Efficacy of a Poly-L-Glutamic Acid-Gemcitabine Conjugate in Tumor-Bearing Mice

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ABSTRACT This study assessed the in vivo antitumor efficacy of a polypeptide-based poly-L-glutamic acid-gemcitabine conjugate (PG-G). PG-G was synthesized by conjugating gemcitabine to poly-L-glutamic acid by a carbodiimide reaction. PG-G was evaluated for its in vivo antitumor efficacy and toxicity using 4T1 murine breast tumor-bearing mice. The antitumor effects of PG-G were superior to those of unconjugated gemcitabine in both single and four-consecutive dosing studies. Tumor regression was observed within 1 day after PG-G administration and continued for 4–5 days. Thereafter, tumors grew at a slower rate compared with the unconjugated gemcitabine treatment group and other control groups. The main toxicity observed from the Berlin test was an apparent reversible weight loss of 10–12%. The unconjugated gemcitabine treatment group also demonstrated a similar, but reduced, weight loss trend. The present study demonstrates that the PG-G formulation exhibits a significant antitumor activity in the aspects of tumor growth inhibition and shrinkage that is more robust than the parent drug and other control groups. Thus, the PG-G dose regimen may be optimized to minimize side effects and render it a potential anticancer therapeutic. Drug Dev Res 73: 120–129, 2012. © 2012 Wiley Periodicals, Inc.

Key words: cancer; drug delivery systems; polymeric drugs; prodrug; gemcitabine; poly-L-glutamic acid

INTRODUCTION

Gemcitabine (2′-deoxy-2′,2′-difluorocytidine; GEMZAR™, Eli Lilly and Company, Indianapolis, IN, USA) is a nucleoside analog that is approved for the treatment of several solid tumors and is a component of standard therapy for several advanced cancers. However, gemcitabine has several limitations. These include unfavorable hydrolytic stability, short half-life, and high water solubility. Hence, various prodrugs of gemcitabine have been developed to improve its pharmacokinetics, reduce toxic effects, and improve efficacy. Polymeric prodrugs are a new class of drug delivery systems that allow the conjugation of a drug to a polymer. These polymers can provide a more sustained release of the drug, protect the drug from enzymatic degradation, and improve the pharmacokinetics of the drug, resulting in an increase in its antitumor efficacy. Poly-L-glutamic acid (PGA) is a naturally occurring nonribonucleic acid (RNA) polymer that is synthesized by Bacillus subtilis. PGA is biocompatible and has been used as a polymer to deliver drugs, DNA, and siRNA. This study assessed the in vivo antitumor efficacy of a polypeptide-based poly-L-glutamic acid-gemcitabine conjugate (PG-G). PG-G was synthesized by conjugating gemcitabine to poly-L-glutamic acid by a carbodiimide reaction. PG-G was evaluated for its in vivo antitumor efficacy and toxicity using 4T1 murine breast tumor-bearing mice. The antitumor effects of PG-G were superior to those of unconjugated gemcitabine in both single and four-consecutive dosing studies. Tumor regression was observed within 1 day after PG-G administration and continued for 4–5 days. Thereafter, tumors grew at a slower rate compared with the unconjugated gemcitabine treatment group and other control groups. The main toxicity observed from the Berlin test was an apparent reversible weight loss of 10–12%. The unconjugated gemcitabine treatment group also demonstrated a similar, but reduced, weight loss trend. The present study demonstrates that the PG-G formulation exhibits a significant antitumor activity in the aspects of tumor growth inhibition and shrinkage that is more robust than the parent drug and other control groups. Thus, the PG-G dose regimen may be optimized to minimize side effects and render it a potential anticancer therapeutic. Drug Dev Res 73: 120–129, 2012.

