Antitumor Activity of Hydrophilic Paclitaxel Copolymer Prodrug Using Locoregional Delivery in Human Orthotopic Non–Small Cell Lung Cancer Xenograft Models

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ABSTRACT

Paclitaxel (Taxol) has demonstrated clinical activity in non–small-cell lung cancer (NSCLC), but its use has not led to marked improvements in survival. This ineffectiveness can in part be attributed to inadequate delivery of effective drug levels to the lung via systemic administration and to drug resistance mechanisms. Locoregional drug administration and the use of drug copolymers are possible approaches to address these issues. In this study, we evaluated the activity of a poly(t-glutamic acid)-paclitaxel (PGA-TXL) formulation administered by intratracheal injection to mice bearing orthotopic human NSCLC tumors (H460, H358). H460 cells were found to be sensitive to paclitaxel and PGA-TXL in vitro, in a time- and concentration-dependent manner. In preliminary acute toxicity studies, PGA-TXL administered by intratracheal injection was found to be much less toxic than paclitaxel, as anticipated. Mice into which H460 cells had been implanted by intratracheal injection were given single-dose intratracheal treatments with paclitaxel (1.2 or 2.4 mg/kg) or with PGA-TXL (15 mg/kg, paclitaxel equivalents) 1 week later. When the mice were sacrificed at up to 65 days after tumor implantation, they were evaluated grossly for tumor at bronchial, neck, and lung sites. Control mice had tumors in 90% of all three sites, and all of the control mice had tumors in at least one site. The low- and high-dose Taxol groups had fewer incidences at these three sites (27–33%) and 60–80% of these mice had tumors in at least one site. The PGA-TXL mice displayed a low (13%) incidence at these sites, and only 40% had detectable tumors. In a subsequent survival study with the intratracheal H358 model, control mice had a mean life span of 95 days, whereas both the intratracheal Taxol (2.5 mg/kg, every 7th day for three doses) and the intratracheal PGA-TXL (20 mg/kg, paclitaxel equivalents, every 7th day for three doses) groups had improved survival (mean life spans: 133.5 and 136.5 days, respectively). In pilot studies intended to compare the feasibility of the development of paclitaxel aerosols suitable for clinical application, based either on Cremophor solutions or on PGA backbones, only the latter gave acceptable particle size distributions and flow rates. These results encourage the development and application of Cremophor-free copolymer formulations of paclitaxel for locoregional treatment (e.g., as aerosol) of endobronchial malignant diseases.

INTRODUCTION

Lung cancer is the leading cause of cancer mortality in the United States, Europe, and many other industrialized countries. NSCLC differs from SCLC in that surgery can frequently be curative, albeit in a small subpopulation of patients (1, 2). Hence, treatment of NSCLC, like that of SCLC, is most frequently also directed to metastatic disease. Unfortunately, NSCLC displays the same characteristics as many solid tumors for which single-agent chemotherapy provides only a small response rate, and even combination chemotherapy produces only marginal survival improvements. Paclitaxel is among the numerous drugs that have been evaluated, either alone or in combination, in NSCLC. Even with the most active regimens, median survival has been reported as only ~40 weeks for responders and ~20 weeks for nonresponders (3–5). This discouraging picture underscores the need for new therapeutic approaches for NSCLC.

Multidrug resistance (MDR) is the best characterized mechanism of acquired resistance expressed by tumor cells against a variety of structurally unrelated chemotherapeutic agents (6, 7). Major MDR mechanisms involve the Mr 170,000 transmembrane P-glycoprotein (6) and the Mr 190,000 multidrug-resistance-associated protein (7), expression of the latter being linked particularly to SCLC (8). Additional mechanisms that might also contribute to the chemoresistance of lung cancers include p53 deletion/mutation and bcl-2 overexpression.

The use of drug copolymers has been proposed as one approach to circumvent MDR and to improve the associated drug pharmacokinetics. These conjugates are internalized by endocytosis, which results in their accumulation in perinuclear lysosomes (9). Drug copolymers may have pharmacokinetic advantages over free drugs, because the latter may readily extravasate to normal tissues, whereas the size of the former may restrict such distribution. However, the leaky, irregular vasculature of solid tumors may be traversed, allowing better
tumor localization and less toxicity, compared with free drugs (10, 11).

Localized aerosol delivery of therapeutic agents to the respiratory tract is an attractive approach to the management of a number of pulmonary diseases. Jet nebulizers have been used to deliver both aqueous preparations of hydrophilic drugs and liposomal formulations of lipophilic drugs (12, 13). Thus, agents as diverse as macromolecular proteins, DNA plasmids, or low-molecular-weight oligonucleotides and lipophiles have been delivered by such means. Preclinical and clinical studies have generally supported the concepts that administration by this route (a) results in minimal systemic concentrations and toxicity, and (b) generates biologically effective pulmonary, but not peripheral, blood drug concentrations.

