Tumor-Penetrating Microparticles for Intraperitoneal Therapy of Ovarian Cancer

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ABSTRACT

Intraperitoneal chemotherapy prolongs survival of ovarian cancer patients, but its utility is limited by treatment-related complications and inadequate drug penetration in larger tumors. Previous intraperitoneal therapy used the paclitaxel/Cremophor EL (polyethoxylated castor oil) formulation designed for intravenous use. The present report describes the development of paclitaxel-loaded microparticles designed for intraperitoneal treatment (referred to as tumor-penetrating microparticles or TPM). Evaluation of TPM was performed using intraperitoneal metastatic, human ovarian SKOV3 xenograft tumor models in mice. TPM were retained in the peritoneal cavity and adhered to tumor surface. TPM consisted of two biocompatible and biodegradable polymeric components with different drug release rates; one component released the drug load rapidly to induce tumor priming, whereas the second component provided sustained drug release. Tumor priming, by expanding interstitial space, promoted transport and penetration of particulates in tumors. These combined features resulted in the following advantages over paclitaxel/Cremophor EL: greater tumor targeting (16-times higher and more sustained concentration in omental tumors), lower toxicity to intestinal crypts and less body weight loss, greater therapeutic efficacy (longer survival and higher cure rate), and greater convenience (less frequent dosing). TPM may overcome the toxicities and compliance-related problems that have limited the utility of intraperitoneal therapy.

A majority of ovarian cancer patients present with stage III or IV disease, accompanied by local metastasis. The standard of treatment is surgical debulking, followed by intravenous chemotherapy with platinum and taxane analogs. Concomitant intraperitoneal chemotherapy has been under development for several decades. Multiple studies have demonstrated significant targeting advantage for intraperitoneal chemotherapy in patients, with peritoneal cavity-to-systemic blood ratios of drug exposure (measured as area-under-concentration time curves, AUC) ranging from 12 for cisplatin to 1000 for paclitaxel (Zimov et al., 1987; Markman et al., 1992; Kerr and Los, 1993). Adding intraperitoneal chemotherapy to intravenous plus intraperitoneal therapy to become a standard of care (Markman and Walker, 2006; Ozols et al., 2006).

Toxicities of intraperitoneal therapy are generally related to procedures for administration and the drugs administered (Markman et al., 1992; Elias and Sideris, 2003; Wenzel et al., 2007). The use of intraperitoneal catheter is associated with higher risk of infection and fever and occasionally physical damages to peritoneal tissues (e.g., perforation). Whereas hematologic toxicity is a major toxicity for drugs rapidly absorbed into the systemic circulation (e.g., cisplatin, carboplatin, melphalan, etoposide), local toxicity is dose-limiting for drugs that are slowly absorbed (e.g., paclitaxel, mitoxantrone, doxorubicin) or drugs that induce chemical peritonitis (e.g., mitomycin, 5-fluorouracil, oxaliplatin) or ileus (e.g., docetaxel) (Alberts et al., 1988; Demicheli et al., 1985; Markman et al., 2001; Armstrong et al., 2006a; Goldberg, 2006; the most recent National Cancer Institute Cooperative Group trial (GOG 172) in stage III patients with <1-cm tumors showed a 16-month longer overall survival. However, toxicities and other issues have prevented concomitant intravenous plus intraperitoneal therapy to become a standard of care (Markman and Walker, 2006; Ozols et al., 2006).

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ABBREVIATIONS: AUC, area under concentration; BrdU, bromodeoxyuridine; C_{max}, maximal concentration; C_{max,tissue}, highest tissue concentration; HPLC, high-performance liquid chromatography; ILS, increase in life span; PBS, phosphate-buffered saline; PLG, poly(DL-lactide-co-glycolide); TPM, tumor-penetrating microparticles; PDI, polydispersity index; MST, median survival time; ANOVA, analysis of variance; PVA, poly(vinyl alcohol); CI, confidence interval.
Elias and Sideris, 2003; Howell et al., 1984; Markman et al., 1992; Monk et al., 1988; Morgan et al., 2003; O’Dwyer et al., 1991; Zimm et al., 1987). The GOG 172 trial showed that three times more patients on the intraperitoneal plus intravenous arm did not complete the assigned six-treatment cycle compared with the intravenous arm (38 vs 17%). For the former, 20% terminated early due to catheter-related complications (infection, blocked or leaky catheter, port access problems), 22% terminated due to other toxicities (gastrointestinal toxicities, including abdominal pain or stomach cramp, dehydration, renal/metabolic, catheter-unrelated infection), and 9% terminated due to patient refusal (Armstrong et al., 2006a).

For intraperitoneal chemotherapy, residual tumor size is the most significant prognostic indicator, with a better prognosis and longer survival interval in patients with smaller tumors (≤0.5 cm) compared with larger tumors (≥2 cm) (Alberts et al., 1996; Topuz et al., 1998; Markman et al., 1998). These findings led to the recommendation of using intraperitoneal therapy in optimally, surgically debulked stage III patients with tumors of less than 1 cm (Armstrong et al., 2006a). The tumor size restriction is probably due to the limited drug penetration into larger tumors. This notion is supported by the observations that while cisplatin and carboplatin were about equally effective in ovarian cancer patients present with only positive margins (<0.5 cm), the analog that shows inferior penetration and seven times lower drug levels in rodent tumors (i.e., carboplatin) also shows inferior activity in patients with larger tumors (1–3 cm) (Los et al., 1990, 1991; Markman et al., 1993).

During intraperitoneal therapy, drug delivery to peritoneal tumors is from two sources. Recirculation of drug absorbed from the peritoneal cavity via the systemic circulation is a minor source due to the relatively low concentration in blood. The primary source is drug diffusion or convection through tumor interstitium. Our laboratory has developed the tumor priming technology that uses an apoptosis-inducing drug (paclitaxel or doxorubicin) to expand the interstitial space and thereby promote the interstitial transport of particulates (Zheng et al., 2001; Kuh et al., 1999; Jang et al., 2001b; Chen et al., 1998; Jang et al., 2001a). The finding that tumor priming is effective in vitro, in the absence of blood flow or vascularization, indicates that expansion of interstitial space is a major mechanism for enhanced transport. Tumor priming can also reduce interstitial fluid pressure and thereby decompress tumor microvessels and enhance extravasation and convection-mediated transport (Griffon-Etienne et al., 1999). Tumor priming is tumor-selective, due to the greater susceptibility of tumor cells to apoptosis compared with normal cells (Lu et al., 2007).

