

Continuous Docetaxel Chemotherapy Improves Therapeutic Efficacy in Murine Models of Ovarian Cancer

Raquel De Souza¹, Payam Zahedi¹, Eduardo H. Moriyama², Christine J. Allen¹, Brian C. Wilson², and Micheline Piquette-Miller¹

Abstract

Ovarian cancer is known as the silent killer for being asymptomatic until late stages. Current first-line treatment consists of debulking surgery followed by i.v. chemotherapeutics administered intermittently, which leads to insufficient drug concentrations at tumor sites, accelerated tumor proliferation rates, and drug resistance, resulting in an overall median survival of only 2 to 4 years. For these reasons, more effective treatment strategies must be developed. We have investigated a localized, continuous chemotherapy approach in tumor models of human and murine ovarian cancers using the antineoplastic agent docetaxel. We show here that continuous docetaxel therapy is considerably more efficacious than intermittent therapy, resulting in a greater decrease in tumor burden and ascites fluid accumulation. Immunohistochemical analyses show that continuous chemotherapy abrogates tumor cell proliferation and angiogenesis to the tumor microenvironment, leading to greater tumor cell death than intermittent docetaxel therapy. Overall, our results show greater therapeutic advantages of continuous over intermittent chemotherapy in the treatment of ovarian cancer. *Mol Cancer Ther*; 9(6); 1820–30. ©2010 AACR.

Introduction

Ovarian cancer is the leading cause of death from gynecologic malignancies, with the majority of patients not surviving beyond 5 years postdiagnosis (1). Symptoms of ovarian cancer are nonspecific, such as bloating and constipation, and are usually not evident until late stages of the disease, when 75% of cases are diagnosed (2, 3). As early diagnosis remains a challenge, effective treatment strategies are needed to manage the disease. Current therapeutic approaches result in a median overall survival of only 2 to 4 years (3). First-line therapy of advanced ovarian cancer consists of surgical debulking of large tumors followed by carboplatin and paclitaxel administered i.v. at the maximum tolerable doses (MTD; ref. 4). This leads to low drug concentrations at tumor sites and high levels in healthy tissues, resulting in dose-limiting toxicities (5, 6). An alternative strategy is to maintain low drug levels in the systemic circulation through localized delivery, consequently decreasing toxic side effects, and increasing local drug concentrations in the peritoneum, where ovarian cancer tumors and ascites reside. This can be achieved through i.p. administration. Based on clinical trials that have evaluated this treatment strategy, the

National Cancer Institute has recommended that i.p. chemotherapy be considered for the treatment of advanced ovarian cancer (7). A major obstacle hindering the implementation of i.p. chemotherapy is the need for a catheter for drug delivery into the peritoneal cavity. In large clinical studies, catheter complications including obstructions, infections, and bowel perforations accounted for 70% of treatment termination cases (8, 9).

Another major problem with current therapy is the administration of short bursts of chemotherapy on a 3-week cycle. Treatment-free periods are required after each dose to allow healthy tissues to recover from the cytotoxic damage (10), during which surviving tumor cells reenter the cell cycle and resume proliferation at a progressively accelerating rate (11). Decreasing the treatment-free period by administering lower doses more frequently diminishes the accelerated proliferation observed during intermittent therapy (12). This strategy, termed metronomic chemotherapy, targets not only tumor cells but also rapidly proliferating endothelial cells that cause neoangiogenesis essential for tumor viability (10). In contrast, during periods between intermittent chemotherapy, neoangiogenesis resumes and new blood vessels are established.

In this study, we have eliminated treatment-free periods to study the effects of continuous chemotherapy delivered locally to the peritoneal cavity. We previously developed an implantable polymer-lipid film capable of continuous release of paclitaxel, which showed efficacy in an ovarian cancer xenograft model (13, 14). The need for surgical implantation and slow biodegradation led to the development of an injectable paste with similar

Authors' Affiliations: Departments of ¹Pharmaceutical Sciences and ²Medical Biophysics, University of Toronto, Ontario, Canada

Corresponding Author: Micheline Piquette-Miller, 144 College Street, Toronto, Ontario, Canada M5S 3M2. Phone: 416-946-8594; Fax: 416-978-8511. E-mail: m.piquette.miller@utoronto.ca

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properties composed of chitosan, phospholipids, and laurinaldehyde, termed PoLi_{gel}, which showed good biocompatibility and biodegradation (15, 16). Although paclitaxel is used in first-line therapy, its analogue docetaxel (DTX) has shown advantages, including slower cellular efflux and higher solubility, allowing for higher intracellular concentrations (17). In clinical trials, DTX resulted in equivalent response rates and has shown activity against paclitaxel-refractory carcinomas (4, 18, 19). *In vitro* studies have shown that DTX has higher affinity for microtubules, and greater apoptosis induction and potency at inhibiting microtubule depolymerization than paclitaxel (17, 18, 20). Thus, it is plausible that DTX may become part of first-line therapy. Continuous delivery of DTX holds much promise because this drug acts during specific phases of the cell cycle. Only a small percentage of the tumor cell population is vulnerable to DTX at a given time. Continuous DTX exposure allows cycling cells to reach the vulnerable phases, potentially resulting in greater tumor cell death.