It is well accepted that the Cremophor component in Taxol has undesirable effects, including toxicities and perturbations of paclitaxel pharmacokinetics (14, 15). Furthermore, the physical-chemical nature of Cremophor is an impediment to the application of Taxol as an aerosol for the treatment of lung neoplasms. Moreover, Cremophor might enhance the absorption of paclitaxel through the mouth, esophagus, and upper airway if Taxol were delivered via an aerosol, possibly increasing local and systemic toxicity. As an alternative to this excipient, liposomal formulations of paclitaxel have been prepared and some of these have been extensively characterized, in fact, to the point of current clinical evaluation (16–24). Although one study used aerosol delivery of liposomal paclitaxel to treat pulmonary metastases of a renal cancer, no liposomal formulation has yet been evaluated using locoregional delivery to treat NSCLC.

Paclitaxel has also been formulated as a water-soluble conjugate with a poly-l-glutamic acid backbone (PGA-TXL; ref. 25). Characterization of PGA-TXL has revealed remarkable in vivo properties in several tumor models, including reduced toxicity, greater tumor localization, and superior antitumor efficacy compared with Taxol, including cures in Taxol-resistant in vivo ref. 25). Characterization of PGA-TXL has revealed remarkable in vivo properties in several tumor models, including reduced toxicity, greater tumor localization, and superior antitumor efficacy compared with Taxol, including cures in Taxol-resistant in vivo ref. 25).

Cell Lines

Human H358 (p53 null) and H460 (wild-type p53) NSCLC cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, in a humidified atmosphere of 5% CO2 and 95% air. The H358 model was previously shown to have a very high (~100%) intrapulmonary implantation rate (29); likewise, the H460 model has been used as an orthotopic model after implantation in the pleural space (30), or intratracheal inoculation has been used (31).

Cytotoxicity Assay

Subconfluent monolayers of the NSCLC cell lines were established in wells of 96-well plates. After overnight incubation, the cells were exposed to a range of concentrations of paclitaxel or PGA-TXL. After further incubation for up to 96 hours, the cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); after solubilizing the incorporated dye, the absorbance at 570 nm was determined. Survival was calculated as the absorbance in wells of treated cells normalized to controls.

Tumor Implantation Procedure

Male and female nude mice (nu/nu; 6–8 weeks old and weighing 18–22 g) were purchased from Harlan Sprague Dawley. After at least 1 week of postshipment acclimatization, they were anesthetized either with Nembutal intraperitoneally (40–60 mg/kg) or by using an isoflurane induction chamber with ~5% isoflurane, followed by maintenance at 1.5 to 3.0% isoflurane delivered via a nose-only exposure unit. The anesthetized mice were fixed on a small animal surgical board with their backs to the board by tying their legs and teeth. The skin outside of the trachea was opened with sterile scissors to achieve an incision ~6–8 mm long, along, or parallel to, the trachea. A syringe containing the tumor cell suspension and fitted with a 30-gauge needle was carefully inserted into the trachea. A single-cell suspension of 1 to 2 × 106 NSCLC cells (H358 or H460) in 80 uL of medium was injected into the upper airway via the trachea, and the needle was carefully withdrawn to facilitate complete delivery to the bronchi and to minimize deposition at the injection site or in the trachea itself. The wound was bathed with 70% EtOH and then was sealed with a wound clip, which was removed a week later. The mice were allowed to recover from the implantation procedure before subsequent treatment.

MATERIALS AND METHODS

Synthesis of PGA-TXL

To a solution of 100 mg of PGA (Mf, 42,100; Sigma-Aldrich) in 5 mL of anhydrous N,N-dimethylformamide, 26 mg of paclitaxel (~30 μmol; Hande Tech, Houston, TX), 30 μL of dicyclohexylcarbodimide (1.0 mol/L solution in dichloromethane), and a catalytic amount of dimethylaminopyridine were added. The reaction was allowed to proceed at room temperature overnight. The mixture was poured into CHCl3, and then diethylether was added. The precipitated compound was filtered, washed several times with diethylether, and dried. The solid product was converted to the sodium salt by dissolving the crude product in a 0.5 mol/L NaHCO3 solution. The aqueous solution of PGA-TXL was dialyzed against distilled water, filtered, and lyophilized to obtain the final product. The yield was ~92%, and the paclitaxel content was 20% (w/w), estimated by using UV measurement and based on a standard curve generated with known concentrations of paclitaxel in methanol (λ = 228 nm).