The present study extended our earlier findings to the development of two-component, biocompatible, biodegradable, polymeric [poly(ε-caprolactone-co-glycolide), PLG], and paclitaxel-loaded microparticles (referred to as tumor-penetrating microparticles or TPM) for intraperitoneal treatment. TPM was designed to address several major limitations of intraperitoneal therapy and, as shown in the results, has the following properties.

a) The size of particles was optimized to reduce clearance and to enable wide distribution in the cavity (i.e., minimal sedimentation).

b) The microparticles adhered to tumor surface.

c) TPM consisted of two types of paclitaxel-loaded microparticles with different drug release rates; i.e., a rapid release component to enable tumor priming (referred to as priming TPM) and promote interstitial transport of remaining particles, and a slow release component to provide sustained drug levels (referred to as sustaining TPM) and to reduce the need of frequent dosing or indwelling catheter. The fractionated drug release approach lowers the drug exposure toxicity to host tissues compared with the bolus all-at-once dose presentation, as was the case for the intravenous paclitaxel formulation used in previous intraperitoneal therapy (paclitaxel solubilized in Cremophor EL (polyethylene castor oil)/ethanol, referred to as paclitaxel/Cremophor).

Materials and Methods

Chemicals and Reagents. Paclitaxel (Hande Tech, Houston, TX), cephalomannine (National Cancer Institute, Bethesda, MD), and 3H[3H]paclitaxel (specific activity, 10.6 Ci/mmol; National Cancer Institute or Moravek Biochemicals, Brea, CA) were found to be >99% pure by high performance liquid chromatographic (HPLC) analysis. Cefotaxime sodium was purchased from Hoechst-Roussel Inc. (Somerville, NJ); gentamicin was from Solo Pak Laboratories (Franklin Park, IL); and all other cell culture supplies were from Invitrogen (Grand Island, NY). PLG was purchased from Birmingham Polymers (Birmingham, AL); OCT embedding matrix was from Bayer Corp. (Emeryville, CA); Cremophor EL and poly(vinyl alcohol, PVA) was from Sigma-Aldrich (St. Louis, MO); and HPLC solvents were from Thermo Fisher Scientific (Walhamb, MA). All chemicals and reagents were used as received.

Preparation of Paclitaxel- or Fluorophore-Loaded PLG Microparticles. TPM were loaded with paclitaxel and consisted of two components, i.e., priming and sustaining TPM, prepared with different compositions of PLG copolymers. Priming TPM was used to produce rapid drug release and tumor priming and comprised 50:50 L/G (inherent viscosity of 0.17 dL/g in hexafluoroisopropanol). Sustaining TPM was used to provide sustained release of paclitaxel and comprised 75:25 L/G (inherent viscosity of 0.67 dL/g in hexafluoroisopropanol). TPM were prepared using solvent evaporation method as described previously (Tsai et al., 2007). In brief, PLG and paclitaxel were codissolved in 5 ml of methylene chloride and emulsified in 20 ml of 1% PVA aqueous solution by homogenization for 30 s. The emulsion was mixed with 500 ml of 0.1% PVA and stirred at 1000 rpm at room temperature, and ambient pressure was used to evaporate the methylene chloride. The residual microparticles pellet was collected by centrifugation, washed three times with deionized water to remove residual PVA, lyophilized, and stored at 4°C. The size of microparticles was determined using scanning electron microscopy. At least 300 particles were measured. Number-based mean diameter (Dn) and volume-based mean diameter (Dv) were obtained. Polydispersity index (PDI) is a measurement of distribution and was calculated as \( \frac{D_{100}}{D_{50}} \times \frac{D_{50}}{D_{10}} \), where \( D_{100}, D_{50}, \) and \( D_{10} \) were the respective volume diameters at 90, 50, and 10% cumulative volumes. For a representative batch of priming TPM, \( D_n = 3.6 \mu m, D_v = 5.7 \mu m, \) and PDI = 0.78, and for a representative batch of sustaining TPM, \( D_n = 3.8 \mu m, D_v = 5.2 \mu m, \) and PDI = 0.63. Priming TPM released paclitaxel rapidly (70% drug load in 24 h), whereas sustaining TPM released 1% daily. The paclitaxel loading was 4.1 ± 0.5% (mean ± S.D. of three batches of priming TPM and three batches of sustaining TPM).

Fluorophore-labeled PLG microparticles (4 and 30 μm, without paclitaxel) were prepared with 50:50 L/G PLG copolymers. The smaller microparticles were prepared as described above, with the exception that paclitaxel was replaced by rhodamine or acridine orange, which showed red and yellow fluorescence under UV light, respectively. The larger microparticles were prepared by emulsifying the polymer solution in 20 ml of 1% PVA for 1 min using magnetic stirring instead of homogenization (\( D_n = 28.1 \mu m, D_v = 34.5 \mu m, \) and PDI = 0.65).
Metastatic Intraperitoneal Ovarian Tumor Models. Human ovarian SKOV3 xenograft tumors were maintained in female athymic BALB/c Nu/Nu mice (Charles River/NCl Laboratories; Wilmington, MD). Animals were cared for in accordance with institutional guidelines. SKOV3 tumor cells (ATCC, Manassas, VA) were maintained in McCoy’s media containing 9% fetal bovine serum, 2 mM l-glutamine, 90 μg/ml gentamicin, and 90 μg/ml cefotaxime sodium at 37°C in a humidified atmosphere of 5% CO2 in air. A metastatic subline was established by serial reimplantation of cells collected from peritoneal washings of mice given intraperitoneal injections of the parent cells. Injection of the metastatic cells (2 × 106) into the peritoneal cavity yielded established tumors in 100% animals (n > 50). In late stage (e.g., 6 weeks after injection of tumor cells), some mice showed tumors invading the pericystea of visceral organs, such as liver and kidney. Protein concentration in peritoneal fluid increased from 3% in normal mice to ~6% in tumor-bearing mice in 2 weeks. The volume of peritoneal fluid increased 7 to 10-folds after 4 weeks and contained aggregates of tumor cells (mostly 5–10 cells). Tumor dissemination and disease progression of the metastatic intraperitoneal SKOV3 model showed similarity to the following observations in late-stage ovarian cancer patients: a) tumors appearing in bowel serosa, perihepatic and perisplenic ligaments, diaphragm, mesentery, and omentum; b) high protein concentrations in peritoneal fluid (~4% in late stage disease) due to leakage of serum proteins and/or presence of ascites in the peritoneal cavity; and c) presence of tumor cell aggregates of similar size in ascites fluid (Tauchi et al., 1996).