The present study is the first to propose a continuous, low-dose, localized DTX regimen for the treatment of cancer. We compared the antitumor efficacy of this strategy to intermittent DTX administration in two distinct models of ovarian cancer—a SKOV3 xenograft model that is highly aggressive and is characterized by large solid tumor formation, and a murine ID8 syngeneic model that progresses slowly, is significantly more chemosensitive, and predominantly forms large amounts of ascites fluid. We chose to assess efficacy in both tumor models as late-stage ovarian cancer is both characterized by tumor formation and large volumes of ascites fluid. The PoLi_{gel} injectable formulation loaded with DTX (DTX-PoLi_{gel}) was used as a means for continuous DTX delivery to the peritoneal cavity. Effects of the two treatment strategies on SKOV3 tumor cell death, proliferation rates, and angiogenesis were investigated throughout the treatment period.

Materials and Methods

Cell lines

The SKOV3 human ovarian adenocarcinoma cell line was obtained from the American Type Culture Collection and maintained as previously described (14). For bioluminescence imaging, SKOV3 cells were transfected with the luciferase gene (SKOV3-luc) and bioluminescence-viability correlation was done as before (13). The murine ovarian cancer cell line ID8 was a kind gift from Dr. Jim Petrik (University of Guelph, Ontario, Canada; ref. 21). ID8 cells were maintained as described elsewhere (21). All cell lines were grown in monolayers at 37°C/5% CO₂ and 90% relative humidity.

Cytotoxicity of DTX

The IC₅₀ values of SKOV3-luc and ID8 cells were determined for use in *in vitro* experiments. Cells were seeded

onto 96-well plates (5,000 cells/well) and exposed to 0.13 to 1×10^5 ng/mL DTX in DMSO for 24, 48, and 72 hours ($n = 3$). Cell viability was determined by MTT.

In vitro activity of DTX-PoLi_{gel}

The DTX-PoLi_{gel} formulation is a blend of water-soluble chitosan, laurinaldehyde, egg phosphatidylcholine, and DTX at final material ratios of 1:4:1:0.71 (w/w/w/w), which was prepared as previously described (15, 16). SKOV3-luc and ID8 cells were seeded onto six-well plates (4×10^5 cells/well) and incubated with 5, 10, 20, and 30 μ L of drug-free PoLi_{gel} or DTX-PoLi_{gel} corresponding to total DTX concentrations of 93, 186, 372, and 558 μ mol/L for 24, 48 and 72 hours ($n = 3$). Cell viability was determined by MTT.

Preliminary evaluation of DTX-PoLi_{gel} efficacy

All animal studies were approved by the University of Toronto Animal Care Committee or the University Health Network Animal Care Committee and adhered to guidelines of the Canadian Animal Care Council. Six to 8-week-old female severe combined immunodeficient (SCID) mice (Charles River) received 1×10^6 SKOV3-luc cells in 200 μ L medium i.p. Seven days later, mice were injected i.p. with saline (controls) or DTX-PoLi_{gel} [total DTX doses of 16, 24, and 32 mg/kg, released at a rate of 3.6% per day] ($n = 6$ /group). Mice were monitored daily for signs of lethargy, weight loss, and abdominal distention. End points requiring humane euthanasia included excessive muscle wasting according to the “body conditioning scoring system” (22), abdominal distention, hypothermia, inactivity, and weight loss in excess of 20%. After a 21-day treatment period, tumor weight was assessed postmortem. Antitumor efficacy was calculated as: [(mean tumor weight nontreated) – (mean tumor weight treated)]/(mean tumor weight nontreated) $\times 100\%$. Tumor and plasma DTX content was determined by high-performance liquid chromatography using an established method (16).

Antitumor efficacy of continuous and intermittent DTX in SKOV3-luc xenografts

Six- to 8-week-old female SCID mice (Ontario Cancer Institute) received 1×10^6 SKOV3-luc cells in 200 μ L serum-free medium i.p. Seven days later, animals were grouped ($n = 12$ /group) as follows: (a) continuous therapy through DTX-PoLi_{gel}, (b) intermittent therapy with Taxotere (Sanofi Aventis), (c) drug-free PoLi_{gel} controls, and (d) nontreated controls. Group 1 received a single i.p. injection of DTX-PoLi_{gel} (total DTX dose of 32 mg/kg, released at a rate of 3.6% per day). Because the DTX-PoLi_{gel} releases 25% of its load weekly (16), group 2 received one i.p. injection of 8 mg/kg Taxotere weekly for a total of 32 mg/kg. Group 3 received a single i.p. injection of drug-free PoLi_{gel} in an equal volume as administered to group 2. Group 4 received one weekly i.p. injection of sterile saline. On days 7, 10, 14, 17, 21, and 24 postinoculation, tumor burden was assessed by bioluminescence

as before (13). Animals were monitored as described above. On days 14, 21, and 24, mice were sacrificed ($n = 4/\text{group}$) for tumor immunohistochemistry. The study was terminated on day 24 when control animals reached end points. Changes in tumor volume for each mouse at every time point were calculated and averaged to illustrate percent changes within each group.