5 J. Klostergaard, unpublished observations.

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Drug Administration Procedure

Mice that had previously received implanted tumor cells as described above or that were naïve hosts, received formulations of paclitaxel or PGA-TXL as aqueous solutions via the intratracheal route. The intratracheal drug injection procedure was essentially identical to that described above for tumor cell implantation.

Acute Toxicity Studies

Using the intratracheal route of administration, we gave groups of naïve 6-to-7-week-old ICR mice (male and female, Harlan) single injections of paclitaxel formulations of various concentrations prepared by dissolving known amounts of paclitaxel in Taxol (6 mg of paclitaxel/mL in Cremophor). These concentrations were selected so that the injection volume would be ~80 µL for a typical mouse weight of ~20 g. The lowest dose delivered was 0.8 mg/kg, with escalation up to 19.2 mg/kg conducted in separate groups. The dose versus percentage of death in each group was plotted, and the resulting curves were simulated with a best-fit mathematical expression to derive an MTD, LD_{10}, LD_{50} and LD_{90}. A similar approach was taken with PGA-TXL but with a dose range from 10 to 70 mg/kg (paclitaxel equivalents) and with dissolution of the conjugate in warmed PBS. The treated mice were observed for several weeks, to monitor any bodyweight changes and possible deaths.

Antitumor Efficacy Studies: Gross Pathology and Survival

Either H358 or H460 cells were implanted in nude mice by intratracheal injection, as described above. Treatments were initiated at 7 to 12 days after tumor implantation. In one arm of the H460 studies, mice from each of the three groups (control, paclitaxel, and PGA-TXL) were sacrificed 1 to 2 days after the intratracheal drug treatment that had occurred, 12 days after tumor implantation, and lung tissues were subjected to histopathology and immunohistochemistry studies (see above).

In the main H460 study, intratracheal treatments were administered 7 days after tumor implantation. Treatments consisted of either 1.2 or 2.4 mg/kg of paclitaxel or 15 mg/kg (paclitaxel equivalents) of PGA-TXL. Only those mice surviving at least 2 weeks after the drug treatment and two surgeries were included in the experiment. On death or sacrifice at 65 days, the mice were evaluated for gross presentation of tumor at bronchial, neck, and lung sites. The number of sites with tumor was noted, as was the number of mice apparently free of tumor by this criterion.

For the H358 survival study, intratracheal treatments consisted of multiple-dose Taxol (2.5 mg/kg) or PGA-TXL (20 mg/kg; paclitaxel equivalents), administered every 7th day for three doses beginning 7 days after tumor implantation. The day of death/humane sacrifice was noted and increased life span (%ILS) was calculated and used as a basis for inter-group comparisons.

Aerosol Studies

To determine aerosolization rates, PGA solutions with concentrations of 12.5, 25, and 100 mg/mL were made by dissolving the sodium salt of PGA (M_m = 42,000) in distilled water; the latter solution corresponded to a PGA equivalent concentration of 25 mg/mL paclitaxel of a 20% (w/w) paclitaxel equivalent formulation of PGA-TXL. The Taxol solution was made by dissolving 60 mg of paclitaxel in 10 mL of Cremophor (Sigma), identical to the clinical formulation. About 4 g of each solution was added to the jet nebulizer (REF 8900, Salter Labs, Issaquah, WA). The nebulizer, the added solution, and the nebulizer plus the solution were all preweighed before the aerosolization procedure. The aerosol was generated by compressed medical air (Techair, White Plains, NY) at a flow rate of 5 or 9 L/min in a closed chemical hood. At 1, 5, and 9 minutes after aerosol generation, the whole nebulizer, including the solution inside, was weighed again. The weight loss divided by the time passed was defined as the aerosol generation rate. The volumes were calculated based on the density of each solution.

To determine the aerosol particle size, aerosols were generated as described above. The output of the nebulizer was linked to a 7-stage cascade impactor (In-Tox Products, Moriarity, NM). This instrument is designed so that a disk on each stage collects the portion of the aerosol within a particular droplet size range, e.g., stage 1, 5.0–3.03 µm, stage 2, 3.03–1.84 µm, etc. The exit port of the impactor was linked to a flow meter and a vacuum pump in series. The flow rate for the whole system was controlled at 5 or 9 L/min. About 4 mL of the solutions were added to the reservoir of the nebulizer. The condensed aerosol samples were collected from 1.0 to 1.5 minutes, from 3 to 3.5 minutes, and from 5 to 5.5 minutes. The preweighed discs on each stage were weighed again. The weight difference on a particular disk
After 48 hours of drug exposure, the IC$_{50}$ for PGA-TXL was 30–100 nmol/L, and for Taxol, it shifted to 3–10 nmol/L. By 72–96 hours, the IC$_{50}$ for PGA-TXL was 30 to 100 nmol/L, and for Taxol, it was 30–100 nmol/L (H9262). Incubation continued for 24, 48, 72, and 96 hours before MTT staining. The survival of the Taxol- and PGA-TXL-treated cells compared with the untreated controls at each time point is shown in Fig. 1.

