysaccharide hydroxyl group into suitable derivatives that can react with amines or thiols [15]. In this study, the activation of dextran was conducted by mixing dextran with 4-nitrophenyl chloroformate to produce the corresponding carbonate moiety [13] (Scheme 2). The carbonate content in the activated dextran was determined by hydrolyzing the activated dextran that dissolves in NaOH aqueous solution. When the activated dextran was dissolved into 0.1 N $NaOH_{(aq)}$, a yellow color solution was obtained. Then the absorption of the solution was monitored by UV–visible spectroscopy and the amount of carbonate in dextran was calculated using Beer's law. For the purpose of realizing the reaction conditions, the time dependence of the degree of activation of dextran at 0°C was determined. As the time proceeded, the proportion of *p*-nitrophenyl carbonates increased accordingly. After reaching the maximal value at about 24 h, it slowly decreased. Moreover, dextran activation was carried out at 0°C for 30 min at the beginning and then the dextran was warmed back to room temperature. As a result, the content of *p*-nitrophenyl carbonate increased with time until 30 min and then started to decrease; afterwards, the amount remained at less than half of the maximal value at 12 h. These results reveal that the reaction time and temperature affects the activation degree of dextran. They also possibly suggest the presence of side-reactions, i.e. slow hydrolysis of the active species, or reaction of the active carbonate with polymeric hydroxyls with the formation of inter- or intra-molecular carbonate structures [13]. In order to avoid the excessive formation of those side-products, the activation reaction of dextran was carried out at −10°C for 30 min and the amount of carbonate in dextran, calculated with Beer's law, was about 11 mol%. We were also able to produce dextran of a higher activation degree by controlling the reaction time and temperature. However, the highly activated product was almost insoluble in water, so it could not be used for the subsequent reaction.