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POLY-L-GLUTAMIC ACID-GEMCITABINE DRUG CONJUGATE

USA) is a water-soluble nucleoside analog of deoxycytidine with broad-spectrum cytotoxicity against solid human tumors [Hertel et al., 1990; Braakhuis et al., 1995; Csoka et al., 1995]. Gemcitabine is approved for the treatment of ovarian, breast, nonsmall cell lung and pancreatic cancers, and is either used as a single agent or in combination with other anticancer therapeutics. It exerts its cytotoxic effect via active diphosphate and triphosphate metabolites, of which the former acts as a competitive inhibitor of ribonucleotide reductase [Heinemann et al., 1990] and the latter incorporates into DNA and disrupts normal DNA synthesis and excision repair via masked chain termination mechanisms that eventually lead to apoptosis [Huang et al., 1991; Huang and Plunkett, 1995]. The anticancer efficacy of gemcitabine is often limited by its relatively short plasma half-life (8–17 min), which is due to the extensive deamination of gemcitabine to inactive 2′,2′-difluorodeoxyuridine by the enzyme cytidine deaminase, which is found mainly in the blood, liver, and kidneys [Immordino et al., 2004]. Thus, gemcitabine is often administered intravenously at high doses to achieve an adequate therapeutic plasma drug concentration [Immordino et al., 2004]. However, high-dose administration may result in nonspecific toxicity in patients, including myelosuppression, pulmonary toxicity, renal impairment, and hepatic toxicity [Fossella et al., 1997]. Therefore, improving the in vivo stability and delivery of gemcitabine is of critical importance. The conjugation of chemotherapeutic drugs to biocompatible polymers may reduce pharmacokinetic elimination and improve targeting and accumulation of drugs in solid tumors through an enhanced permeability and retention (EPR) effect [Duncan and Spreafico, 1994]. This effect may improve drug availability to tumors, reduce nonspecific drug distribution, and consequently reduce systemic toxicity and improve the therapeutic index of the drug [Duncan et al., 2005].

Conjugation of gemcitabine to a α,β-poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA) polymer together with folate via succinyl and diglycolyl linkers was attempted by Cavallaro et al. [2006]. The preliminary evaluation of the PHEA-gemcitabine-folate conjugates revealed improved aqueous and plasma stability and exhibited in vitro cytotoxicity on a panel of tumor cell lines.

Recently, we reported the synthesis of poly-L-glutamic acid-gemcitabine (PG-G) in an attempt to reduce synthetic complexity without the use of linkers. In its polymer–drug conjugate form, PG-G was found to be well protected against degradation by cytidine deaminase in vitro showing improved plasma stability, and it was active against in vitro human breast cancer cell lines and murine 4T1 metastatic mammary tumor cells [Kiew et al., 2010]. Therefore, the objectives of this study are to characterize the effects of the improved plasma stability of gemcitabine in its PG-G form on tumor regression, hematologic profile, and toxicity in 4T1 breast tumor-bearing mice.

MATERIALS AND METHODS

Materials

Poly-L-glutamic acid sodium salt (PGNa; molecular weight by multangle laser light scattering = 97 800, number average molecular weight by multangle laser light scattering = 41 400) and phosphate buffered saline were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gemcitabine was supplied by the 21st Century Global E-commerce Network (Westham, Pevensey, East Sussex, UK). Diethyl ether and absolute ethanol were purchased from BDH (Poole, Dorset, UK). Snakeskin™ dialysis tubing (molecular size cut off 10 000) was supplied by Pierce (Rockford, IL, USA). Murine 4T1 cells (ATCC number, CRL-2539) were obtained from ATCC (Manassas, VA, USA). BALB/c mice were supplied by the Center for Animal Studies, Faculty of Medicine, University of Malaya (Kuala Lumpur, Malaysia).

Preparation and Characterization of PG-G

PG-G was synthesized according to a previously described protocol [Kiew et al., 2010] except that the preparation was conducted under aseptic conditions. Briefly, all glassware was sterilized using dry heat, distilled water was autoclaved, and reagents were filter sterilized prior to synthesis. The synthesized PG-G conjugate was characterized by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE), ultraviolet (UV), and nuclear magnetic resonance (NMR) spectroscopy. The purity and gemcitabine content of PG-G were determined using high performance liquid chromatography (HPLC) as described previously [Kiew et al., 2010].