The responses to Taxol (Fig. 1A) and PGA-TXL (Fig. 1B) were concentration- and time-dependent. At 24 hours, even the highest dose of PGA-TXL (1 mmol/L) could not achieve an IC$_{50}$, whereas the same concentration of Taxol did achieve it. After 48 hours of drug exposure, the IC$_{50}$ for PGA-TXL was ~300–1000 nmol/L, and the IC$_{50}$ for Taxol was achieved with 10–30 nmol/L Taxol. By 72–96 hours, the IC$_{50}$ for PGA-TXL was ~30–100 nmol/L, and for Taxol, it shifted to ~3–10 nmol/L (Fig. 1). Thus, the H460 cells were quite sensitive to paclitaxel, more so as Taxol than as PGA-TXL, as might be expected for a small free drug compared with a large drug copolymer, and at levels that are considered clinically achievable.

Generally similar time- and paclitaxel concentration-dependent responses occurred with p53 null H358 cells (data not shown), although with slightly greater resistance. At 24 hours, the IC$_{50}$ for both PGA-TXL or Taxol exceeded 1 mmol/L. At 48 hours, the IC$_{50}$ for PGA-TXL still exceeded 1 mmol/L, whereas it was ~30–100 nmol/L for Taxol. By 72 hours, the IC$_{50}$ for PGA-TXL was ~30 to 100 nmol/L, and for Taxol, it was ~3 to 10 nmol/L, similar to the pattern with H460 cells at this time point (Fig. 1). The IC$_{50}$ for paclitaxel for these cells has been reported to be ~110 nmol/L after 96-hour exposure (32).

**RESULTS**

**Cytotoxic Activity of Paclitaxel and PGA-TXL In vitro.** H460 cells (p53 wild-type) were seeded in 96-well cell culture plates overnight before exposure to Taxol or PGA-XL at a concentration range spanning 1 μmol/L to 0.3 nmol/L (paclitaxel equivalents). Incubation continued for 24, 48, 72, and 96 hours before MTT staining. The survival of the Taxol- and PGA-TXL-treated cells compared with the untreated controls at each time point is shown in Fig. 1.

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**Acute Toxicity Studies with Intratracheal Administration of Paclitaxel and PGA-TXL.** Groups of 6-to-7-week-old, male and female, ICR mice under anesthesia were given single intratracheal injections of paclitaxel formulations, beginning with the lowest dose delivered, 0.8 mg/kg, and escalating in separate groups to 19.2 mg/kg. These mice were then monitored for the next 2 weeks, and bodyweight changes were noted, as well as any deaths.

All of the dose levels resulted in an initial loss of bodyweight, which was reversible in all surviving mice. Any deaths were attributed to surgical stress and/or drug toxicity, and these generally occurred within 3 or 4 days of treatment. The dose versus percentage of death in the group is shown (Fig. 2). The tentative MTD was 2.4 mg/kg of paclitaxel, because it was the highest dose not resulting in any mouse deaths. Administration of 4.8, 9.6, 14.4, and 19.2 mg/kg dose levels resulted in 36, 33, 80, and 100% death, respectively, in each group of 10–20 mice (Table 1).

To determine the PGA-TXL MTD, we used an essentially identical approach, except that the starting dose was higher: 10 mg/kg, paclitaxel equivalents. Dose levels of up to 30 mg/kg (150 mg/kg of the 20% paclitaxel conjugate) were apparently well tolerated, with a reversible bodyweight nadir within 2 or 3 days of treatment (Fig. 3). By comparison, the bodyweight changes in mice receiving 9.6 mg/kg Taxol are shown; in this study, one of four mice died after Day 2 (Fig. 3). Sham mice subjected to the anesthesia and surgery but given an equal volume of PBS rather than drug, demonstrated a lesser nadir in a similar time frame (Fig. 3), attributable, therefore to the surgical procedure alone. Doses of PGA-TXL higher than 60 mg/kg (paclitaxel equivalents) were very toxic, with deaths occurring within 8 hours. The rapidity of death suggested the possibility that the high viscosity of the solution precipitated embolization in the trachea and/or bronchi. Alternatively, this response may reflect an acute local toxic reaction, similar to what was observed with lower doses (see histologic evaluation of H460 model below).

