Paclitaxel-Loaded Gelatin Nanoparticles for Intravesical Bladder Cancer Therapy

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ABSTRACT

Purpose: The present report describes the development of paclitaxel-loaded gelatin nanoparticles for use in intravesical therapy of superficial bladder cancer. The commercial formulation of paclitaxel contains Cremophor, which forms micelles and thereby entraps the drug and reduces its partition across the urothelium.

Experimental Design: Paclitaxel-loaded gelatin nanoparticles were prepared using the desolvation method, and their physicochemical and biological properties were characterized.

Results: The size of the particles ranged from 600 to 1,000 nm and increased with the molecular weight of the gelatin polymer. Under optimal conditions, the yield was >80%, and the drug loading was 0.7%. Wide-angle X-ray diffraction analysis showed that the entrapped paclitaxel was present in an amorphous state, which has higher water solubility compared with the crystalline state. Identical, rapid drug release from nanoparticles was observed in PBS and urine, with ~90% released at 37°C after 2 hours. Treatment with a protease (i.e., Pronase) rapidly degraded the nanoparticles, with half-lives of 23.8 minutes, 0.6 minute, and 0.4 minute in the presence of 0.01, 0.05, and 0.25 mg/mL Pronase, respectively. The paclitaxel-loaded nanoparticles were active against human RT4 bladder transitional cancer cells; the IC50 paclitaxel-equivalent concentrations were nearly identical to those of aqueous solutions of paclitaxel, i.e., ~30 nmol/L (equivalent to ~25 ng/mL) for 2-hour treatments and ~4 nmol/L for 96-hour treatments. In dogs given an intravesical dose of paclitaxel-loaded particles, the drug concentrations in the urothelium and lamina propria tissue layers, where Ta and T1 tumors would be located, were 7.4 ± 4.3 μg/g (mean ± SD; 3 dogs; 9 tissue sections), which were 2.6× the concentrations we reported for dogs treated with the Cremophor formulation.

Conclusions: Paclitaxel-loaded gelatin nanoparticles represent a rapid release, biologically active paclitaxel formulation that can be used for intravesical bladder cancer therapy.

INTRODUCTION

Fifty-five thousand new cases of superficial bladder cancer are diagnosed annually in the United States. The current treatment consists of transurethral tumor resection of visible tumors, followed by intravesical chemotherapy to reduce disease recurrence and/or progression (1). Intravesical chemotherapy provides the advantage of selectively delivering drugs in high concentration to the tumor-bearing bladder while minimizing the systemic exposure. However, the response of intravesical chemotherapy is incomplete and variable among patients. Studies from our laboratory have shown that failure of treatment with mitomycin C and doxorubicin is due in part to the inability of the drugs to penetrate the bladder tissue and in part to the low drug activity against the more aggressive tumors (2–8). The hypothesis that enhanced drug delivery improves the treatment outcome has been verified in a phase III trial showing that enhancing the delivery of mitomycin C nearly doubled the recurrence-free rate in superficial bladder cancer patients (9). Collectively, these findings indicate that the two important properties of a drug used in intravesical therapy are activity against bladder cancer and ability to penetrate bladder tissues. As discussed below, paclitaxel represents an attractive candidate.