Antitumor efficacy of continuous and intermittent DTX in ID8 xenografts

Six- 8-week-old female C57Bl6 mice (Charles River) received 2.5×10^6 ID8 cells in 200 μL PBS i.p. After 14 days, the animals were divided ($n = 10/\text{group}$) and treated as described for SKOV3-luc xenografts. This model predominantly forms large volumes of ascites; thus, abdominal girth was measured on days 14, 21, 25, 28, 32, and 35 postinoculation using a measuring tape. Assessment by bioluminescence using ID8-luc cells was not successful due to weak signal and/or interference by the dark skin color of the mice, as shaving of abdominal hair caused no improvement. Animals were monitored as described above. The study was terminated on day 35, as control

and intermittently treated mice reached end points. Ascites fluid volume was measured postmortem.

Immunohistochemistry

SKOV3-luc tumors collected from mice on days 14, 21, and 24 were processed and immunostained for Ki67, Casp3, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), are described elsewhere (13), whereas CD-31 immunostaining consisted of incubation with a CD-31 primary antibody (1/50 dilution overnight), followed by steps described for Ki67 and Casp3. A Nikon Coolpix 990 camera mounted onto a Nikon Eclipse e400 microscope was used to photograph a minimum of 10 fields per slide at $\times 100$ magnification, entirely covering each section. Structures were quantified and scored as before (13).

In vitro tumor cell proliferation

IC_{50} DTX concentrations (24 h) determined for ID8 and SKOV3-luc cells were used as the total DTX concentration per cycle. Cells were plated onto six-well plates (4×10^5 cells/well) and incubated overnight. Plates of

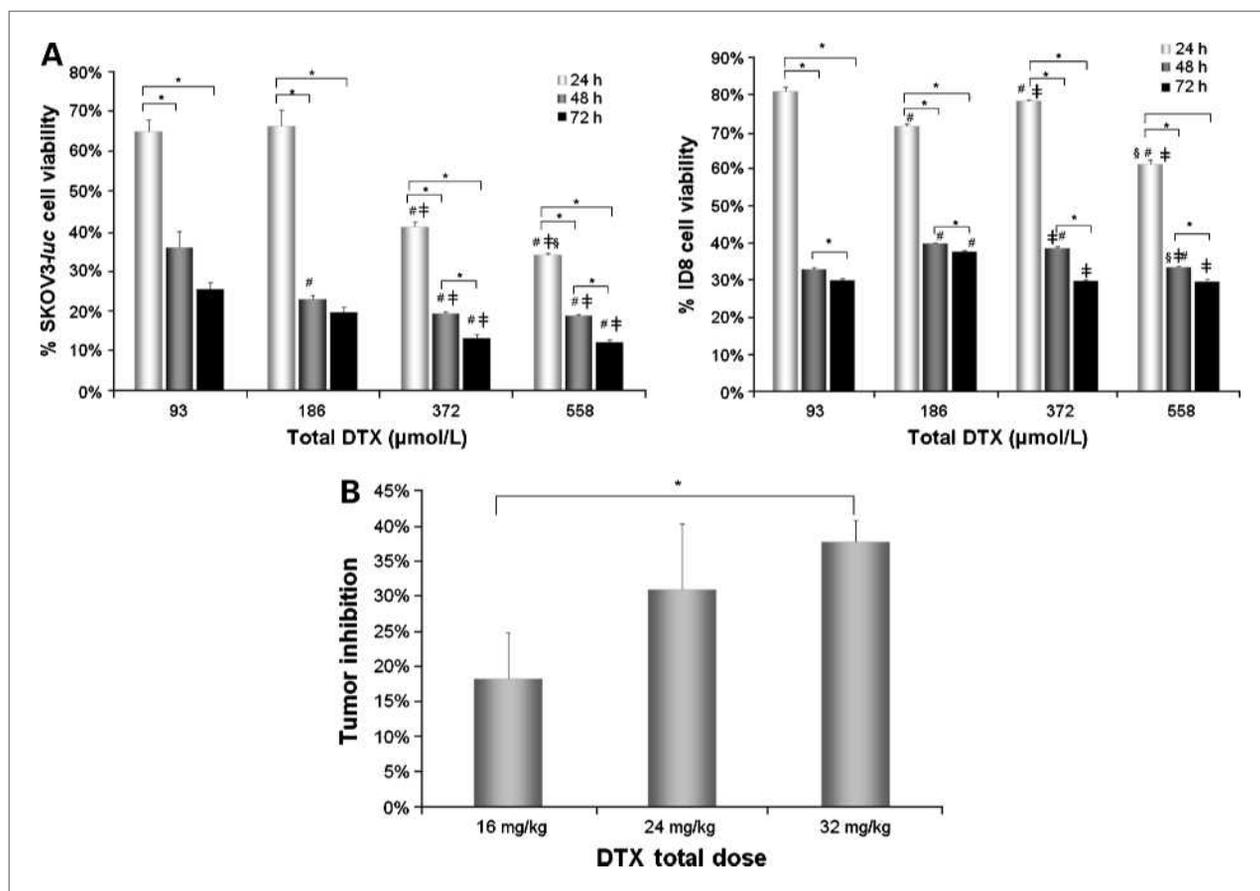
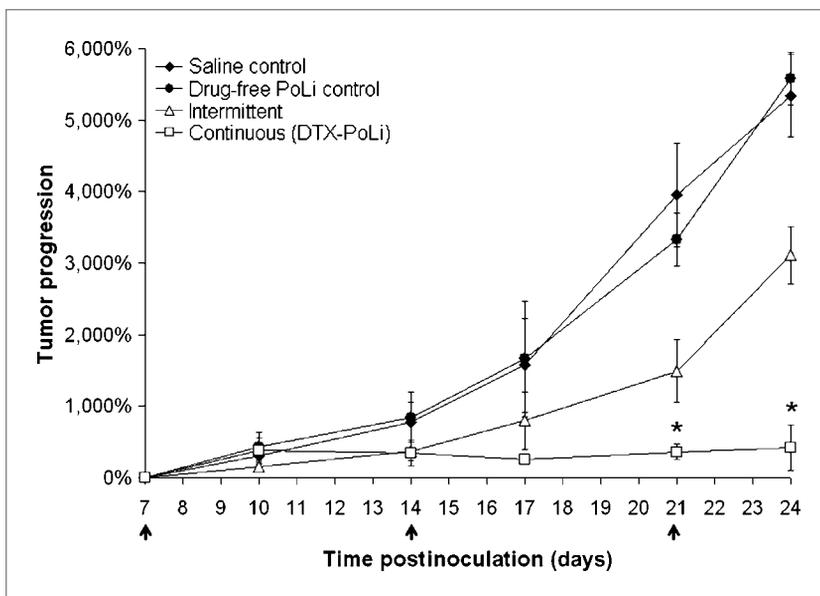