**Treatment of the H460 Model: Histologic and Immunohistochemical Evaluation of Tumors.** H460 cells were implanted by intratracheal injection in nude mice. Twelve days
later, groups of these mice were treated with a single intratracheal injection of either paclitaxel (1.2 mg/kg) or PGA-TXL (7.5 mg/kg, paclitaxel equivalents), or PBS for controls. Two mice each from the control and treatment groups were sacrificed 1 to 2 days after treatment, and sections of lung tissue were examined for tumor burden by using H&E staining. Only the control lung tissues were positive for tumor by this criterion; no tumor cells were evident at this degree of resolution in the lung sections of mice from either treatment group (Fig. 4). There were no signs of drug toxicity in the trachea or esophagus of either treatment group. In the paclitaxel-treated group, there was evidence in either lung of edema and lymphocytic and plasma cell infiltration leading to congestion. These changes were slightly milder in the lungs of the PGA-TXL group. Immunohistochemical staining of these tissues demonstrated that these tumor cells in the lungs of control mice were positive for PCNA expression, a marker of cells in cycle (Fig. 5). These results suggest foremost that either of the intratracheal drug treatment regimens rapidly induces substantial tumor cell death; furthermore, these treatments do so without marked histopathologic evidence for local normal tissue drug toxicity.

Treatment of H460 Model: Treatment Efficacy Judged by Gross Pathologic Examination. H460 cells were implanted by intratracheal injection in nude mice. One week later, groups of these mice were treated with a single intratracheal injection of paclitaxel (1.2 or 2.4 mg/kg), PGA-TXL (15 mg/kg, paclitaxel equivalents), or PBS for the controls. Subsequently, either on death during the course of the study or on sacrifice of all remaining mice alive at 65 days after tumor implantation, the bronchi, neck epithelium, and lung tissues were assessed for tumor incidence evident by gross pathological examination. The results are shown in Table 2.

All of the control mice (5 of 5) had tumors, whereas in the paclitaxel-treated groups, 60% (3 of 5) of the mice (low-dose paclitaxel) or 80% (4 of 5) of the mice (high-dose paclitaxel) had tumors, and only 40% (2 of 5) of the PGA-TXL-treated mice had tumors. Control mice presented with tumors at 60% of the sites (9 of the 15 possible sites for the five mice); because none of the mice was tumor-free, this reflects the tumorigenicity of H460 cells at these sites and the technical efficiency of the intratracheal tumor implantation procedure. The low-dose paclitaxel group had tumors at 27% (4 of 15 possible sites), whereas the high-dose paclitaxel group had tumors at 33% (5 of

Table 1  Acute toxicity in ICR mice after intratracheal single-dose treatment with paclitaxel and PGA-TXL

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total mice treated</th>
<th>No. of mice deceased</th>
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<tr>
<td>Paclitaxel</td>
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<tr>
<td>2.4 (mg/kg)</td>
<td>12</td>
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<td>4.8</td>
<td>14</td>
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<tr>
<td>9.6</td>
<td>15</td>
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<tr>
<td>19.2</td>
<td>10</td>
<td>10</td>
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<tr>
<td>PGA-TXL</td>
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<tr>
<td>30 (mg/kg, paclitaxel eqs.)</td>
<td>11</td>
<td>0</td>
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<td>40</td>
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<td>60</td>
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<tr>
<td>70</td>
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Abbreviation: eqs., equivalents.

later, groups of these mice were given single intratracheal injections of Paclitaxel/Taxol (TXL) formulations under anesthesia, beginning with the lowest dose delivered, 0.8 mg/kg, and escalating in separate groups to 19.2 mg/kg. Data for 2.4 mg/kg and above are shown. With PGA-TXL, an essentially identical approach was used, except that the starting dose was 10 mg/kg paclitaxel equivalents. Data for 30 mg/kg and above are shown. These mice were then monitored for the next 2 weeks or more, and any deaths were noted. The percentage of death in each group versus dose is shown.

Fig. 2  Groups of male and female ICR mice were given single intratracheal injections of paclitaxel/Taxol (TXL) formulations under anesthesia, beginning with the lowest dose delivered, 0.8 mg/kg, and escalating in separate groups to 19.2 mg/kg. Data for 2.4 mg/kg and above are shown. With PGA-TXL, an essentially identical approach was used, except that the starting dose was 10 mg/kg paclitaxel equivalents. Data for 30 mg/kg and above are shown. These mice were then monitored for the next 2 weeks or more, and any deaths were noted. The percentage of death in each group versus dose is shown.