Paclitaxel shows higher activity compared with other antitubulin compounds, such as vinblastine, against human bladder cancer cells (10). A phase II study has shown that 24-hour intravenous infusion of paclitaxel produced a 42% partial and complete response rate of advanced and/or metastatic bladder cancer (11). In histocultures of human bladder tumors, a 2-hour treatment with paclitaxel was sufficient to cause inhibition of tumor cell proliferation and induction of apoptosis, with a greater apoptotic effect in the more rapidly proliferating tumors (12). In addition, paclitaxel, because of its lipophilicity, can penetrate the urothelium more readily than other commonly used drugs such as doxorubicin and mitomycin C (13). Paclitaxel is tightly bound to intracellular macromolecules such as tubulin and microtubule (14, 15), thereby resulting in significant drug accumulation (70–1,500 fold) in tumor cells, as well as drug retention (~10%) in tumor cells after removing the drug from the extracellular matrix (16, 17). The intracellular drug accumulation and retention contribute to the antitumor activity, particularly the delayed activity, of paclitaxel (16). The duration of drug instillation during intravesical bladder therapy is typically limited to 2 hours, after which time the drug is drained from the bladder. Hence, significant drug retention in tumors...
offers the opportunity of extending drug action beyond the 2-hour treatment duration. Finally, paclitaxel is known to cause apoptosis by p53-dependent and -independent pathways and is therefore less dependent on the p53 status (i.e., wild-type or mutated) as compared with other agents such as mitomycin C, which depends on a functional p53 pathway for apoptosis induction (18, 19). Hence, paclitaxel presents a theoretical advantage in the treatment of bladder cancer that shows a high frequency of p53 mutation (20). These characteristics make paclitaxel an attractive candidate for intravesical therapy.

The paclitaxel formulation approved by the Food and Drug Administration (FDA) for human use (e.g., Taxol) uses Cremophor to solubilize the drug. Our laboratory has shown in dogs that Cremophor, by entrapping paclitaxel in micelles, reduces the free fraction of paclitaxel and consequently lowers the drug penetration into the bladder tissue (21). Hence, the currently available paclitaxel formulation is not suitable for intravesical therapy. We have explored two approaches to overcome this problem. The first was to use a surface-active agent that is capable of disrupting the micelle structure and thereby increases the free fraction of paclitaxel; results in dogs show that dimethyl sulfoxide (DMSO) disrupts Cremophor micelles and restores the favorable bladder delivery of intravesical paclitaxel. However, because DMSO also increased the urine production rate and increased drug removal by the perfusing capillaries, the restorative effect of DMSO was incomplete (22).

Our second approach was to develop a new formulation that readily releases paclitaxel, e.g., complete release within 2 hours. The present study describes the preparation of paclitaxel-loaded gelatin nanoparticles and their biological activity in human bladder cancer cells. Gelatin was chosen because of its biocompatibility; gelatin is widely used as a stabilizer in vaccines and has been approved by FDA for extravascular administration (23). In addition, the hydrophilicity of gelatin is expected to facilitate the fluid penetration into the particles and thereby enhance the diffusion-mediated drug release. The submicron particle size of gelatin nanoparticles further enhances the particle degradation rate and thereby enhances the paclitaxel release rate. The in vivo bladder tissue penetration characteristics were also investigated.

MATERIALS AND METHODS

Chemicals. Paclitaxel and [1H]paclitaxel (specific activity, 16.2 μCi/mmole) were obtained from the National Cancer Institute (Bethesda, MD). Type A gelatin from porcine skin, Tween 20, sodium sulfate, sodium metabisulfite, Pronase, and glutaraldehyde (25% in water) were purchased from Sigma Chemical Co. (St. Louis, MO); cefotaxime sodium was purchased from Hoechst-Roussel (Somerville, NJ), gentamicin was purchased from Solo Park Laboratories (Franklin Park, IL), and all other cell culture supplies were purchased from Life Technologies, Inc. (Grand Island, NY).

Preparation of Paclitaxel-Loaded Gelatin Nanoparticles. Nanoparticles were prepared using several preparations of gelatin with different bloom numbers (75–100, 175, and 300) and using the desolvation method (24). A higher bloom number corresponds to a higher molecular weight of the polymer. Gelatin (200 mg) was dissolved in 10 mL of water containing of 2%

Tween 20. The solution was heated to 40°C with constant stirring at 300 rpm. To this solution, 2 mL of a 20% aqueous solution of sodium sulfate was added slowly, followed by 1 mL of isopropanol containing 2 mg of paclitaxel. A second aliquot of sodium sulfate solution (5.5–6 mL) was added until the solution turned turbid, which indicated the formation of gelatin aggregates. Approximately 1 mL of distilled water was then added until the solution turned clear. An aqueous solution of glutaraldehyde (25%, 0.4 mL) was added to cross-link the gelatin. Sodium metabisulfite solution (12%, 5 mL) was added 5 minutes later to stop the cross-linking process. After 1 hour, the crude product was purified on a Sephadex G-50 column. The nanoparticle-containing fraction was lyophilized in a freeze-drier over a 48-hour period.