The tripeptide spacer Gly-Leu-Gly was synthesized based on solution-phase peptide synthesis methods. The NH group protected glycine (Boc-Gly) was used as the starting material, to which leucine and the other glycine were connected to it one-by-one via amide bonding. The protection group Boc was then disposed of by trifluoroacetic acid. After Gly-Leu-Gly was produced, it was coupled with the activated dextran though amide bonding. The NMRl spectra of the spacer component Gly-Leu-Gly and dextran–Gly-Leu-Gly are shown in Fig. 1. In Fig. 1B, besides the strong absorptions of the hydroxyl group between 4.2 and 5.2 ppm,
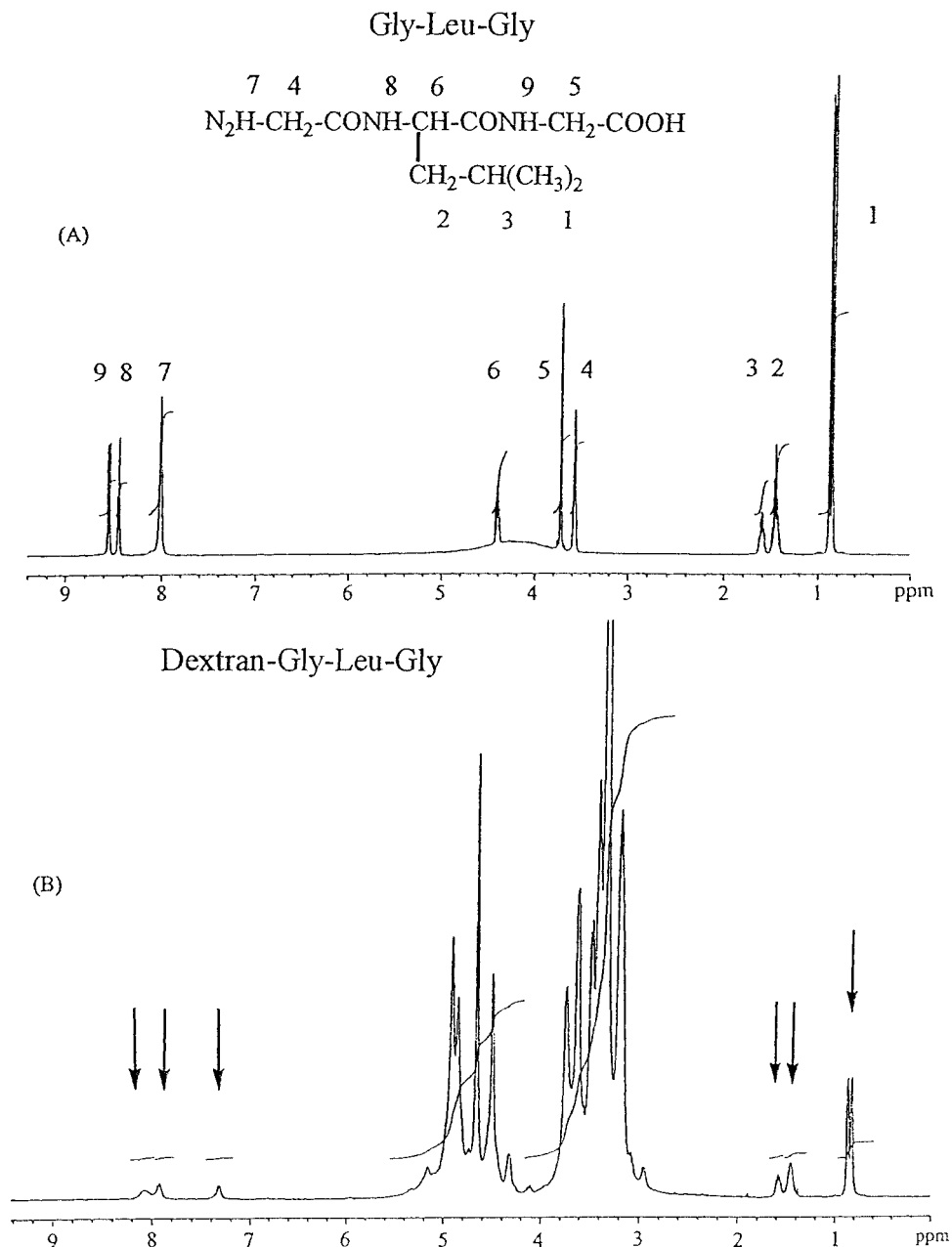
Figure 1. ^{1}H-NMR spectra $[(CD_3)_2SO]$ of (A) Gly-Leu-Gly and (B) dextran–Gly-Leu-Gly.

some smaller absorption peaks approximately match the absorption of Gly-Leu-Gly, as shown in Fig. 1A. These peaks are thought to be due to the tripeptide spacer residue. The absorption of amino groups of the tripeptide spacer between 7 and 9 ppm had shifted slightly from low field (Fig. 1A) to high field (Fig. 1B); this

$$\textbf{6} \ + \ \text{ADR} \ \xrightarrow[\text{pH8}]{\text{EDC/H}_2\text{O}}$$

(7)
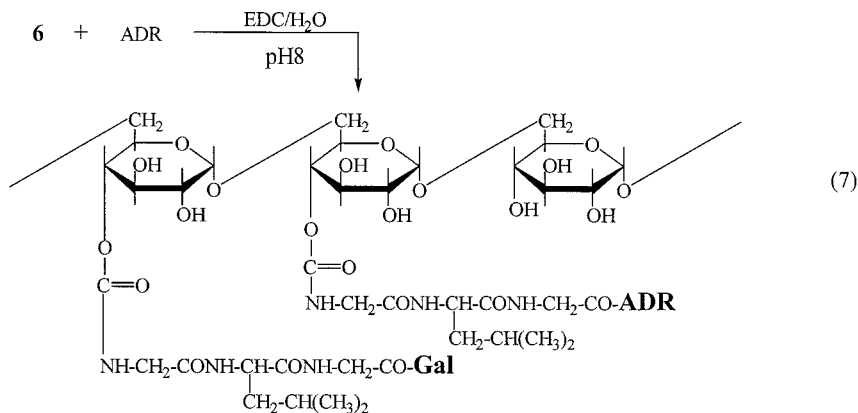
**Scheme 3.** Synthesis of the dextran–Gly-Leu-Gly-ADR-galactosamine conjugate.