Dose Preparation and Administration. Paclitaxel/Cremophor EL was prepared by dissolving paclitaxel in Cremophor EL:ethanol (1:1) and diluted with normal saline immediately before injection. TPM were suspended in either phosphate-buffered saline (PBS, pH = 7.4) or physiological saline with 0.01% Tween 80 and injected intraperitoneally into animals using 25-gauge needles. Anesthesia was attained using inhalation isoflurane (diluted to 15% in light glass slides and stored at 4°C. Samples were injected into the cleanup column and eluted with a two-component TPM group. All treatments consisted of a mixture of priming TPM, sustaining TPM, or two-component TPM (1:1 priming/sustaining). The total paclitaxel dose was 20 mg/kg for Cremophor EL and single component TPM groups and 40 mg/kg for the two-component TPM group. All treatments consisted of a mixture of 3H-labeled and nonradiolabeled drug (1.6 μCi/20 mg paclitaxel).

Tumors were flash-frozen and cut into 20-μm sections. To minimize potential data variation due to unavoidable differences in tumor size and shape, comparison of drug penetration used tumor sections obtained at equal depths; i.e., sequential sections were obtained at each 200-μm depth. Autoradiographic images were captured by Quest Pharmaceutical Services, LLC (Newark, DE). Commercially available, precalibrated microscale tritium autoradiography standards (0.1–109.4 nCi/mg; Amersham Biosciences Corp., Piscataway, NJ) were placed onto glass slides holding tumor sections. Slides were scanned using Typhoon imaging system (Amersham Biosciences Corp., Piscataway, NJ) for 1 week at room temperature, and signals were scanned using Typhoon imaging system (Amersham Biosciences Corp.). The limit of detection was 1 to 2 nCi/mg. Pixel gray-scale analysis was performed using Optimas software. Drug concentration as a function of distance from tumor periphery was quantified by averaging the gray-scale intensities in four perpendicular directions spanning the length of the tumor; this procedure was designed to minimize the location-related variability within a tumor. Background radioactivity was determined by measuring signals in tumors obtained from control animals, which were typically <10% of the intensity in experimental groups. After correcting for background, drug concentrations were calculated using standard curves, and AUC depth curves from periphery to 2-mm depth were calculated using the trapezoid rule.

HPLC Analysis of Paclitaxel. Paclitaxel was extracted from aqueous samples with ethyl acetate using cephalomannine as the internal standard and analyzed with column-switching HPLC assay, as described previously (Song and Au, 1995). For measuring paclitaxel levels in TPM, microparticles were dissolved in methylene chloride and analyzed without extraction. HPLC stationary phase consisted of a cleanup column (Nova-Pak C18, 4-μm particle, 3.9 × 75 mm; Waters, Milford, MA) and an analytical column (Bakerbond C18, 5-μm particle, 4.6 × 250 mm; Mallinckrodt Baker, Phillipsburg, NJ). Samples were injected into the cleanup column and eluted with a...
mobile phase consisting of 37.5% acetonitrile at a rate of 1.0 ml/min. The analytical mobile phase consisted of 49% acetonitrile and was passed through the analytical column at 1.2 ml/min. Paclitaxel was detected by UV absorbance at 229 nm; the lower limit of detection was 1 ng per injection.

### Antitumor Activity

The in vivo therapeutic efficacy in tumor-bearing mice was measured as increase in overall survival time. Drug treatment was initiated on day 28, which equaled half of the median survival time of the control group. Increase in life span (ILS) was calculated as [(median survival time of treatment group − 28 days)/(median survival time of control group − 28 days)] × 100%. Mice received physiological saline, paclitaxel/Cremophor EL, or TPM at equal milligram and equitoxic dose.

Post mortem autopsy was performed to evaluate the cause of death. Typically, deaths that occurred within 10 days after treatment were considered treatment-related deaths (e.g., >15% body weight loss, internal hemorrhage due to faulty injections). Deaths that occurred at later times and accompanied by presence of large tumor nodules (e.g., >4 mm) and/or tumor infiltration into organs were considered deaths due to disease progression.

An earlier phase I trial of intraperitoneal paclitaxel-loaded poly(DL-lactide-co-ethyl phosphate) particles (average size, 53 μm) revealed extensive, diffuse adhesions in a patient (Armstrong et al., 2006b). Hence, we evaluated whether TPM caused adhesion using the same definition in an earlier animal study; i.e., an abnormal connection between intra-abdominal contents that could not be disrupted by gentle separation is considered adhesion, and microparticles attached to an intra-abdominal surface but not causing apposition of two surfaces are not considered adhesion (Kohane et al., 2006).