Experimental Animals

Female BALB/c mice were housed at 20–25°C with a 12-hour light–dark cycle. A standard pellet diet and water were provided ad libitum. Experimental work was conducted at the Centre for Animal Studies, University of Malaya Medical Center, Kuala Lumpur, Malaysia, in accordance with institutional guidelines (animal ethics approval reference number: PM/19/08/2004/CLY (R)).

In Vivo Antitumor Efficacy Study

Murine 4T1 cells (1 × 10⁶ cells) in Hank’s buffered saline were orthotopically injected into the mammary fat pouch. The experimental animals were then randomized into the following groups: control (saline), gemcitabine alone at fixed dose, and PG-G at fixed dose. PG-G was synthesized according to a previously described protocol [Kiew et al., 2010] except that the preparation was conducted under aseptic conditions. Briefly, all glassware was sterilized using dry heat, distilled water was autoclaved, and reagents were filter sterilized prior to synthesis. The synthesized PG-G conjugate was characterized by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE), ultraviolet (UV), and nuclear magnetic resonance (NMR) spectroscopy. The purity and gemcitabine content of PG-G were determined using high performance liquid chromatography (HPLC) as described previously [Kiew et al., 2010].

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pad of the mice (18–20 g, 9 weeks old) [duPre’ et al., 2008]. When the tumors reached a palpable size of 7 mm in diameter (170 mm³) after tumor inoculation, mice were administered by tail vein injection either PG-G (20–40 mg gemcitabine equivalent/kg body weight), PGNa (amount equivalent to that of 40 mg gemcitabine equivalent/kg), or gemcitabine (20–80 mg/kg) in normal saline or normal saline alone (0.2 mL) in a single dose or four consecutive doses at 3- or 7-day intervals. Animals were examined and weighed daily, and tumor volume was determined by caliper measurements [tumor volume, mm³ = (length (mm) × width (mm) × height (mm))/2] [Li et al., 1998]. Results were expressed as the percentage of tumor volume reduction/increase [% tumor volume reduction/increase = ((tumor volume (mm³) – initial tumor volume (mm³))/initial tumor volume (mm³) × 100%]. During the study, the mean tumor diameter did not exceed 13 mm, which is less than or equal to a 540% change in tumor volume.

Hematologic Profile

At the conclusion of the in vivo antitumor efficacy study, the mice were sedated with diethyl ether, and blood was collected from the jugular vein [Hoff, 2000]. The blood was then analyzed for erythrocyte (red blood cell) count, hemoglobin concentration, hematocrit percentage, mean corpuscular volume, platelet count and volume, and white blood cell (WBC) count using a GEN-S Beckman Coulter Hematology Analyzer (Villepinte, Roissy, France) [Coudert et al., 2000].

Statistical Analyses

Results from the different study groups were expressed as the mean ± standard error of the mean (SEM) (n = 10). Statistical analyses were determined by one-way analysis of variance (ANOVA) or two-way ANOVA studies followed by Bonferroni’s post hoc test or Student’s t-test (Prism 5, Graphpad Software, San Diego, CA, USA), where P < 0.05 was selected as the cutoff for statistical significance.

RESULTS

Preparation and Characterization of PG-G

The polypeptide-based drug conjugate PG-G was synthesized and then characterized (Fig. 1). The molecular weight distribution (number-average molecular weight by multiangle laser light scattering, 41 400; SDS-PAGE, 60–70 kDa), UV (λmax, 268 nm), and NMR (1H NMR (D2O), δ (ppm): 6.13 (d, 1H, H-5), 8.07 (d, 1H, H-6), 6.08 (t, 1H, H-1′), 3.54 (m, 1H, H-4′), 3.80 (m, 2H, H-5′), 2.11 (m, 2H, H-γ), 1.78, 1.88 (dt, 2H, H-β), 4.15, 4.17 (dd, 1H, H-α); 13C NMR (D2O), δ (ppm): 161.02 (C4–NH2), 65.84 (C5′), 174.35 (C3′), 34.42 (C-γ), 28.83 (C-β), 54.30 (C-a), 182.31 (C-e)) characteristics of the synthesized PG-G conjugate were consistent with our earlier report [Kiew et al., 2010]. The gemcitabine content in PG-G was 10–11 molar percent as estimated by HPLC, and HPLC analysis also demonstrated that there was no unconjugated gemcitabine contaminant in the synthesized conjugate (Fig. 2).