should be influenced by the hydrogen bonding interaction between the amide group of Gly-Leu-Gly and the hydroxy groups of dextran. The content of Gly-Leu-Gly in the dextran derivative was approximately 6 mol%, which was determined by measuring the ratio of the peak area of Gly-Leu-Gly versus dextran in the NMR spectrum.

The attachment of ADR to the dextran derivative was accomplished using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as the coupling reagent under aqueous conditions [16] (Scheme 3). The amount of ADR, determined by detection and calculation via UV spectra, was about 3 mol%. In a previous report, this reaction was allowed to proceed for 48 h at room temperature and the pH value was adjusted to between 5 and 6 [16]. However, in the present study we found that the bonding ratio at pH 8 (3.1 mol%) was higher than that at pH 5.5 (1.2 mol%). Such a result was probably due to the process of reduction of ADR from the reagent that had to be performed under basic conditions. Thus, the reaction of attaching ADR to dextran was carried out at pH 8 in this study.

Release of ADR from the dextran–ADR conjugate

The hydrolysis of the dextran–ADR conjugate was carried out by incubating the conjugate with papain (or PBS) at 37°C. ADR residues were analyzed once an hour by HPLC using UV detection at 204 nm and the time dependende of the release of ADR was plotted. The results of the hydrolysis of DGLGA by papain and PBS is shown in Fig. 2. As time proceeded, ADR was released under treatment with papain; the total release was approximately 43 mol% at 48 h and then the release of ADR slowed down. When treated with PBS, the release of ADR was almost negligible. DC_6A was also incubated with papain and the results are also shown in Fig. 2, in comparison with DGLGA. DC_6A presented a rather low releasing effect in the degradation of papain.

In the development of targetable lysosomotropic drug delivery systems, the drug carrier linkage is of utmost importance. All macromolecular carriers ultimately
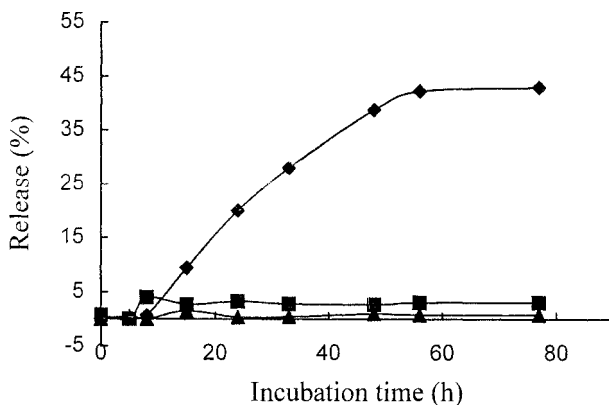
Figure 2. Time dependence of the release of ADR after incubation of the DGLGA conjugate with papain ($\blacklozenge$), in pH 7.4 buffer solution ($\blacksquare$), and the incubation of DC$_6$A with papain ($\blacktriangle$).

arrive in the lysosomal compartment of the cell following their pinocytic capture. Drug–polymer linkages are designed to be hydrolyzed by lysosomal enzymes and to be resistant to attack in other body compartments. Kopecek *et al.* reported in a detailed study that lysosomal enzyme degraded the oligopeptide side-chain in HPMA copoymer. They concluded that in the lysosome of liver cells, cathepsin B is the most important thiol protease that plays a major role in degrading natural and synthetic polypeptides [17]. Papain is a cysteine protease and has enzymatic specificity similar to cathepsin B [18]. Therefore, papain could be used as a model enzyme to carry out the enzymatic hydrolysis of dextran–ADR conjugates, as was used in this study.