Characterization of Paclitaxel-Loaded Nanoparticles. A mixture of gelatin nanoparticles and distilled water (~50 μL) was placed on foil paper, dried, coated with gold, and observed under a Philips XL 30 scanning electron microscope (SEM). For particle size distribution, more than 1,000 nanoparticles were examined using SEM images taken from four to six fields. The production yield was calculated from the weight of freeze-dried gelatin nanoparticles and expressed as a percentage of the starting weight of gelatin.

Wide-angle X-ray diffraction spectra of pure paclitaxel, mixtures of paclitaxel (2%, in weight percentage) and blank gelatin nanoparticles, and paclitaxel-loaded nanoparticles (1.62% loading) were obtained using Scintag PAD-V diffractometer. The samples were scanned from 5 degrees to 60 degrees with a scanning rate of 1 degree per minute.

Determination of Paclitaxel Loading in Gelatin Nanoparticles. Two milligrams of paclitaxel-loaded nanoparticles were dispersed in 0.5 mL of PBS and digested with 0.5 mL of Pronase (1 mg/mL in PBS) in a metabolic shaker at 37°C. After about 1 hour (or when a clear solution was obtained), the internal standard, cephalomannine (50 μL of 20 μg/mL methanol), was added, followed by extraction with 2 volumes of 3 mL of ethyl acetate. The ethyl acetate layers were pooled, dried under a stream of air, and reconstituted in acetonitrile. The paclitaxel concentrations in the extracts were analyzed using high-pressure liquid chromatography [HPLC (see below)] and compared with the concentrations in reference samples to determine the paclitaxel loading. The reference samples, consisting of mixtures of blank gelatin nanoparticles and known amounts of paclitaxel, were processed as described in the nanoparticles.

Release of Paclitaxel from Gelatin Nanoparticles. Paclitaxel nanoparticles (12 mg) were dispersed in 100 mL of PBS or dog urine and incubated at 37°C. Serial samples (1 mL) were withdrawn and centrifuged for 15 minutes at 143,000 × g using a Beckman L-70 ultracentrifuge. The nanoparticle-free supernatant (400 μL) was removed and extracted with 3 mL of ethyl acetate twice. The ethyl acetate extract was analyzed for paclitaxel concentration by HPLC.

Adsorption of Paclitaxel to Nanoparticles. Adsorption of paclitaxel to nanoparticles was determined by incubating a trace amount of [1H]paclitaxel (0.022 μCi) with empty gelatin nanoparticles for 5 hours at 37°C. Aliquots were collected for analysis of the total radioactivity. After incubation, the mixture was centrifuged for 15 minutes, and the resulting pellet contain-
ing the nanoparticles and the supernatant fraction were collected. The pellet was digested with Pronase (1 mL of 1 mg/mL). The radioactivity of the original mixture and digested pellet and supernatant was analyzed using a liquid scintillation analyzer (1600TR; Packard, Meriden, CT). The adsorption of paclitaxel onto gelatin nanoparticles was calculated as the percentage of radioactivity in the pellet.

**Enzymatic Degradation of Gelatin Nanoparticles.** Gelatin nanoparticles containing 0.74% paclitaxel were dispersed in PBS (pH 7.4; final concentration, 10 mg of nanoparticles per mL) and incubated with Pronase (final enzyme concentrations of 0.01, 0.05, and 0.25 mg/mL). Degradation of nanoparticles was monitored by measuring the absorbance at 540 nm using a DU-640 spectrophotometer (25). Under these conditions, the absorbance was proportional to the concentration of nanoparticles.