In Vivo Antitumor Efficacy

Single injections of PG-G (20–40 mg gemcitabine equivalent/kg) at day 0 in the 12-day study induced a dose-dependent tumor volume reduction within 24 hours (Fig. 3A). A gradual tumor volume reduction of 40.2 ± 13.6% and 61.0 ± 7.3% (mean ± SEM; n = 10) was observed at day 5 in both 20 and 40 mg gemcitabine equivalent/kg treatment groups, respectively. This tumor volume reduction was significantly higher than that of the unconjugated gemcitabine treatment and control groups (PGNa and normal saline) (ANOVA; P < 0.05). The tumors then regrew from day 6; however, the rate of tumor regrowth in the PG-G
treated group was less than that observed in the unconjugated gemcitabine treatment and control groups (ANOVA; \( P < 0.05 \)). In contrast to PG-G treatment, tumor growth in the unconjugated gemcitabine treatment and control groups was progressive (Fig. 3A). In addition, a single dose of PG-G (40 mg gemcitabine equivalent/kg) was also found to be significantly more effective in suppressing tumor growth when compared with a four-consecutive-dose treatment regimen of gemcitabine (40 mg/kg) at 3-day intervals over 12 days (ANOVA; \( P < 0.05 \)) (Fig. 3C).

In the four-consecutive-dose study, PG-G (20–40 mg gemcitabine equivalent/kg at 7 days intervals) resulted in a similar pattern of tumor volume reduction and regrowth when compared with the single injection study, with the primary difference being that the growth pattern was cyclical and related to dosing (Fig. 3B). Administration of PG-G also induced a rapid tumor volume reduction within 24 hours that continued for 4–5 days, which yielded a maximum tumor volume reduction of 43.2 ± 4.8% and 60.2 ± 4.8% (mean ± SEM; \( n = 10 \)) for 20 mg and 40 mg gemcitabine equivalent/kg. This tumor volume reduction was significantly greater compared with that of unconjugated gemcitabine and control groups at equivalent time points (ANOVA; \( P < 0.05 \)). The tumor then regrew until the next dose was administered. The rate of tumor regrowth of both PG-G treatment groups was significantly less than the other treatment groups (ANOVA; \( P < 0.05 \)). After four consecutive doses, unconjugated gemcitabine (20–80 mg/kg) again failed to exhibit complete tumor growth inhibition compared with PG-G treatment. However, tumor growth was attenuated by unconjugated gemcitabine when compared with control groups after administration of the third and fourth doses. The four-consecutive-dose treatment of PG-G at 7-day intervals did not eradicate the tumor mass in mice, and the tumor volume remained more or less unchanged (Fig. 3B).