An extensive study on the release of a model compound from poly[N-(2-hydroxy-propyl) methacrylamide] (HPMA) by changing the peptide spacers has also been reported [19]. The release rate of *p*-nitrophenol (a model compound) from the polymer was strongly dependent on the length and sequence of the peptide spacers between the main chain and *p*-nitrophenol. Compared with the case of a dipeptide spacer, conjugates with tripeptide sequences led to much higher release rates (approximately 3–8 times) and their rates were dependent on the specific sequence. For tetrapeptides, the release rates were much accelerated and also dependent on the sequence. In the present study, we introduced a tripeptide chain into the dextran–ADR conjugate as the lysosomal enzyme degradable spacer. The tripeptide spacer Gly-Leu-Gly has sufficient length and the sequence followed the rules of oligopeptide design [20]. Therefore, DGLGA released ADR by the specific hydrolysis of papain but did not release ADR in pH 7.4 phosphate buffer solution, which is analogous situation in the blood stream. DC$_6$A, with a hexamethylene spacer that could not be digested by enzyme, shows an insignificant release of ADR under incubation with papain.

Cytotoxicity of the dextran–ADR conjugate against tumor cells in vitro

The lysosomotropic agent concept [6] explains how the polymeric-drug conjugate enters cells through the endocytosis route and is digested by the enzymes in lysosome to release the drug. Therefore, dextran–drug conjugates, after pinocytosis into the cells, will eventually end up within the lysosomal compartment. The cytotoxicity effect of these conjugates was determined by MTT assay against Hep-3B and SiHa cells. Hep-3B (or SiHa) cells were cultured for 72 h under exposure to the polymeric conjugates. The results for Hep-3B are shown in Fig. 3. ADR shows the highest growth-inhibitory effect against Hep-3B cells among the five conjugates, for it enters cells by diffusion, or facilitated the transport pathway without driven energy. The cytotoxicity effect of the other conjugates follows the order DGLGA-Ga > DGLGA > DC_6 ≥ dextran. The intensity of the cytotoxicity of these conjugates could also be separated into two groups; the higher cytotoxicity group conjains DGLGA-Ga and DGLGA, and the lower cytotoxicity group has DC_6A and dextran. DGLGA-Ga and DGLGA have a tripeptide spacer between the polymer carrier and ADR. Such a tripeptide spacer with the sequence Gly-Leu-Gly was designed to match the active subsite of the lysosomal enzyme. Chiu *et al*. [11] investigated this tripeptide as the spacer in a poly(amino acid)–drug conjugate, in which the model drug was released at moderate rate. Thus, the enzyme-degradable spacer promoted the drug release effect of DGLGA-Ga and DGLGA. However, the spacer that connects dextran and ADR of DC_6A is a hexamethylene group, which could not be digested by the lysosomal enzyme at all. Therefore, it shows no significant growth inhibition effect towards tumor cells and the result is similar to that of dextran (Fig. 3). In addition, the macromolecular carrier itself may influence the activity of the conjugate. Even when it is not connected with any drugs, dextran shows a slight effect on the growth inhibition of tumor cells. Forster and Lloyd [21] also point out that the permeability limit of lysosomal membranes lies approximately in the size of dipeptides. The accumulation of dextran molecules in lysosomes gives rise to problems of osmotic balance of lysosomal membranes and, consequently, malfunction of the cells. As a result, the accumulation of dextran has little effect on the growth inhibition of tumor cells.

Hepatocytes have a receptor for galactose-terminated glycoproteins, which efficiently takes them up from the circulation [22, 23]. Thus, the galactosamine residues in an anticancer pro-drug are usually utilized as the targeting moiety, based on the ability of acutely targeting at the surface of the hepatoma cell [24]. In the present study, the higher cytotoxicity effect of DGLGA-Ga than DGLGA might be due to its faster rate of cellular uptake (Fig. 3).