Fig. 3  Female nude mice were given single intratracheal injections of PGA-TXL at 30 mg/kg (150 mg/kg of the 20% paclitaxel conjugate) or Taxol (TXL) at 9.6 mg/kg. Sham mice were subjected to the anesthesia and surgery but were given an equal volume of PBS rather than PGA-TXL or TXL. Mean bodyweights ± SEM are shown. SEM bars are obscured by symbols for means. PGA-TXL-treated mice demonstrated a substantial but reversible bodyweight nadir in the first 2 days, and one of the four TXL-treated mice died, whereas PBS control mice displayed a lesser nadir in a similar time frame, attributable, therefore, to the surgical and anesthetic procedures alone.
15 possible sites). The lower incidences compared with controls suggested some antitumor effects of these paclitaxel treatments. Furthermore, the PGA-TXL group had tumors at only 13% (2 of 15 possible sites). Thus, both the paclitaxel and the PGA-TXL treatments resulted in reduced tumor incidences and, in some cases, tumor burdens that were undetectable according to this criterion.

**Treatment of H358 Model: Survival Study.** H358 cells were implanted by intratracheal injection in nude mice. One week later, groups of these mice were treated with multiple intratracheal injections of either paclitaxel (2.5 mg/kg, every 7 days for 3 doses), PGA-TXL (20 mg/kg, paclitaxel equivalents, every 7 days for 3 doses) or PBS for controls. The mice were monitored over the next several months, and the day of death of each was noted. The results on host survival are shown in a Kaplan–Meyer plot (Fig. 6).

**Fig. 4** H460 cells were implanted by intratracheal injection in nude mice. Twelve days later, groups of these mice were treated with a single intratracheal injection of either paclitaxel (1.2 mg/kg), PGA-TXL (7.5 mg/kg, paclitaxel equivalents), or PBS for controls. Two mice each from the control and treatment groups were sacrificed 1 to 2 days after treatment, and sections of lung tissue were examined for tumor burden by using H&E staining. Control group (A, high power and B low power), paclitaxel group (C, high power) and PGA-TXL group (D and E, high power) are shown. Low magnifications, ×100; high magnifications, ×400. Tumor cells in TXL and PGA-TXL groups were undetectable at this time point.

**Fig. 5** Lung sections (from tumor-positive control mice only), described in the legend for Fig. 4, were subjected to immunohistochemical staining for PCNA expression in tumor, a marker of cells in cycle. Low magnifications, ×100; high magnifications, ×400.
All of the mice in each group eventually succumbed to their tumor burden, again reflecting the tumorigenicity of H358 cells in this orthotopic model and the technical efficiency of the intratracheal tumor implantation procedure. Control mice had a mean life span of 95 days, whereas both the intratracheal paclitaxel and intratracheal PGA-TXL groups had improved survival (mean life spans: 133.5 and 136.5 days, respectively; \( P < 0.03 \) and \( P < 0.04 \), respectively). These data demonstrate that locoregional delivery of either paclitaxel formulation results in substantial tumor control of this orthotopic model and improvements in host survival.

**Aerosol Characterization.** Solutions of paclitaxel in Cremophor (Taxol) or of PGA were subjected to aerosolization techniques with a nebulizer, and the resulting particle size distributions were characterized by a cascade impactor. These solutions had quite distinct aerosol characteristics. The accumulative portion of the droplets in the total collection, with increasing droplet size, versus the aerosol droplet size (mean \( \pm \) SD) from three independent measurements, is shown for Taxol and for PGA (Fig. 7). Approximately 50% of the PGA solution gave rise to droplets of <5 \( \mu \)m in diameter, whereas only \( \sim 10\% \) of the Cremophor-based solution was in this range, with \( \sim 90\% \) in the 5 \( \mu \)m range or larger. Thus, a much larger fraction of the PGA solution resulted in an appropriate aerosol, compared with Taxol.

An additional important advantage for PGA versus Cremophor was apparent in the rate of aerosol generation. The data shown in Table 3 are the mean \( \pm \) SD of the 1, 5, and 9 minutes aerosolization rates from three independent experiments. Far higher (\( \sim 80-400\)-fold) rates were achievable with the PGA solution compared with the Cremophor solution, an important consideration in preclinical evaluation and even moreso in clinical application.