**In vitro Biological Activity Evaluation.** Human RT4 cancer cells were obtained from American Type Culture Collection (Manassas, VA). These cells were derived from a transitional cell papillary bladder tumor. Cells were cultured in McCoy’s medium supplemented with 9% fetal bovine serum, 2 mmol/L L-glutamine, 90 μg/mL gentamicin, and 90 μg/mL cefotaxime sodium at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were harvested from subconfluent cultures using trypsin and resuspended in fresh medium. Cells with >90% viability, as determined by trypan blue exclusion, were used. Cells were seeded in 96-well microtiter plates (~2,000 cells per well) and allowed to attach to the plate surface for 24 hours.

We have shown that paclitaxel produces immediate and delayed cytotoxicity (16). For immediate effect evaluation, cells were incubated with 0.2 mL of culture medium containing an aliquot of an aqueous solution of paclitaxel (referred to as free paclitaxel) or paclitaxel-loaded nanoparticles at equivalent paclitaxel doses for 48 and 96 hours, and the drug effect was measured immediately after treatment. For delayed effect evaluation, cells were treated similarly for 15 minutes and 2 hours, washed once with PBS, and then incubated with drug-free medium for a total of 96 hours, at which time the drug effect was measured. The number of cells remaining after treatment was measured using the sulforhodamine B assay (26). The sigmoidal concentration-response curves were analyzed to obtain the drug concentrations producing 50% inhibition (IC50), using nonlinear regression as described previously (16).

Stock solutions of free paclitaxel were prepared by first dissolving paclitaxel in EtOH, followed by serial dilution using culture medium. The final EtOH concentration was <0.1%, which does not affect cell growth (27).

**In vivo Evaluation of Paclitaxel Penetration into Bladder Tissues.** This study was conducted in four male beagle dogs. The animal protocol was approved by the Institutional Animal Care and Use Committee. All animals were fasted overnight and allowed access to water. The surgical procedures were as described elsewhere. In brief, a jugular vein was catheterized for the collection of systemic blood samples, and a cephalic vein was catheterized for the administration of anesthetics. A urethral catheter was inserted for the collection of urine samples and the administration of drug solution. All experiments were conducted in the morning between 7 and 10 a.m. Animals were anesthetized for the duration of the experiment. After emptying urine from the bladder, an intravesical dose of paclitaxel-loaded gelatin nanoparticles, containing 1,000 μg of paclitaxel dispersed in 20 mL of physiologic saline, was instilled into the bladder. One dog was given only the vehicle (blank nanoparticles), and its bladder was used to prepare the standard curve samples. Animals were euthanized with an overdose of intravenous pentobarbital immediately after removal of the bladder.

Serial urine samples were collected before and during instillation and immediately before surgical removal of the bladder. To analyze the total paclitaxel concentration, urine samples were incubated with 1 mg/mL Pronase (1:1) at 37°C for 1 hour, and 50 μL of the internal standard (cephalomannine, 20 μg/mL) were then added. The mixture was extracted with 6 mL of ethyl acetate and centrifuged at 3,000 rpm for 10 minutes. The supernatant was transferred and evaporated to dryness under a stream of air. The residue was reconstituted and injected into the HPLC system.

The excised bladder was cut into three sections (i.e., left and right lateral sides and dome). The tissue sections were snap-frozen in liquid nitrogen on a flat stainless steel plate cooled on dry ice. The procedures between removing the urine and freezing the bladder tissue were completed as rapidly as possible (i.e., in <5 minutes). The rapid removal and freezing were necessary to maintain the concentration gradient between urine and tissue and to avoid washout of the drug during tissue processing. The paclitaxel concentrations in bladder tissues were determined. Frozen bladder wall tissues were cut in parallel to the urothelial surface into 10- or 20-μm slices using a cryotome, as described previously (22). The first two sections were discarded to avoid contamination of tissue by urine, which contained a high drug concentration. The subsequent sections, representing a tissue depth of 40 to 300 μm, were used to analyze the total paclitaxel concentrations. Briefly, tissues were weighed, spiked with 50 μL of the internal standard (cephalomannine, 20 μg/mL), and homogenized with 3 mL of ethyl acetate. The homogenizer probe was washed with a second portion of ethyl acetate to recover residual adhering tissue. The two ethyl acetate fractions were combined and centrifuged at 3,000 rpm for 10 minutes. The supernatant was transferred and evaporated to dryness under a stream of air. The residue was reconstituted and injected into the HPLC system.