SiHa cells are a squamous carcinoma cell line of the cervix [25], in which there is no lysosomal enzyme, as in hepatocytes. In the cytotoxicity experiments against SiHa cells, free ADR drug displayed the highest growth-inhibitory effect; DGLGA-Ga, DGLGA, DC_6A, and dextran have similar cytotoxicity effects without a significant effect of cell killing (Fig. 4). This shows that regardless of whether there is an oligopeptidyl spacer in the drug–polymer conjugate or not, the cytotoxicity
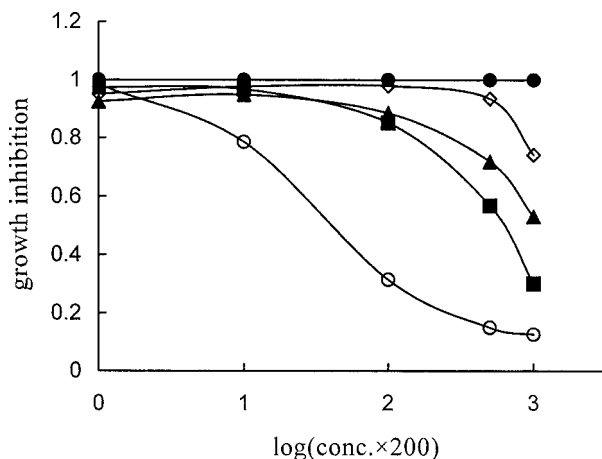
Figure 3. Cytotoxicity effect of DGLGA-Ga (■), DGLGA (▲), DC_6A (◇), free ADR drug (○), and the control group (●) against Hep-3B hepatoma cells *in vitro*. The cells were exposed for 72 h to the conjugate. Untreated cells were used as the control group.
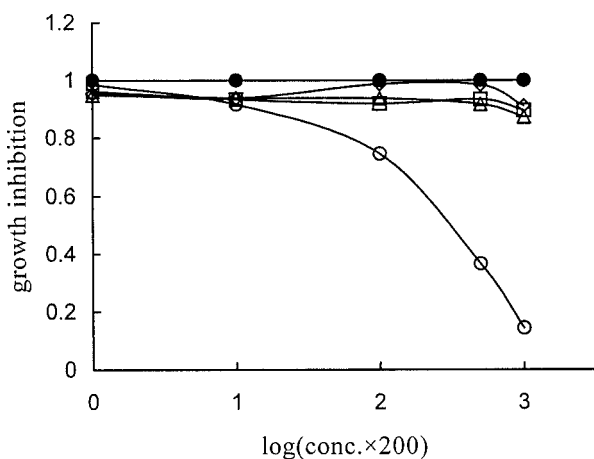

Figure 4. Cytotoxicity effect of DGLGA-Ga (□), DGLGA (△), DC_6A (◇), free ADR drug (○), and the control group (●) against SiHa cells *in vitro*. The cells were exposed for 72 h to the conjugate. Untreated cells were used as the control group.

effects are all the same. This result reflects the requirement of a suitable enzyme for the digestion of oligopeptide, which helps to release ADR from dextran–drug conjugates.

Cytotoxicity of the dextran–ADR conjugate in vivo

The *in vivo* cytotoxicity effect of DGLGA, DGLGA-Ga, DC_6A, and free ADR drug was examined in order to compare their ability to suppress the growth of tumor cells in Balb-C mice and to prolong the survival time of mice. The murine colon carcinoma cell line CT-26 was implanted in mice. This cell line contains many