### Gastrointestinal Toxicity

Gastrointestinal toxicity of intraperitoneal paclitaxel was monitored by changes in body weight and labeling index of intestinal crypts. Tumor-free mice were given intraperitoneal injections of paclitaxel/Cremophor EL (single dose of 40 mg/kg/day, 3 doses of 40 mg/kg on 3 consecutive days), priming TPM at 40 mg/kg (single dose), or two-component TPM at 120 mg/kg (1:2 priming/sustaining, single dose). Control group received physiological saline. Mice in single dose groups were euthanized at 24 h after treatment, and mice in multiple dose and two-component TPM groups were euthanized at 120 h after the initial treatment. At 1 h before euthanization, a DNA precursor bromodeoxyuridine (BrdU, 100 mg/kg) was injected intravenously. A segment of small intestine (jejunum) was excised, flushed with physiological saline, embedded in paraffin, and processed for immunostaining by BrdU using previously described methods (Gan et al., 1996).

### Statistical Analysis

Survival data were analyzed with the log rank test between different treatment groups and Kaplan-Meier plots. Analysis used SAS software (SAS Institute, Cary, NC). For the analysis of particle penetration data and intestinal crypt BrdU-labeling data, comparisons between two groups used unpaired Student’s t test and comparisons between three groups used one-way ANOVA with post hoc Tukey’s test. Two-sided P values of less than 5% were considered statistically significant.

### Results

#### Effects of Particle Size on Intraperitoneal Distribution and Tumor Localization

We first studied the distribution and retention of microparticles in peritoneal cavity in tumor-free mice. Mice were treated with free rhodamine (dissolved in PBS), free rhodamine plus unlabeled microparticles (4 μm), or rhodamine-labeled microparticles (4 μm). In the first two groups, the fluorescence was evenly distributed throughout the abdominal cavity at early time points (e.g., 15 min) but rapidly declined to a level not distinguishable from background autofluorescence at 24 h; the results for the second group are shown in Fig. 1A (top). In contrast, mice treated with rhodamine-labeled microparticles showed clusters of strong fluorescence signals localized in the folds of gastrointestinal tract and other tissues at 15 min and remained detectable on the surface of diaphragm, omentum, and mesentery at 24 h (Fig. 1A, bottom).

The above data confirm our earlier finding that drug carriers significantly affect drug disposition within the peritoneal cavity (Tsai et al., 2007) and indicate microparticles as a useful tool to promote drug retention and distribution during intraperitoneal therapy. The 4-μm microparticles were selected for subsequent studies in tumor-bearing mice. The results showed that these particles (rhodamine-labeled) were localized on the surface of tumor nodules (Fig. 1C) and visibly absent on the surface of peritoneum and other intraperitoneal organs (not shown), indicating preferential adherence of microparticles to tumor surface.

### Effects of Tumor Priming Treatments on Particle Penetration

This study compared the efficiency of priming TPM and paclitaxel/Cremophor EL for tumor priming, using micron-sized drug-free fluorescent latex beads as the penetrant. The control group treated with intraperitoneal blank particles showed restricted dispersion of latex beads on tumor periphery, whereas both tumor priming groups showed deeper penetration, wider dispersion, and greater total uptake of latex beads (Fig. 2A). Among the two priming groups, results of quantitative image analysis showed significantly greater uptake as well as enhanced dispersion of latex beads in priming TPM group compared with paclitaxel/Cremophor EL, especially at the later time point of 240 h (Fig. 2B), indicating greater and more sustained tumor priming for TPM.

### In Vivo Tumor Targeting by TPM

This study compared the tumor targeting of intraperitoneal paclitaxel/Cremophor EL and priming TPM at equal milligram doses. Because drug penetration is dependent on tumor size (e.g., easier penetration in smaller tumors) and tumor location (e.g., greater drug exposure for tumors in contact with peritoneal fluid), the comparison was limited to tumors located on the omentum and of comparable sizes (3–6 mm diameter). HPLC analysis of total paclitaxel concentrations showed different kinetics for the two treatments (Fig. 3). The paclitaxel/Cremophor EL treatment resulted in concentrations (C_max) that peaked at an earlier time (24 h) and declined more rapidly (below the detection limit of 0.5 μg/g at 72 h), whereas TPM yielded slower uptake (C_max at 72 h) and slower decline (remained detectable for at least 28 days). TPM also yielded 4-fold higher C_max and 16-fold higher AUC time curve.

### TPM Enhanced Paclitaxel Penetration and Spatial Distribution in Tumors in Vivo

We tested the hypothesis that two-component TPM, due to the tumor priming property of priming TPM and the sustained drug release from sustaining TPM, improves the drug penetration and retention in tumors. Figure 4A shows the micrographs of sections of omental tumors excised from animals 72 h after treatment;
Fig. 1. Intra-abdominal distribution of polymeric microparticles. A, distribution. Tumor-free mice were given intraperitoneal injections of rhodamine dissolved in vehicle (0.01% Tween 80 in PBS) plus blank microparticles (top) or rhodamine-labeled microparticles (bottom). Rhodamine appears red under UV light. B, effect of particle size. Tumor-free mice were given intraperitoneal injections of acridine orange-labeled microparticles with average diameters of 4 or 30 μm. Acridine orange appears yellow under UV light. The smaller particles were dispersed throughout the cavity and on mesenteric membrane and omentum that are common sites of local metastases of ovarian tumors. The larger particles were localized in lower abdomen and were absent on mesenteric membrane and omentum. Arrow indicated injection site. C, localization of 4 μm particles on tumors. Mice were implanted with intraperitoneal human ovarian SKOV3 xenograft tumors. After tumors were established (day 42), a mouse was given an intraperitoneal dose of rhodamine-labeled microparticles. Three days later, the animal was anesthetized, and the abdominal cavity was exposed. Photographs were taken in the region of omentum and mesentery under UV light (left) and room light (right). Note the large tumor on omentum (~13 mm longest diameter) and multiple small tumors on mesenteric membrane (1–3 mm longest diameter). Red color under UV light indicated localization of rhodamine-labeled particles on tumor surface.