**In Vivo Toxicity**

PG-G was administered to mice systemically via the tail vein. The most apparent side effect was a body
Fig. 3. (A) Percent change in tumor volume of 4T1 tumor-bearing BALB/c mice relative to the initial tumor volume over 12 days after receiving a single intravenous dose of poly-L-glutamic acid-gemcitabine (PG-G) (20 \( \text{mg/g}\) or 40 \( \text{mg/g}\) gemcitabine equivalent/kg), gemcitabine (20 \( \text{mg/kg} \), 40 \( \text{mg/kg} \), or 80 \( \text{mg/kg} \) gemcitabine equivalent/kg \( \bullet \)), PG-G acid sodium salt (PGNa) (the amount of PGNa was equivalent to that present in PG-G of 40 mg gemcitabine equivalent/kg \( \bullet \)) and normal saline (0.2 mL \( \text{ml} \)) at day 0 (↑). (B) Percent change in tumor volume of 4T1 tumor-bearing BALB/c mice relative to the initial tumor volume over 28 days after receiving four intravenous doses of PG-G (20 \( \text{mg/g} \) or 40 \( \text{mg/g} \) gemcitabine equivalent/kg), gemcitabine (20 \( \text{mg/kg} \), 40 \( \text{mg/kg} \), or 80 \( \text{mg/kg} \) gemcitabine equivalent/kg \( \bullet \)), or normal saline (0.2 mL \( \text{ml} \)) at days 0, 7, 14, and 21 (↑). (C) Comparison of percent change in tumor volume of 4T1 tumor-bearing BALB/c mice relative to the initial tumor volume over 12 days after receiving a single intravenous dose of PG-G (40 mg gemcitabine equivalent/kg \( \Delta \)) at day 0 (↑) or four intravenous doses of gemcitabine (40 mg/kg \( \Delta \)) at days 0, 3, 6, or 9 (↓). Each data point is expressed as the mean ± standard error of the mean \( \text{SE} \) \( n=10 \).
weight loss that was based on the Berlin test of typical symptoms, such as apathy, horrent fur, behavior changes, and body weight loss [Koudelka et al., 2010]. In the single-injection study, dose-dependent body weight loss was observed within 1 day after the administration of PG-G (20 mg and 40 mg gemcitabine equivalent/kg) and continued for 4–5 days (9.9 ± 0.9 and 10.7 ± 3.7% weight loss [mean ± SEM; n = 10] at nadir for the respective groups). Thereafter, the body weight progressively increased (Fig. 4A). Other signs of toxicity, such as horrent fur and behavioral changes, were not observed. A similar weight reduction was also found in the gemcitabine treatment groups receiving 40 mg/kg and 80 mg/kg gemcitabine (5.2 ± 1.1% and 9.2 ± 2.3% weight loss at nadir for the respective groups).

In the four-consecutive-dose study, PG-G (20–40 mg gemcitabine equivalent/kg at 7-day intervals) resulted in a similar pattern of body weight loss upon drug conjugate administration (9.6 ± 1.4 and 11.6 ± 1.9% weight loss at nadir for the respective groups at week 1), followed by body weight regain (Fig. 4B). The pattern of body weight loss and regain was cyclical and related to the drug conjugate administration pattern. Similar to the single-injection study, the PG-G (20–40 mg gemcitabine equivalent/kg at 7-day intervals) treatment group did not demonstrate horrent fur or behavioral changes. Weight reduction was also found in the gemcitabine treatment groups receiving 40 mg/kg and 80 mg/kg gemcitabine (4.7 ± 1.6 and 6.1 ± 1.0% weight loss at nadir for the respective groups).

In the single-injection study, both PG-G and unconjugated gemcitabine induced dose-dependent elevations in both WBCs and platelets as measured at day 12. In contrast, erythrocyte count, hemoglobin concentration, percent hematocrit, and platelet volume were not affected in the PG-G or unconjugated gemcitabine treatment groups compared with controls (Table 1).

Similarly, in the four-consecutive-dose study, the PG-G (20 mg–40 mg gemcitabine equivalent/kg at 7-day intervals) treatment groups also demonstrated elevated WBC and platelet counts compared with control groups on day 28. In contrast, the unconjugated gemcitabine treatment and control groups showed similar values of erythrocyte count, hemoglobin concentration, percent hematocrit, platelet count and volume, and WBC count (Table 2).

In the PGNa and normal saline control groups, the hematological parameters observed, except total WBC, were consistent with earlier findings in normal BALB/c mice [Nemzek et al., 2001]. The 4T1 tumor-bearing mice used in this study showed a 10-fold higher WBC count. Further examination showed that within the total WBC count, the number of monocytes (about 0.5 × 10³/μl) and lymphocytes (about 9.6 × 10³/μl) were similar, and the neutrophil count (63.1 × 10³/μl) was
10-fold higher compared with the numbers published in Nemzek et al. [2001] (monocytes, [0.7 ± 0.06] × 10^3/µl; lymphocytes, [10.5 ± 0.5] × 10^3/µl; neutrophils, [5.7 ± 0.4] × 10^3/µl). The elevated neutrophil count (total WBC) is likely to be a consequence of the leukemoid reaction caused by the production of colony-stimulating factor in 4T1 breast tumor BALB/c mice [duPre’ and Hunter, 2007].