enzymes with similar capability to the cells existing in liver and lung [26] and its distinct shape is easy to detect. A single dose (5 mg ADR-equiv./kg) of each polymeric ADR conjugate, as well as free ADR, was administered intraperitoneally on day 4 after tumor cells were implanted in the mice. The survival time of the mice was monitored and is shown in Fig. 5. Tumor growth was not suppressed by any of the conjugates and no conjugate was able to extend the survival time of the mice. This is possibly due to an insufficient drug dosage.
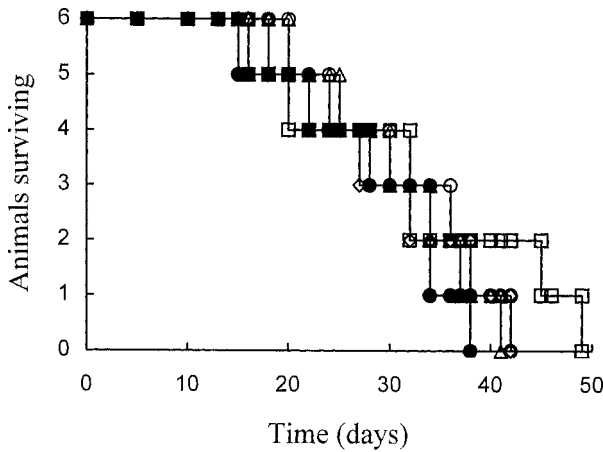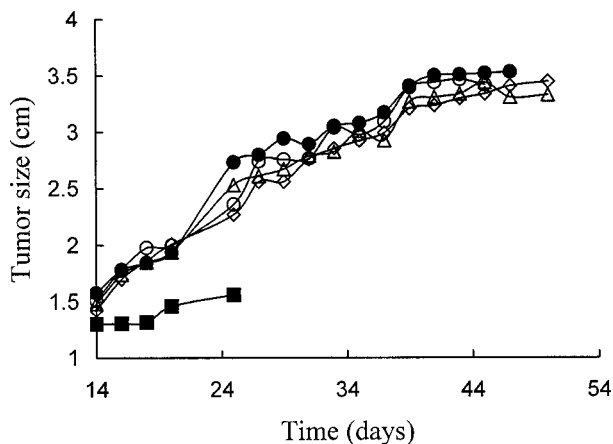

Figure 5. Effect of the treatment of Balb-C mice carrying i.p. CT-26 with a single dose (5 mg ADR eqiv./kg) of DGLGA-Ga ($\diamond$), DGLGA ($\triangle$), DC$_6$A ($\bigcirc$), free ADR drug ($\square$), and the control group ($\bigcirc$) on the survival time. The mice carrying CT-26 in the control group were not treated with any conjugate.
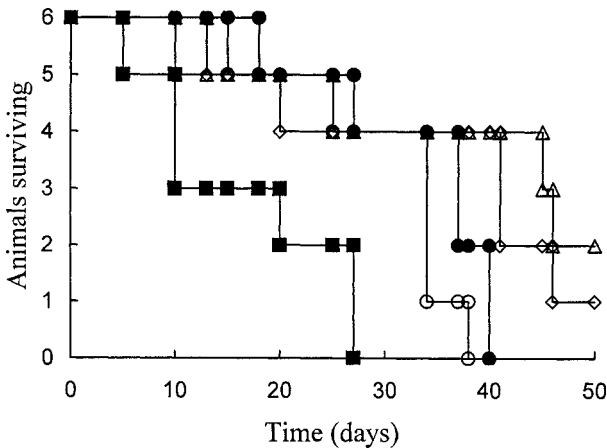
Figure 6. Effect of the treatment of Balb-C mice carrying i.p. CT-26 with a single dose (20 mg ADR eqiv./kg) of DGLGA-Ga ($\diamond$), DGLGA ($\triangle$), DC$_6$A ($\bigcirc$), free ADR drug ($\blacksquare$), and the control group ($\bullet$) on the survival time. The mice carrying CT-26 in the control group were not treated with any conjugate.