Fig. 2. Tumor priming promoted penetration of latex beads in intraperitoneal SKOV3 tumor nodules. Mice bearing intraperitoneal SKOV3 tumors were given an intraperitoneal injection of a tumor priming treatment with either paclitaxel/Cremophor EL or priming TPM (40 mg/kg), followed by an intraperitoneal injection of fluorescent latex beads (2 μm diameter) given 48, 144, and 216 h later. The dose of latex beads was 40 mg/kg (2% solid, 10-fold dilution in normal saline, 0.5 ml per 25 g mice). Control group received blank, drug-free microparticles (i.e., no tumor priming pretreatment). A, representative tumor sections showing amount and dispersion of latex beads in tumors. Beads showed red fluorescence (shown as white dots in the black-and-white pictures). White lines indicate the outer perimeter of tumor nodules. 100x magnification. B, quantitative image analysis results. The amounts of latex beads in tumors are expressed as (total fluorescence intensity) normalized by (tumor area); a higher value indicates a greater amount. The bead dispersion results are expressed as percentages of tumor occupied by beads; a higher value indicates a greater dispersion. Solid bars, blank particles; open bars, paclitaxel/Cremophor EL, hatched bars, TPM. Error bars show 95% confidence intervals. *, elevated bead amounts or dispersion in paclitaxel/Cremophor EL group compared with blank particle group at corresponding time points (p < 0.05, Student’s t test). †, elevated bead amounts or dispersion in TPM group compared with paclitaxel/Cremophor EL or blank particle groups (p < 0.05, ANOVA with post hoc Tukey’s test).
Two-component TPM group showed dose-adjusted AUC compared with paclitaxel/Cremophor EL group, whereas drug delivery, priming TPM group showed significantly higher component TPM (followed by priming TPM (5-fold decline over 168 h) and two-component in paclitaxel/Cremophor EL group (14-fold decrease over 168 h), indicating TPM penetration into tumors. AUTOMATICALLY RESUMED CLUSTER paragraphs further in text.

Figure 3. Tumor targeting advantage of TPM: HPLC results. Mice bearing intraperitoneal SKOV3 tumors were given intraperitoneal injections of either paclitaxel/Cremophor EL or TPM (10 mg/kg). At predetermined times, tumors located on omentum were removed and analyzed for paclitaxel concentrations using HPLC. Compared with paclitaxel/Cremophor EL, TPM yielded 4-fold higher maximal concentration (13 versus 3.2 μg/g) and 16-fold higher AUC (82 versus 5 μg × day/g).

Clusters of microparticles were observed in tumor interior, indicating TPM penetration into tumors. We next used autoradiography to compare the spatial distribution of [3H]paclitaxel. The comparison was restricted to omental tumors of comparable sizes (as in the HPLC study) and to tumor midsections having the longest diameter. Applying these criteria across different treatment groups minimized differences due to unavoidable heterogeneities in tumor size and shape.

Figure 4B shows the autoradiograms in tumors removed from animals treated with paclitaxel/Cremophor EL, priming TPM, or sustaining TPM (all at 20 mg/kg) or two-component TPM (40 mg/kg, 1:1 priming/sustaining). All four groups showed the highest concentration at tumor surface (C_{max, tissue}), followed by a decline with increasing distance from the periphery. All three groups treated with different compositions of TPM showed higher concentrations and deeper penetration compared with the group treated with the Cremophor EL carrier. Figure 4C shows the concentration-depth profiles obtained from densitometric analysis of the autoradiograms; the data represented relative total concentrations and not the absolute concentrations because the actual weights of tissues on the tape sections could not be determined (Tsai et al., 2007). Due to the scarcity of the radiolabeled drug and the lower yield of sustaining TPM, the two-component and sustaining TPM groups were studied only at the later time points (72 and 168 h). Table 1 summarizes the results. With respect to changes in concentrations over time, paclitaxel/Cremophor EL and TPM groups showed similar patterns, with the highest concentrations attained at the earliest time point of 6 h followed by a decline with increasing time. With respect to rate of drug removal as shown by changes of C_{max, tissue} with time, the decline was most rapid in paclitaxel/Cremophor EL group (14-fold decrease over 168 h), followed by priming TPM (5-fold decline over 168 h) and two-component TPM (<20% decline over 96 h). With respect to total drug delivery, priming TPM group showed significantly higher AUC compared with paclitaxel/Cremophor EL group, whereas two-component TPM group showed dose-adjusted C_{max, tissue} and AUC values that exceeded the individual values or the sums in the former two groups. The higher levels in the three TPM groups, relative to paclitaxel/Cremophor EL, could have resulted from the localization of TPM on tumor surface and interior.

It is noted that the autoradiographic results differed from the HPLC results in that the former showed decreasing concentrations in tumors, whereas the latter showed increasing paclitaxel concentrations from 24 to 72 h. Because the two measurements used different parts of tumors (i.e., HPLC analysis used the whole tumor, whereas autoradiography used only the widest part of a tumor), the different results could be due to unavoidable regional heterogeneities. For example, a greater drug accumulation in tumor periphery would result in a higher total concentration by HPLC. Moreover, autoradiography detected total radioactivity and did not distinguish the unchanged paclitaxel from its metabolites, whereas HPLC measured only the unchanged drug. Collectively, the above data indicate that priming TPM and/or sustaining TPM provided more favorable delivery of paclitaxel to tumors compared with the Cremophor EL carrier and that sustaining TPM produced sustained tumor priming and/or drug retention in tumors.