**Fig. 4.** (A) Percentage weight change of 4T1 tumor-bearing BALB/c mice relative to the initial body weight over 12 days after receiving a single intravenous dose of poly-L-glutamic acid-gemcitabine (PG-G) (20 [□] or 40 [△] mg gemcitabine equivalent/kg), gemcitabine (20 [■], 40 [▲], or 80 [▲] mg/kg), poly-L-glutamic acid sodium salt (PGNa) (the amount of PGNa was equivalent to that present in PG-G of 40 mg gemcitabine equivalent/kg [•]) or normal saline (0.2 mL [○]) at day 0 (↑). (B) Percentage weight change of 4T1 tumor-bearing BALB/c mice relative to the initial body weight over 28 days after receiving four intravenous doses of PG-G (20 [□] or 40 [△] mg gemcitabine equivalent/kg), gemcitabine (20 [■], 40 [▲], or 80 [▲] mg/kg), PGNa (the amount of PGNa was equivalent to that present in PG-G of 40 mg gemcitabine equivalent/kg [•]) or normal saline (0.2 mL [○]) at days 0, 7, 14, and 21 (↑). Each data point is expressed as the mean ± standard error of the mean (n = 10).
### TABLE 1. Effects of Single-Dose PG-G, Gemcitabine, and PGNa Administered on Day 0 on Hematologic Profiles of 4T1 Tumor-Bearing BALB/c Mice Determined at Day 12

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>RBC (×10⁶/µL)</th>
<th>Hb (g/dL)</th>
<th>Hematocrit (%)</th>
<th>MCV (fl)</th>
<th>Platelets (×10³/µL)</th>
<th>MPV (fl)</th>
<th>Total WBCs (×10³/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG-G (20 mg gemcitabine eq/kg)</td>
<td>9.9 ± 0.3</td>
<td>14.6 ± 0.8</td>
<td>47.3 ± 2.7</td>
<td>52.5 ± 0.4</td>
<td>1604.0 ± 72.7*</td>
<td>5.8 ± 0.1</td>
<td>162.0 ± 9.3</td>
</tr>
<tr>
<td>PG-G (40 mg gemcitabine eq/kg)</td>
<td>9.8 ± 0.2</td>
<td>14.9 ± 0.5</td>
<td>46.4 ± 1.9</td>
<td>51.8 ± 1.0</td>
<td>1669.4 ± 119.0*</td>
<td>6.0 ± 0.1</td>
<td>197.8 ± 17.3*</td>
</tr>
<tr>
<td>Gemcitabine (20 mg/kg)</td>
<td>10.3 ± 0.2</td>
<td>14.8 ± 0.8</td>
<td>46.9 ± 1.3</td>
<td>50.1 ± 0.6</td>
<td>1041.4 ± 58.8</td>
<td>5.8 ± 0.1</td>
<td>159.4 ± 4.0</td>
</tr>
<tr>
<td>Gemcitabine (40 mg/kg)</td>
<td>10.4 ± 0.4</td>
<td>14.9 ± 1.1</td>
<td>47.2 ± 2.4</td>
<td>49.6 ± 0.6</td>
<td>1163.4 ± 122.4</td>
<td>5.7 ± 0.1</td>
<td>253.3 ± 4.5*</td>
</tr>
<tr>
<td>Gemcitabine (80 mg/kg)</td>
<td>9.7 ± 0.2</td>
<td>14.1 ± 0.5</td>
<td>46.8 ± 0.9</td>
<td>49.2 ± 0.7</td>
<td>1230.2 ± 137.1</td>
<td>5.6 ± 0.1</td>
<td>254.9 ± 2.6*</td>
</tr>
<tr>
<td>PGNa</td>
<td>10.2 ± 0.3</td>
<td>14.6 ± 0.4</td>
<td>47.4 ± 2.0</td>
<td>50.6 ± 0.6</td>
<td>1067.2 ± 78.7</td>
<td>5.6 ± 0.1</td>
<td>156.7 ± 22.5</td>
</tr>
<tr>
<td>Normal saline</td>
<td>9.9 ± 0.4</td>
<td>14.3 ± 1.0</td>
<td>44.6 ± 3.4</td>
<td>51.1 ± 1.0</td>
<td>1025.6 ± 80.1</td>
<td>5.9 ± 0.1</td>
<td>157.4 ± 14.0</td>
</tr>
</tbody>
</table>