Figure 1. *In vitro* (A) and *in vivo* (B) ovarian cancer growth inhibition upon DTX-PoLi_{gel} exposure. A, within each time point: #, different from 93 $\mu\text{mol/L}$ ($P < 0.05$); †, different from 186 $\mu\text{mol/L}$ ($P < 0.05$); §, different from 372 $\mu\text{mol/L}$ ($P < 0.05$). Within each concentration: *, differences between exposure times ($P < 0.05$). Columns, mean ($n = 3$); bars, SEM. B, *, differences among groups ($P < 0.05$). Columns, mean tumor growth inhibition relative to tumor growth in nontreated control mice ($n = 6/\text{group}$); bars, SEM.

Figure 2. *In vivo* efficacy of continuous and intermittent i.p. DTX in a SKOV3-luc xenograft model assessed by bioluminescence. Continuous therapy: DTX-PoLi_{gel} on day 7 (32 mg/kg); intermittent therapy: weekly Taxotere (8 mg/kg; arrows). *, significant differences between treatment groups ($P < 0.05$). Points, mean % change in bioluminescence from day 7 within each group ($n = 16$ /group on day 7, $n = 12$ /group on day 14, $n = 8$ /group on day 21, and $n = 4$ /group on day 24); bars, SEM.



SKOV3-luc cells received one of the following treatments: (a) 4.3 $\mu\text{g/mL}$ DTX as Taxotere (intermittent treatment); (b) Taxotere vehicle (polysorbate 80 and 13% ethanol in water) in an equivalent volume used in "a" (intermittent control), (c) 1.4 $\mu\text{g/mL}$ anhydrous DTX in DMSO (continuous treatment), and (d) DMSO volume equivalent to "c" (continuous control; $n = 3$). ID8 cells were also treated in this manner; however, the intermittent concentration was 0.5 $\mu\text{g/mL}$ and the continuous concentration was 0.17 $\mu\text{g/mL}$. Three 3-day treatment cycles were done. Intermittent treatments and controls were incubated with cells for 12 hours then replaced with fresh medium. Sustained treatments and controls were replaced every 24 hours to ensure equal drug exposure as the intermittent treatment. Proliferation was assessed by the clonogenic assay. Cells were plated at known numbers ($n = 3$), and colony formation was allowed for 7 days (SKOV3-luc) or 4 days (ID8). Colonies were fixed with methanol and stained with 1% crystal violet (Sigma). Plates were scanned, and colonies (>50 cells) were counted using Image J. Plating efficiency (PE) was calculated as [(viable colonies)/(cells plated)], and number of clonogenic cells was calculated as [(cells counted at each time point) \times PE].