**High-Pressure Liquid Chromatography Analysis of Paclitaxel.** Paclitaxel was analyzed using our previously reported column-switching HPLC method (28). Briefly, the HPLC stationary phase consisted of a clean-up column (Novapak C8; 75 × 3.9-mm inner diameter; 4-μm particle size; Waters Associated, Milford, MA) and an analytical column (Bakerbond C18; 250 × 4.6-mm inner diameter; 5-μm particle size; I.D. Baker, Phillipsburg, NJ). The clean-up mobile phase was 37.5% acetonitrile in water at a flow rate of 1 mL/min, and the analytical mobile phase was 49% acetonitrile at a flow rate of 1.2 mL/min. The fraction from 8 to 15 minutes containing paclitaxel and cephalomannine was transferred from the clean-up column into the analytical column. The limit of sensitivity for paclitaxel was 5 ng/mL for urine samples and 5 ng per injection for tissue samples.
Statistical Analysis. Comparison of values between groups was performed using two-tailed Student’s t tests. A value of \( P < 0.05 \) was considered statistically significant.

RESULTS

Characterization of Nanoparticles. SEM photographs of paclitaxel-loaded gelatin nanoparticles are shown in Fig. 1A. The nanoparticles are spherical in shape. Column purification yielded particle sizes ranging from 600 to 1,000 nm. The particle increased with the bloom number. With the 175-bloom gelatin, the sizes of nanoparticles with 1% and 2% theoretical loading were 671 ± 267 and 620 ± 218 nm (mean ± SD), respectively. With the 300-bloom gelatin, the size increased to 976 ± 464 nm. X-ray diffraction analysis was performed to determine whether the entrapped paclitaxel existed in the less water-soluble crystalline state or the more soluble amorphous state. Sharp peaks in X-ray diffraction spectra indicate a crystalline structure. Results in Fig. 1B show sharp peaks for free paclitaxel and the mixture of free paclitaxel and blank gelatin nanoparticles, but not for paclitaxel-loaded gelatin nanoparticles. This indicates that the paclitaxel entrapped in the nanoparticles existed in an amorphous state. Table 1 shows the physical properties of different preparations of paclitaxel nanoparticles. The yield of nanoparticles ranged from 40% to 82% and decreased with increasing molecular weight of gelatin. The actual drug loading was between 33% and 78% of the theoretical loading. Nanoparticles prepared using high molecular weight gelatin formed large aggregates; the size of aggregates ranged from 10 to 30 μm (Fig. 1C). Removal of these aggregates during the column chromatographic purification step resulted in low yield and low drug entrapment efficiency. Low entrapment efficiency was also observed in nanoparticles prepared using low molecular weight gelatin. Optimal and highest yield and entrapment efficiency were achieved using the medium molecular weight gelatin (175 bloom). Subsequent studies used gelatin with 175 bloom to prepare paclitaxel-loaded nanoparticles.

Release of Paclitaxel from Nanoparticles. Release of paclitaxel from nanoparticles into PBS or dog urine was rapid, with 55%, 87%, and 92% released after 15 minutes, 2 hours, and 3 hours at 37°C, respectively (Fig. 2). Results of the adsorption study indicated that 4.5 ± 0.4% (mean ± SD of results of two experiments, three replicates per experiment) of the total amount of paclitaxel loaded in nanoparticles was adsorbed on the nanoparticles.