Comparison of Toxicity of TPM and Paclitaxel/Cremophor EL. Treatment-related toxicity was monitored as body weight loss and inhibition of intestinal crypt-labeling index. The results are shown in Table 2 and Fig. 5. For body weight loss, all of the tested treatments for paclitaxel/Cremophor EL (1 × 40 mg/kg, 4 × 10 mg/kg over 2 weeks, 8 × 15 mg/kg over 4 weeks) and TPM (40 mg/kg priming TPM, 80 mg of sustaining TPM, 120 mg/kg 1:2 priming/sustaining; all given as single dose) produced a maximum of less than 10% body weight loss in 2 days, followed by recovery to the baseline level in 3 to 7 days. In addition, no reduction of intestinal crypt-labeling index was observed for a single dose of paclitaxel/Cremophor EL (1 × 40 mg/kg) or TPM (40 mg/kg priming TPM or 120 mg of two-component TPM), compared with the untreated control group. Reduction of intestinal crypt-labeling index was observed at a more dose-dense schedule of paclitaxel/Cremophor EL (3 daily doses of 40 mg/kg for a total of 120 mg/kg). These results suggest that the intestinal toxicity of paclitaxel was determined to a greater extent by the dosing schedule than by the total dose. The lower toxicity for less dose-dense schedules supports the use of slow release TPM. None of the 26 mice treated with a single dose of TPM (eight mice with 40 mg/kg priming TPM, nine with 80 mg/kg sustaining TPM, and nine with 120 mg/kg two-component TPM) showed adhesion at the end of experiments (between days 42 and 135).

Treatment-induced lethality was observed only in the single dose paclitaxel/Cremophor EL group (2/15 or 13.3%); one animal died within 1 day, and a second animal died 1 week later. The second animal showed significant body weight loss (26%). Neither death appeared to be due to tumor burden as the tumor size was several-times smaller compared with animals that died later from excessive tumor burden. A possible cause of death was accidental needle puncture of peritoneal organs.

In Vivo Efficacy and Toxicity of TPM. We compared the survival benefits of paclitaxel/Cremophor EL and TPM at equal milligram and equitoxic doses (six different treatments). Figure 6A shows the Kaplan-Meier plots, and Tables 2 and 3 summarize the results. Without drug treatments, animals died rather quickly; all animals died within 71 days (referred to as early deaths). We measured treatment bene-
fits in three ways, i.e., reducing early deaths, extending MST, and producing tumor-free cures. Tumor-free cures refer to animals that did not show tumor nodules in the peritoneal cavity on the last day of experiment (163–174 days after tumor implantation). Figure 6B compares the frequency of animal deaths at various time intervals (arbitrarily divided into 71 days for early deaths, followed by increments in 45-day intervals).

Compared with untreated controls, treatments with either paclitaxel/Cremophor EL or TPM significantly reduced the fraction of early deaths and extended MST. In general, increasing the total paclitaxel-equivalent dose and/or treatment frequency delayed disease-related deaths. At the equivalent total dose of 120 mg/kg, a single dose of two-component TPM was equally effective as eight doses of paclitaxel/Cremophor EL, and both treatments were significantly more efficacious compared with all other treatments. The qualitative and quantitative differences among Cremophor EL and TPM formulations are as follows. First, 9 of 33 (27.3%) mice in three paclitaxel/Cremophor EL groups showed early deaths within 71 days, whereas only 1 of 26 mice (3.8%, death on day 70) in the three TPM groups showed early deaths. For paclitaxel/Cremophor EL, no long-term tumor-free survivors were observed for the two groups receiving 40 mg/kg (either single dose or four divided doses over 2 weeks), whereas the group receiving 120 mg/kg in eight divided doses (twice weekly for 4 weeks) showed 25% cures. In contrast, all three TPM groups, irrespective of the dose or drug release rate (40 mg/kg priming TPM, 80 mg/kg sustaining TPM, 120 mg/kg two-component TPM) yielded ~22 to 33% tumor-free cures.

We next tested the hypothesis that a single dose of TPM was as efficacious as multiple doses of paclitaxel/Cremophor

Fig. 4. Effect of tumor priming on spatial drug distribution in tumors: autoradiographic results. Mice bearing intraperitoneal SKOV3 tumors were given intraperitoneal injections of either paclitaxel/Cremophor EL or priming TPM or sustaining TPM (all at 20 mg/kg) or two-component TPM (40 mg/kg) (1:1 priming/sustaining). A, TPM penetration into tumor interior. An omental tumor was removed from a mouse at 72 h after treatment with two-component TPM, sectioned, and stained with hematoxylin and eosin. The image was converted using Adobe Photoshop; TPM appeared as black dots. Top panel shows areas with clusters of TPM (circumscribed with dotted lines). Bottom panel shows the enlarged picture of the boxed area. B, autoradiograms of tumor sections (see Materials and Methods). C, concentration-depth profiles. Autoradiograms shown in B were processed to obtain measurements of total radioactivity using computer-assisted densitometric analysis (see Materials and Methods). Radioactivity was expressed as paclitaxel equivalents, with the highest level set at 100%.

TABLE 1
Drug penetration in peritoneal tumors
Mice bearing intraperitoneal SKOV3 tumors were given intraperitoneal injections of either paclitaxel/Cremophor EL or priming TPM or sustaining TPM (all at 20 mg/kg) or two-component TPM (40 mg/kg) (1:1 priming/sustaining); paclitaxel was radiolabeled. At predetermined times, tumors were excised and processed for autoradiography, and the radiographic signals were converted to drug concentrations, as described under Materials and Methods. $C_{\text{MAX,tissue}}$ is the maximal concentration found in the outer tumor perimeter in all treatment groups. Data are presented as relative concentrations, with the highest level set at 100%. $AUC_{\text{depth}}$ represents area-under-concentration-depth curve. Data are mean ± S.D. ($n = 3$ or 4 animals).