***In vitro* tumor cell death**

Treatments were done as described for "*In vitro* tumor cell proliferation" for one treatment cycle. At each day, MTT was used to assess cell viability.

Statistical analysis

Results are expressed as means \pm SEM. For comparisons among different treatment groups, the Student's *t* test was used with significance assigned at $P < 0.05$.

Results

Activity of continuous i.p. DTX exposure on ovarian cancer cells and tumors

To test whether continuous DTX provided by the injectable DTX-PoLi_{gel} formulation shows activity against ovarian cancer cells, human ovarian adenocarcinoma cells (SKOV3-luc) and mouse ovarian cancer cells (ID8) were exposed to four volumes of drug-free PoLi_{gel} or DTX-PoLi_{gel} at total DTX concentrations of 93, 186, 372, and 558 $\mu\text{mol/L}$, over 24, 48, and 72 hours, at which times cell viability was assessed by the MTT assay. Drug-free PoLi_{gel} did not affect cell viability regardless of concentration or exposure time. Upon exposure, SKOV3-luc cell viability ranged from $97.4 \pm 2.6\%$ to $103.4 \pm 10.8\%$, and ID8 cell viability ranged from $96.5 \pm 8.9\%$ to $99.7 \pm 8.5\%$. On the other hand, DTX-PoLi_{gel} caused a decrease in cell viability in a concentration- and time-dependent manner in both cell lines (Fig. 1A). This shows that continuous DTX exposure has the potential to suppress growth of tumors arising from these cells. Although metronomic DTX has been deemed efficacious (23), this is the first study to use a continuous DTX regimen. To assess *in vivo* efficacy of continuous DTX therapy, groups of SCID mice bearing SKOV3-luc tumor xenografts were injected with increasing doses of i.p. DTX-PoLi_{gel}. At the end of the 21-day treatment period, tumor burden of mice treated with DTX-PoLi_{gel} (continuous DTX) was assessed by weight and compared with that of nontreated controls. Consistent with *in vitro* results, continuous DTX therapy resulted in a dose-dependent tumor inhibition (Fig. 1B), which was $38 \pm 3\%$ at the highest total dose of 32 mg/kg. This dose was chosen for subsequent comparative studies between continuous and intermittent DTX. Blood plasma and tumor DTX content was measured by

high-performance liquid chromatography. At the highest dose of 32 mg/kg, drug levels in tumors were 178 ± 44 times greater than in plasma, which is consistent with the pharmacologic advantage reported for i.p. administered DTX (24). These *in vitro* and *in vivo* observations establish that continuous DTX therapy, as provided by the DTX-PoLi_{gel} formulation, is efficacious against ovarian cancer tumor growth.

SKOV3-luc tumor growth inhibition due to continuous and intermittent i.p. DTX therapy

The antitumor efficacy of continuous and intermittent i.p. DTX therapy was evaluated *in vivo* using the SKOV3-luc human ovarian cancer xenograft model in SCID mice. Bioluminescence imaging, an established method of efficacy assessment, was used to measure changes in tumor burden over time (13, 25). To confirm that this method accurately reflects tumor cell number, SKOV3-luc cells were treated with a range of DTX concentrations, and bioluminescence signal was correlated with cell viability as measured by the MTT assay. A strong linear correlation was observed ($R^2 = 0.93$). In addition, IC_{50} studies showed no difference in DTX chemosensitivity between

native and transfected SKOV3 cells (data not shown). Mice were treated with either continuous DTX through the DTX-PoLi_{gel} or intermittently with Taxotere, such that the cumulative DTX dose during the study period was identical. It is important to note that the weekly 8 mg/kg dose of Taxotere used is subtherapeutic, and it was used to match the 8 mg/kg dose released weekly from 32 mg/kg of DTX-PoLi_{gel}. The average tumor growth in animals treated with intermittent DTX was over seven times greater than in mice treated with continuous DTX (Fig. 2). Drug-free PoLi_{gel} had no effect on antitumor efficacy, as tumor progression was not different from control. The diversion in antitumor efficacy between the two treatment strategies occurred within the second cycle of chemotherapy.