Enzymatic Degradation of Gelatin Nanoparticles. Fig. 3 shows the biphasic kinetics of nanoparticle degradation in the

Table 1 Loading efficiency of paclitaxel-loaded gelatin nanoparticles

<table>
<thead>
<tr>
<th>Gelatin molecular weight (bloom no.)</th>
<th>Yield (%)</th>
<th>Theoretical loading (weight %)</th>
<th>Actual loading (weight %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75–100</td>
<td>75.9 ± 8.0</td>
<td>0.99</td>
<td>0.46 ± 0.08</td>
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<tr>
<td>175</td>
<td>82.7 ± 7.5</td>
<td>0.99</td>
<td>0.74 ± 0.07</td>
</tr>
<tr>
<td>300</td>
<td>52.0 ± 7.5</td>
<td>0.99</td>
<td>0.33 ± 0.05</td>
</tr>
<tr>
<td>175</td>
<td>56.6 ± 9.3</td>
<td>1.96</td>
<td>1.54 ± 0.09</td>
</tr>
<tr>
<td>300</td>
<td>39.7 ± 8.6</td>
<td>1.96</td>
<td>0.76 ± 0.06</td>
</tr>
</tbody>
</table>

NOTE. A higher bloom number indicates a higher molecular weight (mean ± SD of three preparations). Yield is the weight of freeze-dried gelatin nanoparticles obtained after preparation and gel filtration, expressed as a percentage of the starting weight of gelatin.

Fig. 1 Characterization of paclitaxel-loaded gelatin nanoparticles. A, scanning electron micrographs of nanoparticles prepared using 175-bloom gelatin. Drug loading was 1.62%. Particle size was 620 ± 218 nm (mean ± SD). B, wide angle X-ray diffraction spectra. Top, paclitaxel powder. Middle, mixture of paclitaxel (2% in weight) and blank gelatin nanoparticles. Bottom, paclitaxel-loaded gelatin nanoparticles described in A. Arrows indicate the major diffraction peak of crystalline paclitaxel. C, scanning electron micrograph of paclitaxel-loaded nanoparticles using 300-bloom gelatin before column purification. Note the large variation in particle size. Large aggregates, white arrow; small aggregates, black arrow.
presence of a protease, Pronase. Treatment with 0.01 mg/mL Pronase showed a rapid initial degradation of 23% within 20 seconds, followed by a first-order decline with a half-life of 23.8 minutes and a total of 87% degradation at 60 minutes. Increasing the Pronase concentration to 0.05 mg/mL enhanced the degradation dramatically, with a maximal 90% degradation attained at 10 minutes and an initial apparent half-life of 0.6 minute. Further increasing the Pronase concentration to 0.25 mg/mL enhanced the maximal degradation to 100% and resulted in a slight decrease in the apparent half-life to 0.4 minute.

The enzymatic degradation of the gelatin nanoparticles is consistent with a degradation of the glutaraldehyde cross-link by proteolytic enzyme. The enzymatic degradation of paclitaxel-loaded gelatin nanoparticles is more rapid compared with nanoparticles prepared with poly(ε-caprolactone) (50:50) copolymer, which has a degradation half-life of about 60 days (29).

Biological Activity of Paclitaxel-Loaded Nanoparticles in RT4 Cells. Fig. 4 compares the concentration-response curves of free paclitaxel and paclitaxel-loaded nanoparticles. It is noted that the cytotoxicity study reported here used relatively low paclitaxel concentrations (i.e., nmol/L range), at which paclitaxel can be solubilized in water using a minimal amount of EtOH (i.e., <0.1%) without the use of Cremophor or other surface-active agents. Such a solubilization method is not applicable for intravesical therapy, where paclitaxel would be administered at much higher concentrations in at least the μmol/L range.

For the immediate effect, treatments with either free or nanoparticle-entrapped paclitaxel resulted in maximal inhibition of 60% at 48 hours and 84% at 96 hours. The increase in maximum paclitaxel cytotoxicity with treatment time is consistent with our previous observations (16). For the 48- and 96-hour treatments, the respective IC₅₀ values were 11.0 ± 0.4 and 4.0 ± 0.4 nmol/L for free paclitaxel and 9.6 ± 1.1 and 4.0 ± 0.3 nmol/L paclitaxel-equivalents for paclitaxel-loaded nanoparticles (mean ± SD of three experiments for both preparations).