**DISCUSSION**

The present studies demonstrate that a poly-amino acid-based paclitaxel copolymer, PGA-TXL, has substantially lower toxicity than paclitaxel/Taxol, and, moreover, essentially equivalent antitumor efficacy compared with equitoxic paclitaxel/Taxol against an orthotopic human NSCLC xenograft model, when both are administered by intratracheal injection. Further, PGA, but not Cremophor, solutions gave rise to acceptable aerosols in terms of size distribution and flow rates; together, these results suggest that a therapeutic approach that uses aerosol delivery of this novel paclitaxel formulation may warrant further development.

New treatment approaches, molecular targets, and therapeutic agents are all urgently needed to address the limited effectiveness of current treatment modalities for NSCLC. The bases for the poor impact on survival of present chemotherapeutic regimens are not well understood but may be attributable in part to the suboptimal pharmacokinetics of systemically delivered drugs, as well as to the inherent or acquired drug resistance expressed by the tumor cells.

Among treatment avenues being explored are alternatives to systemic drug delivery, and the use of novel non-Cremophor-containing formulations of paclitaxel, a drug that alone or in combination with other drugs induces a limited improvement in NSCLC patient survival. These include conventional phospholipid liposome formulations (18, 20, 22) or long-circulating liposomes containing polyethylene-glycol–derivatized lipids (20). One of these formulations has been evaluated with delivery as an aerosol and has thereby demonstrated superior pulmonary pharmacokinetics compared with systemic (intravenous) administration, as well as antitumor activity in a syngeneic murine renal carcinoma pulmonary metastases model (22). In other recent studies, cationic liposomal formulations of paclitaxel have demonstrated efficient targeting to the tumor vasculature (17, 21). After intravenous infusion, one such formulation, LipoPac, exhibited both vascular targeting as well as a marked ability to reduce tumor growth in an amelanotic hamster melanoma model, in a manner much superior to that observed with free Taxol (17). Some liposomal formulations of paclitaxel have demonstrated reduced toxicity over free Taxol but not enhanced antitumor activity (23, 24).

PGA-TXL is a recently developed, novel paclitaxel copolymer prodgur that also obviates the need for a Cremophor excipient. In preclinical models, it was less toxic and resulted in greater tumor localization compared with free paclitaxel (ref. 25 and unpublished data),5 the latter observation most likely linked

### Table 2  
H460 NSCLC tumor incidences after orthotopic implantation and intratracheal single-dose treatment with paclitaxel and PGA-TXL

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bronchus</th>
<th>Neck</th>
<th>Lung</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4/5</td>
<td>3/5</td>
<td>2/5</td>
<td>9/15</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2 mg/kg</td>
<td>0/5</td>
<td>2/5</td>
<td>2/5</td>
<td>4/15</td>
</tr>
<tr>
<td>2.4 mg/kg</td>
<td>1/5</td>
<td>3/5</td>
<td>1/5</td>
<td>5/15</td>
</tr>
<tr>
<td>PGA-TXL</td>
<td>15 mg/kg</td>
<td>2/5</td>
<td>0/5</td>
<td>2/15</td>
</tr>
</tbody>
</table>

---

**Fig. 6**  
H358 cells were implanted by intratracheal injection in nude mice. One week later, groups of these mice were treated with multiple intratracheal injections of either paclitaxel/Taxol (2.5 mg/kg, every 7 days for 3 doses), PGA-TXL (20 mg/kg, paclitaxel equivalents, every 7 days for 3 doses) or PBS for controls. The mice were monitored over the next several months, and the day of death/humane sacrifice of each was noted. The results in terms of host survival are shown in the Kaplan-Meier plot.
to the so-called enhanced permeability and retention (EPR) effect (9–11, 33). The essential role of covalent coupling of the paclitaxel moiety to the PGA backbone, as opposed to mere admixing of these two components, to achieve the improved therapeutic profile, has been previously noted (25). A previous study with PGA-TXL reported antitumor activity of this conjugate against a human breast adenocarcinoma, MDA-MB-435, selected for lung homing but implanted ectopically in the mammary fat-pad. Using systemic (intravenous) administration of near-MTD levels of PGA-TXL and paclitaxel, we found that PGA-TXL treatment caused tumor regression rates and reduced incidences of pulmonary metastases superior to those observed with paclitaxel (26). Another study of two compartmental models of intraperitoneal human ovarian carcinoma xenografts with locoregional (intraperitoneal) drug administration demonstrated the superior antitumor efficacy of even a single-dose administration of PGA-TXL compared with multiple-dose Taxol (28). Although PGA-TXL has not previously been evaluated in an orthotopic human lung cancer xenograft model, a PGA backbone copolymer of camptothecin (CPT) as been evaluated in a model similar to the ones used in the present study (34). Systemic (intravenous) administration of this CPT construct markedly improved the survival of the hosts bearing H322 intratracheal tumors, whereas these models were resistant to intravenous CPT or cis-diamminedichloroplatinum. Locoregional (intratracheal) drug administration, as used in our studies, was not evaluated by these investigators.