In vivo tumor apoptosis, proliferation, and angiogenesis during continuous and intermittent i.p. DTX therapy

Mechanisms leading to differences in efficacy between continuous and intermittent chemotherapy were investigated by immunohistochemistry to quantify tumor cell death, proliferation, and angiogenesis. These parameters were analyzed in SKOV3-luc tumors extracted over time

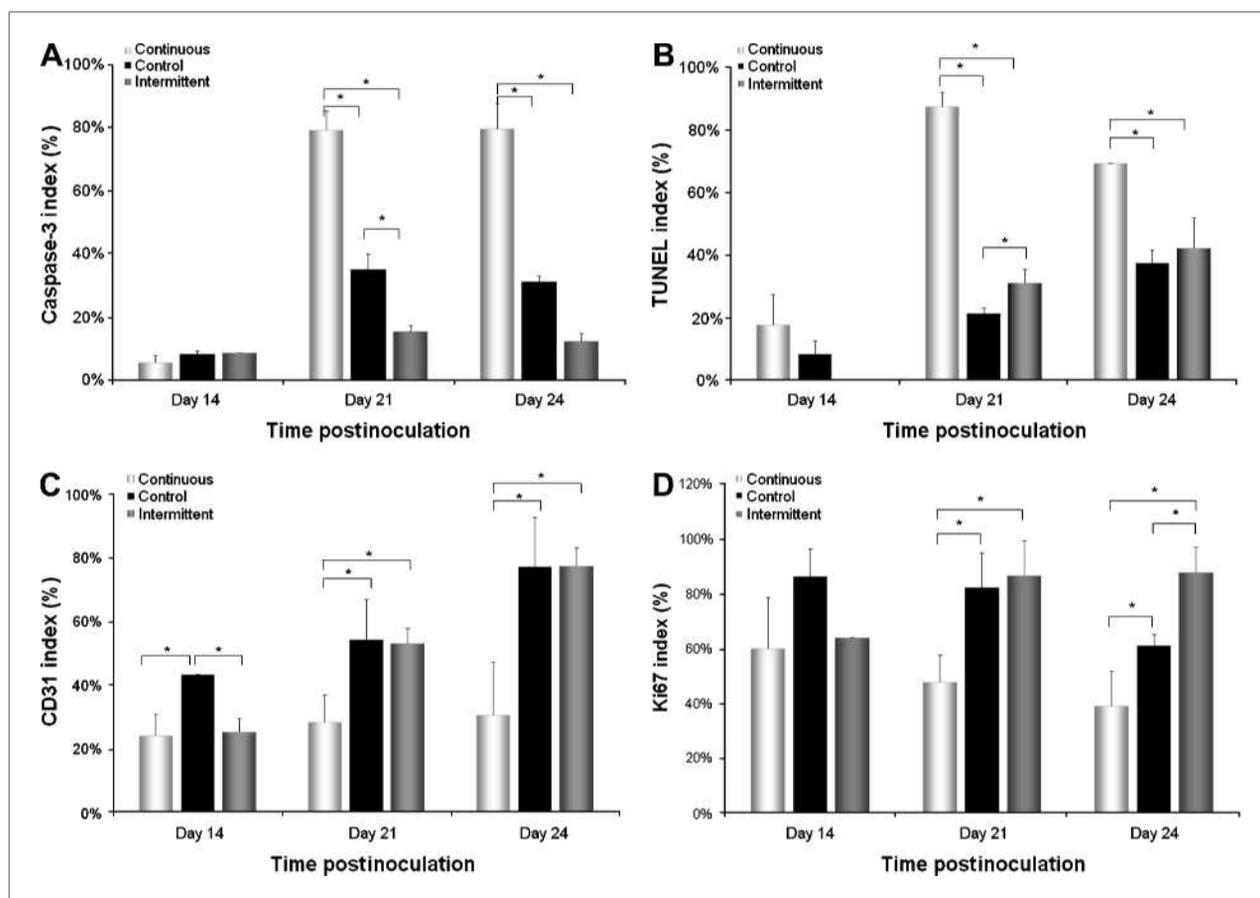


Figure 3. Changes in tumor cell death, proliferation, and angiogenesis over time following continuous or intermittent DTX therapy, illustrated by indices of caspase-3 (A), TUNEL (B), CD31 (C), and Ki67 (D). *, differences among groups ($P < 0.05$). Columns, mean ($n = 4$ /group); bars, SEM.