The mice were administered a single intravenous dose of PG-G (20–40 mg gemcitabine equivalent/kg), gemcitabine (20–80 mg/kg), PGNa (the amount of PGNa was equivalent to that present in PG-G of 80 mg gemcitabine equivalent/kg), or normal saline (0.2 mL) at day 0. Blood was withdrawn from the mice on day 12 for the measurements of hematologic profiles and sacrificed for anatomical examination. The results are expressed as mean ± SEM (n = 10). eq.: equivalent. Student’s t-test: *P < 0.05 compared with the normal saline control.

PG-G, poly-L-glutamic acid-gemcitabine; PGNa, poly-L-glutamic acid sodium salt; RBC, red blood cell; WBC, white blood cell; Hb, hemoglobin; MCV, mean corpuscular volume; MPV, mean platelet volume; SEM, standard error of the mean.

### TABLE 2. Effects of Four Consecutive Doses of PG-G, Gemcitabine, and PGNa Administered on Days 0, 7, 14, and 21 on Hematologic Profiles of 4T1 Tumor-Bearing BALB/c Mice at Day 28

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>RBC (×10⁶/µL)</th>
<th>Hb (g/dL)</th>
<th>Hematocrit (%)</th>
<th>MCV (fl)</th>
<th>Platelets (×10³/µL)</th>
<th>MPV (fl)</th>
<th>Total WBCs (×10³/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG-G (20 mg gemcitabine eq/kg)</td>
<td>9.5 ± 0.4</td>
<td>14.0 ± 0.4</td>
<td>45.8 ± 0.9</td>
<td>52.8 ± 3.2</td>
<td>1278.8 ± 97.8*</td>
<td>6.0 ± 0.2</td>
<td>239.9 ± 24.9*</td>
</tr>
<tr>
<td>PG-G (40 mg gemcitabine eq/kg)</td>
<td>9.9 ± 0.3</td>
<td>15.6 ± 0.6</td>
<td>47.4 ± 2.0</td>
<td>51.2 ± 1.2</td>
<td>1331.4 ± 160.4*</td>
<td>6.0 ± 0.2</td>
<td>247.5 ± 37.3*</td>
</tr>
<tr>
<td>Gemcitabine (20 mg/kg)</td>
<td>9.2 ± 0.5</td>
<td>14.1 ± 0.7</td>
<td>47.0 ± 2.1</td>
<td>52.8 ± 3.1</td>
<td>955.8 ± 78.0</td>
<td>6.0 ± 0.3</td>
<td>175.1 ± 13.4</td>
</tr>
<tr>
<td>Gemcitabine (40 mg/kg)</td>
<td>9.5 ± 0.4</td>
<td>15.4 ± 0.4</td>
<td>47.6 ± 1.8</td>
<td>51.1 ± 1.9</td>
<td>962.0 ± 97.2</td>
<td>6.4 ± 0.2</td>
<td>176.6 ± 17.6</td>
</tr>
<tr>
<td>Gemcitabine (80 mg/kg)</td>
<td>9.5 ± 0.5</td>
<td>15.4 ± 1.1</td>
<td>47.9 ± 2.5</td>
<td>50.3 ± 0.5</td>
<td>944.6 ± 63.3</td>
<td>5.8 ± 0.3</td>
<td>176.9 ± 23.6</td>
</tr>
<tr>
<td>PGNa</td>
<td>8.4 ± 0.6</td>
<td>12.5 ± 1.0</td>
<td>44.0 ± 2.9</td>
<td>52.7 ± 0.9</td>
<td>951.2 ± 55.7</td>
<td>5.9 ± 0.2</td>
<td>172.3 ± 18.5</td>
</tr>
<tr>
<td>Normal saline</td>
<td>8.2 ± 0.6</td>
<td>12.3 ± 0.6</td>
<td>42.7 ± 3.0</td>
<td>51.5 ± 0.9</td>
<td>944.2 ± 48.8</td>
<td>5.9 ± 0.1</td>
<td>171.0 ± 28.3</td>
</tr>
</tbody>
</table>