The differences between the IC₅₀ values for these two preparations are not significant (P = 0.15 for 48 hours and P = 0.71 for 96 hours, unpaired t test).

For the delayed effect (i.e., the effect measured at 96 hours), treatments with either free or nanoparticle-entrapped paclitaxel for 15 minutes and 2 hours resulted in maximal inhibition of 74% to 85%. For the 15-minute and 2-hour treatments, the respective IC₅₀ values were 156.7 ± 6.6 and 33.0 ± 4.8 nmol/L for free paclitaxel and 165.7 ± 33.5 and 31.4 ± 1.8 nmol/L paclitaxel-equivalents for paclitaxel-loaded nanoparticles (mean ± SD of three experiments for both preparations). The differences between the corresponding IC₅₀ values for the two preparations are also not significant (P = 0.70 for 15 minutes and P = 0.64 for 2 hours, unpaired t test).

Urine Pharmacokinetics of Intravesical Paclitaxel-Loaded Gelatin Nanoparticles in Dogs. Fig. 5 shows the decline of urine paclitaxel concentrations, as a function of time, in dogs treated with paclitaxel-loaded gelatin nanoparticles. The concentration at zero time is the concentration of paclitaxel in the dosing solution, and the first sample was obtained at 1 minute. The slight concentration decline (~7%) during the first 10 minutes was due to the dilution of the 20-ml dosing solution by the residual urine (~2 mL). From 10 to 120 minutes, the drug concentration was further reduced by 1.8-fold due to dilution by newly produced urine and due to removal by absorption into the bladder tissue and systemic circulation.

Bladder Tissue Drug Concentrations in Dogs Treated with Paclitaxel-Loaded Gelatin Nanoparticles. The average concentration of total paclitaxel (free plus bound and/or nanoparticle-entrapped drug) in tissue sections comprising the urothelium and lamina propria (300-μm depth from the inner surface of the bladder) was 7.4 ± 4.3 μg/g tissue (mean ± SD of results from three dogs, three tissues per dog).
DISCUSSION

The low aqueous solubility of paclitaxel presented significant difficulty in its formulation. The FDA-approved formulation uses EtOH and Cremophor to solubilize paclitaxel. The life-threatening hypersensitivity of the paclitaxel/Cremophor/EtOH formulation observed in early clinical studies was attributed to Cremophor (30). The hypersensitivity is currently managed by premedication with steroids (31). In addition, as discussed in Introduction, paclitaxel is entrapped in Cremophor micelles. During systemic treatment, Cremophor is metabolized by the liver (32, 33), and paclitaxel is released. However, during regional therapy, in which a drug formulation is delivered directly into the tumor-bearing cavity, paclitaxel remains trapped in micelles. For intravesical therapy, the entrapment of paclitaxel in Cremophor micelles limits drug penetration into bladder tissues (21). We have shown that the addition of a surface agent such as DMSO disrupts the micelles and partially restores favorable paclitaxel penetration into bladder tissues (22). The present study explored a second approach, i.e., establishing a paclitaxel formulation that is suitable for intravesical therapy.

Because of its broad spectrum activity, there has been significant interest in developing different sustained paclitaxel microspheres and nanospheres formulations (34–37). However, these formulations were designed to provide a slow and sustained release of paclitaxel and hence are not suitable for intravesical bladder cancer therapy, in which the drug formulation is typically maintained in the bladder for only a short duration (i.e., 2 hours). For example, the release of paclitaxel from poly(lactic-co-glycolic acid) microspheres was <15% after 3 weeks (34). In another paclitaxel-loaded polymeric microspheres formulation (Paclimer), which was developed for intraperitoneal therapy of ovarian cancer, about 90% of paclitaxel was released over 90 days in vitro (36). In paclitaxel-loaded poly(ethylene oxide)-poly(lactide/glycolide) nanospheres with a size of about 150 nm, about 45% of paclitaxel was released in 3 days (37).