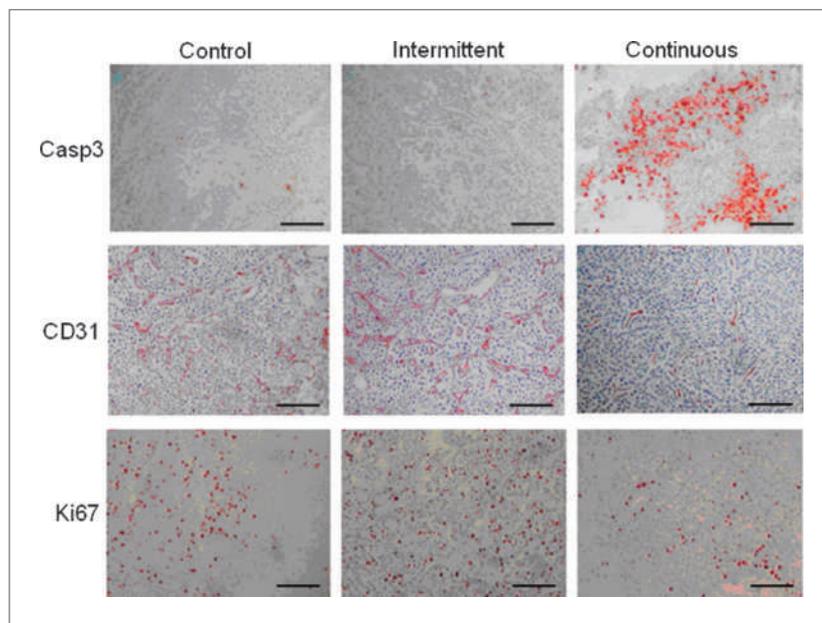


Figure 4. Representative tumor sections following immunohistochemistry, illustrating the effects of intermittent and continuous DTX treatment on tumor apoptosis (Casp3), angiogenesis (CD31), and proliferation (Ki67) on day 24 postinoculation (x100 magnification; scale bars, 10 μ m).

from mice treated with continuous (DTX-PoLigel) and intermittent DTX. Differences in these factors between continuous and intermittently treated mice become apparent on day 21 (Fig. 3), which is also when tumor progression curves diverge significantly (Fig. 2).

Tumor cell apoptosis, measured by caspase-3 activity, was five times greater on day 21 and six times greater on day 24 due to continuous therapy compared with intermittent therapy (Figs. 3A and 4). In fact, intermittent DTX led to 2 to 2.5 times less caspase-3 activity than no treatment on days 21 and 24, which may suggest the development of drug resistance. TUNEL analysis revealed slightly greater cell death in intermittently treated tumors on day 21 when compared with nontreated controls; however, no difference was detected on day 24 (Fig. 3B). Continuous DTX leads to 2.8 and 1.5 times greater cell death than intermittent DTX on days 21 and 24, respectively, although differences are not as pronounced as seen with apoptosis. The TUNEL assay does not distinguish between necrotic and apoptotic cells (26); hence, these results suggest that intermittent DTX may lead to a greater number of necrotic cells than apoptotic ones, although both caspase-3 and TUNEL analyses indicate greater cell death as a result of continuous chemotherapy.

Indices of the endothelial cell marker CD31 were measured to assess angiogenesis, which did not increase as a result of continuous chemotherapy. Intermittent DTX caused a considerable increase in angiogenesis from day 21 to day 24 (Figs. 3C and 4), and at those times, levels were identical to controls, indicating that intermittent therapy has no effect on angiogenesis inhibition. Tumor cell proliferation, quantified by Ki67 activity, decreases during continuous DTX exposure and progressively increases with intermittent therapy, surpassing non-

treated controls 1.5-fold by day 24 (Figs. 3D and 4). This increase in tumor cell proliferation as a result of intermittent therapy was further explored *in vitro*.

***In vitro* cell death and proliferation during continuous and intermittent DTX exposure**

Once changes to tumor biology after each treatment cycle were determined, we sought to understand changes in cell death and proliferation between treatment cycles *in vitro* by performing clonogenic assays immediately before and after each treatment cycle. Striking differences in SKOV3-luc cell proliferation resulted from the two treatment approaches (Fig. 5A). Cells treated with continuous DTX lost their clonogenic potential within the first treatment cycle. Intermittent DTX treatment led to a decrease in the number of colony-forming SKOV3-luc cells immediately after drug exposure (i.e., day 1); however, cells quickly recovered and the number of clonogenic cells approached pretreatment levels on day 3. Proliferation was reduced again upon drug exposure and accelerated again during the treatment-free period. Accelerated proliferation after each treatment was evident in every cycle. In contrast, the effects of intermittent and continuous DTX on ID8 cell proliferation were identical (Fig. 5B). The number of colony-forming ID8 cells decreased steadily upon both treatment strategies, and proliferative potential was lost after two treatment cycles.

In vitro cell death was examined within one treatment cycle. Continuous DTX treatment resulted in a steady decline in SKOV3-luc cell viability over time (data not shown). Intermittent treatment induced the same amount of cell death as continuous treatment on day 1, immediately after intermittent drug exposure. However, during the treatment-free period, cell viability increased following intermittent DTX exposure. At the end of the treatment

cycle, continuous DTX exposure resulted in 37% greater cell death than intermittent exposure. Similar to results obtained from *in vitro* proliferation studies, no significant difference in ID8 cell death resulted from the two treatment strategies (data not shown). In addition, there was no increase in cell death over time due to intermittent exposure, and only a slight decrease in cell viability was observed on day 3 of continuous therapy.