Therefore, the dosage was increased from 5 mg to 20 mg in each polymeric ADR conjugate, and a single dose was administered intraperitoneally to mice after the tumor cells were implanted. The survival time and the tumor size were monitored as shown in Figs 6 and 7, respectively. The mean survival time (MST) and long-term survivors (LTS) are given in Table 1. The survival time of the mice treated by free ADR is shorter than that of the untreated control group. This indicates that ADR exhibits high toxicity to mice in such a dosage. Animals treated with DC_6A had increased in tumor size and had a similar survival time to the untreated control group. This is because DC_6A does not possess high toxicity. The poor therapeutic effect of DC_6A revealed that ADR is not released from the polymer–drug conjugate. Moreover, the toxicity of the ADR component is reduced by the shielding effect of the polymer chain attached to dextran. The tumor growth of animals treated with DGLGA was suppressed to only a small degree (Fig. 7), yet their survival time was much longer than that of the control group and the free ADR drug-treated group. In fact, two mice reached a LTS longer than 50 days. This implies that DGLGA

Figure 7. Effect of the treatment of Balb-C mice carrying i.p. JZT-26 with a single dose (20 mg ADR eqiv./kg) DGLGA-Ga ($\diamond$), DGLG ($\triangle$), DC_6A ($\bigcirc$), free ADR drug ($\blacksquare$), and the control group ($\bullet$) on the tumor size. The mice carrying CT-26 in the control group were not treated with any conjugate.

Table 1.

Effect of i.p. administration of PGA–ADR conjugates on Balb-C mice ($n = 6$) bearing 10^6 CT-26 cells inoculated i.p. on day 0 (dose 20 mg ADR/kg on day 4)

Compound	Survival time (days)	MST (SE) (days)	T/C (%)	LTS (day 50)
Control	18, 27, 37, 37, 40, 40	33.2 (3.6)	—	0/50
ADR	5, 10, 10, 20, 27, 27	16.5 (3.9)	49.7	0/50
DGLGA	13, 25, 45, 46	32.3 (8.0)	97.3	2/50
DGLGA-Ga	10, 20, 41, 41, 46	31.6 (7.0)	95.2	1/50
DC_6A	15, 25, 34, 34, 34, 38	30 (3.5)	90.4	0/50

exhibits a certain degree of activity on therapeutics. This is probably due to the fact that ADR released from the Gly-Leu-Gly spacer of the polymer–drug conjugate is able to eliminate some of the tumor cells. The survival result of mice treated with DGLGA-Ga is similar to that of the DGLGA-treated group. The targeting function of the galactosyl group did not have an effect in the DGLGA-Ga therapeutic case. This is because the galactosyl–dextran conjugates have high affinity only to liver cells, without any affinity to other tissues [30].

CONCLUSION

A dextran–ADR conjugate, DGLGA, having a Gly-Leu-Gly tripeptide spacer was synthesized. In addition, DGLGA-Ga, which is a conjugate that contains a tripeptide spacer and a liver-targeting galactosamine moiety, was prepared. Gly-Leu-Gly was synthesized using the solution-phase peptide synthesis method. The biologically active agent ADR was fixed onto dextran under aqueous conditions in which the pH was adjusted to 8. The hydrolysis results show that DGLGA releases iree ADR specifically in response to papain; in other words, the dextran–ADR conjugate containing the Gly-Leu-Gly spacer is not expected to release ADR in the blood stream (pH = 7.4), but to release effectively after uptake into tumor cells through hydrolysis of the oligopeptide spacer by lysosomal enzyme. Since DGLGA-Ga has the highest cytotoxicity effect compared with the other conjugates *in vitro*, it is responsible for the active targeting of cell-specific affinity of the conjugate for hepatoma cells via galactose receptor-mediated endocytosis. Although the targeting effect of the polymer–drug conjugate was not observed using CT-26 as the cell line in the *in vivo* experiment, the polymer–drug conjugates DGLGA and DGLGA-Ga do exhibit a therapeutic effect on mice. In summary, it is reasonable to propose that dextran–ADR conjugates containing oligopeptide and a targeting moiety can be used for cancer chemotherapy.

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