The present study reports the development of paclitaxel-loaded gelatin nanoparticles that rapidly released the drug in vitro, either in PBS or dog urine (87% in 2 hours without enzymes). For a drug to be released from microparticles and nanoparticles, the release medium must diffuse into a particle and dissolve the entrapped drug, which then diffuses out of a particle (38). Two properties of gelatin nanoparticles are favorable for a faster diffusion. First, compared with micro-

Fig. 4 Biological activity of paclitaxel-loaded gelatin nanoparticles. An aqueous solution of paclitaxel (squares) and paclitaxel-loaded gelatin nanoparticles (circles) was incubated with human bladder cancer RT4 cells. Drug-induced decrease in cell numbers was measured using the sulforhodamine B assay. Values represent the mean ± SD (n = 6 replicates). A, For immediate effect measurements, cells were treated with either free paclitaxel or paclitaxel-loaded nanoparticles for 48 hours (filled symbols) or 96 hours (open symbols). Drug effect was measured immediately after treatment. B, For delayed effect measurements, cells were treated for 15 minutes (filled symbols) or 2 hours (open symbols), incubated in drug-free medium for a total of 96 hours, and then analyzed for drug effect.

Fig. 5 Paclitaxel concentrations in urine during intravesical instillation for 120 minutes. Dogs were given an intravesical dose of paclitaxel-loaded gelatin nanoparticles containing 1,000 µg of paclitaxel, dispersed in 20 mL of physiologic saline. Values represent the means ± SD (n = 3).
particles, the small, submicron size of the nanoparticles decreases the effective diameter for the release medium to reach the drug and for outward drug diffusion. Furthermore, the hydrophilicity of gelatin enhances fluid uptake, whereas the hydrophobicity of polymers such as poly(lactic acid) and poly(lactic-co-glycolic acid) limits fluid diffusion (39), which would also result in a slower drug release. Second, because paclitaxel has a very low aqueous solubility, its physical state within the gelatin matrix determines its dissolution rate. The fact that paclitaxel existed in the more water-soluble amorphous state within the nanoparticles, as shown in X-ray diffraction results, might have contributed to the faster drug release rate.

The paclitaxel-loaded gelatin nanoparticles showed anti-tumor activity comparable with free paclitaxel. This is consistent with the rapid and near complete drug release characteristics of the particles. To determine whether the nanoparticles provide favorable drug delivery to bladder tissues comprising the urothelial surface and lamina propria, which are the locations of superficial bladder tumors targeted by intravesical therapy, we compared the results of the present study with our earlier two studies (13, 22). These earlier studies showed an average drug concentration in these bladder tissues of 4.6 μg/g or 5.4 μmol/g in dogs treated with 500 μg of paclitaxel dissolved in water and 1.4 μg/g or 1.6 μmol/g for dogs treated with 500 μg paclitaxel dissolved in 0.22% Cremophor and 0.21% EtOH (13, 22). The corresponding tissue concentrations adjusted to the 1,000-μg dose used in the current study are 9.2 and 2.8 μg/g. Note that we have shown a linear relationship between the mitomycin C concentration in dosing solution or urine and the drug penetration in human bladder tissues (6). In comparison, the nanoparticles yielded a bladder tissue concentration of 7.4 μg/g, which is about 80% of the concentration derived from the water/EtOH solution and 2.6× the concentration derived from the Cremophor/EtOH formulation.

In summary, we have developed paclitaxel-loaded gelatin nanoparticles that rapidly released paclitaxel, showed significant activity against human bladder cancer cells, and showed higher tissue concentrations compared with the commercial Cremophor/EtOH formulation. These data suggest the nanoparticles as a potentially useful paclitaxel formulation for intravesical treatment of bladder cancer.

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