<table>
<thead>
<tr>
<th></th>
<th>6 h</th>
<th>24 h</th>
<th>72 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel/Cremophor EL</td>
<td>32.4 ± 13.8</td>
<td>19.9 ± 10.5</td>
<td>4.8 ± 1.5</td>
<td>2.3 ± 1.1</td>
</tr>
<tr>
<td>Priming TPM</td>
<td>38.2 ± 14.6</td>
<td>31.2 ± 2.8</td>
<td>16.4 ± 10.6</td>
<td>7.7 ± 3.5</td>
</tr>
<tr>
<td>Sustaining TPM</td>
<td>N.A.</td>
<td>N.A.</td>
<td>42.9 ± 11.4</td>
<td>N.A.</td>
</tr>
<tr>
<td>Two-Component TPM</td>
<td>N.A.</td>
<td>N.A.</td>
<td>100.0 ± 33.2</td>
<td>82.7 ± 10.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>6 h</th>
<th>24 h</th>
<th>72 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel/Cremophor EL</td>
<td>11.5 ± 6.0</td>
<td>6.7 ± 1.2</td>
<td>2.8 ± 1.2</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>Priming TPM</td>
<td>14.5 ± 7.0</td>
<td>9.6 ± 1.9</td>
<td>7.5 ± 3.6</td>
<td>5.2 ± 3.0</td>
</tr>
<tr>
<td>Sustaining TPM</td>
<td>N.A.</td>
<td>N.A.</td>
<td>9.5 ± 2.6</td>
<td>N.A.</td>
</tr>
<tr>
<td>Two-Component TPM</td>
<td>N.A.</td>
<td>N.A.</td>
<td>28.0 ± 17.0</td>
<td>31.9 ± 6.0</td>
</tr>
</tbody>
</table>

N.A., not available.
EL. At the equal milligram dose of 40 mg/kg, a single dose of priming TPM had comparable activity as a single dose of paclitaxel/Cremophor EL. Furthermore, a single dose of priming TPM, but not the single dose of paclitaxel/Cremophor EL, yielded significantly longer survival time compared with the twice-weekly schedule of paclitaxel/Cremophor EL (4 × 10 mg/kg). At the equal milligram dose of 120 mg/kg, a single dose of two-component TPM (120 mg/kg, 1:2 priming/sustaining) had comparable activity as eight doses of paclitaxel/Cremophor EL (8 × 15 mg/kg twice weekly over 4 weeks).

**Discussion**

The present study provided several findings that may be applied to improving intraperitoneal therapy. First, tumor priming using paclitaxel/Cremophor EL or TPM promoted the transport of micron-sized particles in tumor nodules and, hence, may improve the efficacy of intraperitoneal treatment of bulky disease. Second, the properties of TPM were selected, such that they were localized on the surface of tumor nodules and penetrated tumor interior. This, together with the two-component feature providing gated and sustained drug release, resulted in significantly greater tumor-targeting advantage. TPM formulation was developed based on the desired pharmacodynamics for successful intraperitoneal therapy; the two-component feature is unique compared with other commercially available formulations of paclitaxel. For example, compared with paclitaxel/Cremophor EL, TPM yielded higher and more sustained paclitaxel concentrations (16-times higher AU_{\text{depth}}), lower host toxicity (less body weight loss and lower toxicity to intestinal crypts), and greater therapeutic efficacy (longer survival) at equal milligram or equitoxic doses. We propose that the lower toxicity of TPM is probably a result of the fractionated dose presentation compared with the bolus injection of the entire dose all at once, as in the case for paclitaxel/Cremophor EL. Another important benefit of TPM is its apparent ability to eliminate the need of frequent dosing, given that a single dose of TPM (40 or 120 mg/kg) was equally or more effective compared with multiple divided doses of paclitaxel/Cremophor EL (4 × 10 or 8 × 15 mg/kg). Taken together, these findings support our contention that TPM, specifically tailored to the unique properties of peritoneal cavity and intraperitoneal tumors, represent a potentially useful strategy for intraperitoneal therapy of ovarian cancer.

We previously reported the effects of carriers on the peritoneal clearance of paclitaxel (Tsai et al., 2007). A major clearance mechanism for drug-containing carriers that are not readily transported through the peritoneum is drainage through the lymphatics. The smaller nanosize Cremophor EL and polymeric formulations are readily cleared through the lymphatics, whereas TPM, which has a diameter of 4 μm approaches or exceeds that of subdiaphragmatic lymphatic openings (~3 μm), is better retained in the peritoneal cavity. The present study further showed that the size of TPM affected its distribution in the peritoneal cavity; smaller TPM (4 μm) was widely distributed in the peritoneal cavity and adhered to tumor surface, whereas larger TPM (e.g., 30 μm) localized in the lower abdomen.

Several factors affect the pharmacokinetic-pharmacodynamic relationship of intraperitoneal therapy. First, as shown by other investigators (see Introduction) and confirmed in the present study, penetration of most drugs (without using TPM) into tumors is limited to the periphery. TPM adhered to tumor surface, penetrated, and resided in tumor interior. The intratumoral heterogeneity in drug penetration and distribution makes it difficult to extrapolate efficacy based on tumor pharmacokinetics, in vitro drug release rate, or dosing intensity and dosing rate. In addition, intraperitoneal metastatic tumors of-

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**TABLE 2**

Comparison of antitumor activity and overall toxicity

Mice were implanted with metastatic intraperitoneal SKOV3 tumor cells. Treatment was initiated on day 28 after tumor implantation, which was ~50% of the MST of control group. Treatment consisted of physiological saline (Control), paclitaxel/Cremophor EL (single dose of 40 mg/kg or multiple, twice-weekly doses at 4 doses of 10 mg/kg or 8 doses at 15 mg/kg), priming TPM (single dose of 40 mg/kg), sustaining TPM (single dose of 80 mg/kg), or two-component TPM (120 mg/kg total dose, 1:2 priming/sustaining). Increase in life span or ILS was calculated as (median survival time of treatment group − 28 days)/(median survival time of control group − 28 days) × 100% minus 100. In some treatment groups (with long-term cures), the upper limit of 95% CI was not reached (experiments were terminated on day 163 or 174).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight Change</th>
<th>Cure</th>
<th>MST (95% CI) Days</th>
<th>ILS Day</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>−0.8</td>
<td>0</td>
<td>52 (48–58)</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Paclitaxel/Cremophor EL (40 mg/kg)</td>
<td>17</td>
<td>−8</td>
<td>0</td>
<td>91 (85–93)</td>
<td>63</td>
<td>163</td>
</tr>
<tr>
<td>Paclitaxel/Cremophor EL (10 × 4 mg/kg)</td>
<td>8</td>
<td>−3.5</td>
<td>0</td>
<td>72 (63–77)</td>
<td>44</td>
<td>83</td>
</tr>
<tr>
<td>Paclitaxel/Cremophor EL (15 × 8 mg/kg)</td>
<td>8</td>
<td>−4.7</td>
<td>25</td>
<td>109 (49–174+)</td>
<td>81</td>
<td>258</td>
</tr>
<tr>
<td>Priming TPM (40 mg/kg)</td>
<td>8</td>
<td>−6.9</td>
<td>25</td>
<td>81 (76–163+)</td>
<td>53</td>
<td>121</td>
</tr>
<tr>
<td>Sustaining TPM (80 mg/kg)</td>
<td>9</td>
<td>−0.8</td>
<td>22</td>
<td>103 (101–107)</td>
<td>75</td>
<td>213</td>
</tr>
<tr>
<td>Two-Component TPM (120 mg/kg)</td>
<td>9</td>
<td>−7.2</td>
<td>33</td>
<td>117 (113–63+)</td>
<td>89</td>
<td>271</td>
</tr>
</tbody>
</table>