Collectively, these studies were an incentive for the present study, in which the PGA-TXL conjugate was compared with paclitaxel/Taxol by using locoregional delivery to treat two orthotopic models of human NSCLC xenografts. Both the H460 (p53 wild-type) and H358 (p53 null and high HER-2/new-expressing (32) cell lines were paclitaxel sensitive, although H460 seemed more so (Fig. 1; ref. 33). Nevertheless, H358 ectopic xenografts are reported to be paclitaxel sensitive and to display activated caspase-3 after paclitaxel treatment, as determined by immunohistochemical assays (32). In contrast, paclitaxel-mediated cell death in vitro in H460 cells has been shown to be caspase independent (35). Other studies have reported responses to paclitaxel in human NSCLC ectopic heterotransplants that correlate with the clinically reported response rates to Taxol (36).

The issues of locoregional versus systemic administration for the evaluation of drugs in preclinical NSCLC models are inevitably linked to the particular aspect of the disease presentation that is reflected in the preclinical model itself. Ectopic models (32), particularly those involving readily accessible superficial (e.g., subcutaneous) sites, facilitate monitoring of tumor response to treatment but may not accurately reflect key tumor-stromal dynamics representative of lung tumors or the pharmacokinetic issues relevant to clinical NSCLC. Even orthotopic models may differ in important tumor-stromal interactions and pharmacokinetic characteristics, depending on whether the tumors propagate in the lumen or on the airway epithelial surface of the lung. Most orthotopic NSCLC models use pleural injection (30, 37–43), which may be most relevant to locally invasive or metastatic disease in the lung. Other investigators, however, have used techniques similar to ours to implant tumors in the lung via tracheotomy (29, 31, 44, 45). Since our approach uses intratracheal routes for both tumor implantation and drug administration, this method appears most appropriately directed to early endobronchial disease and is realistically predicated on a scenario of early detection. Although this method of administration directs treatment to where the initial epithelial cellular transformations are likely to have occurred in response to airborne carcinogens, this scenario is all too infrequently captured in clinical practice. This situation will hopefully evolve as validated early-detection methods are developed and implemented.

In summary, the intratracheal route of drug administration can be considered a preclinical counterpart, or forerunner, of aerosol administration, which is already in wide clinical use. Our study provides evidence that locoregional (intratracheal) administration of PGA-TXL is well-tolerated in this preclinical NSCLC model and induces considerable antitumor activity. Thus, we propose that aerosolization of the poly(aminoc acid)–backbone-based PGA-TXL formulation is a feasible next step in the preclinical development of this approach to NSCLC treatment. In contrast, the Cremophor excipient in Taxol is a major impediment to an aerosol delivery strategy for this formulation of paclitaxel. According to our data (Table 3; Fig. 7), with Cremophor solutions, flow rates are unacceptably slow; further, as much as 90% of the aerosolized Cremophor solution forms droplet size.

Table 3  Aerosol generation rates of PGA and Cremophor solutions

<table>
<thead>
<tr>
<th>Flow rate†</th>
<th>Cremophor</th>
<th>PGA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.5 ± 0.3</td>
<td>199 ± 7</td>
</tr>
<tr>
<td>9</td>
<td>5.1 ± 0.5</td>
<td>423 ± 35</td>
</tr>
</tbody>
</table>

NOTE. Values are in mg/min, mean ± SEM.
* 25 mg/mL.
† L/min.
5-μm or larger droplets, which is too large for effective accumulation in the lung and would likely lead to unacceptable local toxicities in the nasal passages and mouth.

NOTE ADDED IN PROOF
After our studies were initiated, we became aware that a review of interim results of a two-stage phase II clinical trial of XYOTAX, the clinical formulation of PGA-TXL, in NSCLC patients indicated that continuation of the trial was warranted based on tolerability and disease control (46). Although this trial used intravenous administration of drug, our results suggest that future clinical trials with aerosol delivery could be considered.

ACKNOWLEDGMENTS
The animal studies described herein were approved by the M. D. Anderson Cancer Center (MDACC) Institutional Animal Care and Use Committee, and have observed the New York Academy of Sciences Ad Hoc Committee on Animal Research guidelines.

REFERENCES


Antitumor Activity of Hydrophilic Paclitaxel Copolymer Prodrug Using Locoregional Delivery in Human Orthotopic Non–Small Cell Lung Cancer Xenograft Models

Yiyu Zou, Hao Fu, Sukhen Ghosh, et al.


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