The mice were administered four intravenous doses of PG-G (20–40 mg gemcitabine equivalent/kg), gemcitabine (20–80 mg/kg), PGNa (the amount of PGNa was equivalent to that present in PG-G of 80 mg gemcitabine equivalent/kg), or normal saline (0.2 mL) on days 0, 7, 14, and 21. Blood was withdrawn from the mice on day 28 for the analysis of hematologic profiles. The results are expressed as mean ± SEM (n = 10). eq.: equivalent. Student’s t-test: *P < 0.05 compared with the normal saline control.

PG-G, poly-L-glutamic acid-gemcitabine; PGNa, poly-L-glutamic acid sodium salt; RBC, red blood cell; WBC, white blood cell; Hb, hemoglobin; MCV, mean corpuscular volume; MPV, mean platelet volume; SEM, standard error of the mean.
DISCUSSION

To improve the antitumor efficacy of gemcitabine, we utilized a polypeptide polymeric backbone to conjugate gemcitabine and form a PG-G drug conjugate. This study demonstrated that PG-G suppresses tumor progression in a 4T1 mouse breast tumor model that resembles stage 4 human metastatic breast cancer [Pulaski and Ostrand-Rosenberg, 1998]. The most striking effect of PG-G was the rapid tumor regression within 1 day of treatment, which continued for 5 days and resulted in up to 60% tumor regression. The rapid tumor regression observed suggests that the EPR effect of the breast tumor vasculature promotes accumulation of the macromolecular PG-G conjugates in the tumor interstitium [Duncan and Spreatfi, 1994; Duncan et al., 2005] and that gemcitabine has been successfully released from the polymeric backbone to exert its cytotoxic effect. In addition, the improved plasma stability of gemcitabine in the PG-G form, as reported in our previous study [Kiew et al., 2010], reduces the degradation of gemcitabine and promotes the higher accumulation of PG-G conjugates in the tumor vasculature. Moreover, active uptake of the PG-G macromolecule by 4T1 breast tumor cells most likely occurs via endocytosis, which may further contribute to the observed enhanced antitumor effects [Kiew et al., 2010]. Although tumor regrowth was observed after 5 days following the cessation of treatment, the inhibition of tumor regrowth was significantly higher in the PG-G group than in the unconjugated gemcitabine or control groups for at least 12 days after drug administration in the single-injection study. PG-G administered as four consecutive doses at 3- or 7-day intervals also clearly demonstrated that PG-G had a superior antitumor activity compared with unconjugated gemcitabine and control treatment groups.

To determine the potential in vivo toxicity of PG-G, we assessed the PG-G, unconjugated gemcitabine, and control treatment groups using Berlin tests [Koudelka et al., 2010] and hematologic parameters. The main toxicity of PG-G, as determined by the Berlin test, was an apparent reversible weight loss. The unconjugated gemcitabine treatment group also demonstrated a similar but lower trend of weight loss. Interestingly, the pattern of weight loss in PG-G treatment groups mirrored the change in tumor volume, suggesting that the change in body weight could be partially accounted for by the regression of tumor mass.

In conclusion, in our previous study, we reported the synthesis, hydrolytic and plasma stability, extended release profile, and in vitro cytotoxicity of the polypeptide drug conjugate PG-G [Kiew et al., 2010]. We now present findings on its superior antitumor efficacy in 4T1 murine breast tumor-bearing BALB/c mice. Although reversible weight loss was observed in the PG-G treatment groups, it may be possible to minimize this side effect by adjusting the dose and treatment duration, and hence, PG-G may be developed as a favorable gemcitabine prodrug for clinical applications. This study will serve as a basis for the assessment of the PG-G conjugate over longer periods in future in vivo studies.

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