**TABLE 3**

Statistical analysis continued from Table 2

Statistical significance for the differences between groups was analyzed using the log rank test.

<table>
<thead>
<tr>
<th>Group Comparison</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs each of six treatment groups</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Single vs multiple divided doses of paclitaxel/Cremophor EL (40 mg/kg)</td>
<td>0.30</td>
</tr>
<tr>
<td>Priming TPM vs single dose of paclitaxel/Cremophor EL</td>
<td>0.54</td>
</tr>
<tr>
<td>Priming TPM vs multiple doses of paclitaxel/Cremophor EL (40 mg/kg)</td>
<td>0.045</td>
</tr>
<tr>
<td>Priming TPM vs Two-component TPM</td>
<td>0.13</td>
</tr>
<tr>
<td>Two-component TPM vs single dose of paclitaxel/Cremophor EL</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Two-component TPM vs multiple doses of paclitaxel/Cremophor EL (120 mg/kg)</td>
<td>0.45</td>
</tr>
</tbody>
</table>
Ten comprise a) loosely attached tumor cells that typically grow rapidly and b) tumors embedded or attached to intraperitoneal structures, such as mesenteric membrane or omentum that typically grows slowly (unpublished observation). Because it is likely that rapidly growing tumors result in early deaths and slowly growing tumors result in deaths at later times, successful treatments against rapidly growing cells will be more effective in reducing early deaths, whereas successful treatments against slowly growing tumors will be effective in producing long-term cures. This hypothesis is supported by several findings in the present study. First, administration of 40 mg/kg paclitaxel/Cremophor EL all at once in a single dose would be more likely to produce greater effects against the rapidly growing cells compared with administration in four divided doses over a period of 2 weeks; this is consistent with the finding that the single dose schedule was more effective in reducing the early deaths. Second, long-term cures, suggestive of eradication of the slowly growing tumors embedded in intraperitoneal tissues, were possible only for extended treatments (i.e., 4 weeks for paclitaxel/Cremophor EL or in the form of sustained release formulations such as TPM). Third, TPM (priming TPM, 40 mg/kg), which yielded greater and more sustained drug concentrations in slowly growing omental tumors compared with paclitaxel/Cremophor EL (1 × 40 mg/kg), did not reduce early deaths but produced long-term cures, whereas paclitaxel/Cremophor EL did not produce cures. Additional studies to evaluate the three-way relationship between drug release/dosing rate, tumor growth rate, and treatment efficacy may improve the utility of intraperitoneal therapy.

Locoregional administration of PLG copolymer is generally well tolerated in humans, because intramuscular administration of PLG microparticles elicited mild tissue response followed by complete recovery (Visscher et al., 1985; Shive and Anderson, 1997). On the other hand, 1 of 13 patients in the phase I trial of intraperitoneal paclitaxel-loaded particles made with a different polymer [i.e., poly(DL-lactide-coethyl phosphate)] and with a relatively large size (53 μm) showed extensive, diffuse adhesions completely obliterating the peritoneal space in the lower abdomen and pelvis (Armstrong et al., 2006b). A recent animal study evaluated whether intraperitoneal injection of microparticles (5–250 μm diameter) made of PLG with different molecular masses (7–90 kDa) caused adhesion of abdominal tissues. The results show higher frequency of adhesion for high molecular mass PLG microparticles (e.g., 90 kDa) (Kohane et al., 2006). The present study showed that TPM comprised low molecular mass polymers (8–40 kDa) and that having a relatively small size (4 μm) did not cause adhesion in all of the 26 mice examined. Another possibility is that paclitaxel suppresses adhesion, as reported in the phase I trial of intraperitoneal paclitaxel-loaded particles made with a different polymer [i.e., poly(DL-lactide-coethyl phosphate)] and with a relatively large size (53 μm) showed extensive, diffuse adhesions completely obliterating the peritoneal space in the lower abdomen and pelvis (Arm’s).
previously (Jackson et al., 2002). Whether TPM produces adhesion in humans needs to be investigated. Another potentially interesting finding is the selective tumor-adhering property of TPM. We speculate that this may be a result of interaction between PLG and tumor surface. Other carriers such as activated carbon particles also showed selective adherence to surface of intraperitoneal Yoshida sarcoma (Hagiwara et al., 1990). As tumor adherence may provide a tumor-selective delivery platform, further investigations on polymer-tumor interactions are warranted.

In summary, two-component TPM was designed to address the key challenges in intraperitoneal treatment of ovarian cancer. The present study demonstrated the several significant advantages of two-component TPM over the commercially available paclitaxel/Cremophor EL. These advantages may help to eliminate the need of indwelling catheter, minimize the local toxicity, and improve the compliance of patients and medical staff. The use of multiparameters with different drug release rates presents an additional theoretical advantage in that the combination of rapid and slow drug presentation enables the control of tumor cells with different growth rates. Finally, the good safety records of paclitaxel and PLG copolymers in humans support the clinical evaluation of two-component TPM.

Acknowledgments

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References


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Paclitaxel-loaded crosslinked hyaluronic acid films for the prevention of postural