ID8 ascites inhibition during continuous and intermittent i.p. DTX therapy

In addition to studies in the SKOV3-luc xenograft model, the antitumor efficacy of continuous and intermittent i.p. DTX therapy was also evaluated *in vivo* using the ID8 syngeneic murine ovarian cancer model in C57Bl6 mice. Three main differences exist from the SKOV3-luc model: (a) ID8 is a slower growing, more chemosensitive cell line; (b) C57Bl6 host mice have an intact immune

system; and (c) ID8 is a tumor model characterized by large amounts of ascites fluid formation, whereas SKOV3 cells mainly form large solid tumors. Disease progression was assessed throughout the treatment period by abdominal girth measurements and ascites fluid volume, established methods for assessing efficacy in tumor models characterized by ascites fluid accumulation (27). As described above, *in vitro* experiments showed no difference between the two different strategies in ID8 cell death and proliferation. To our surprise, continuous DTX therapy significantly deterred ascites fluid formation as assessed by abdominal girth, which became evident during the second treatment cycle, whereas intermittent therapy showed no efficacy (Fig. 6A). At the end of the 35-day study period, or 21 days posttreatment initiation, the volume of ascites fluid in mice treated with continuous DTX was reduced by >15 times compared with non-treated mice and 10 times compared with intermittently

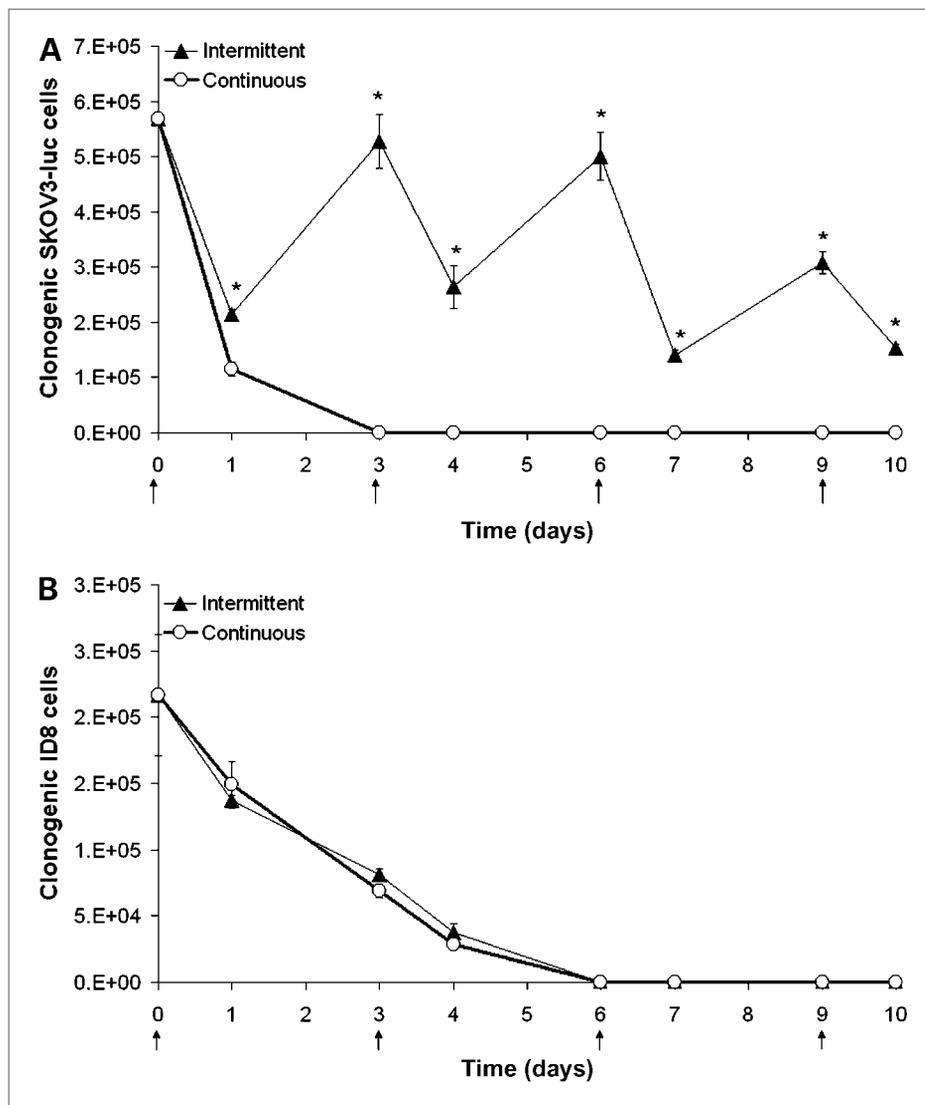


Figure 5. Changes in SKOV3-luc (A) and ID8 (B) cell proliferation during intermittent and continuous DTX therapy. *, differences ($P < 0.05$). Arrows, 12-h intermittent DTX administration. Points, mean ($n = 3$); bars, SEM.

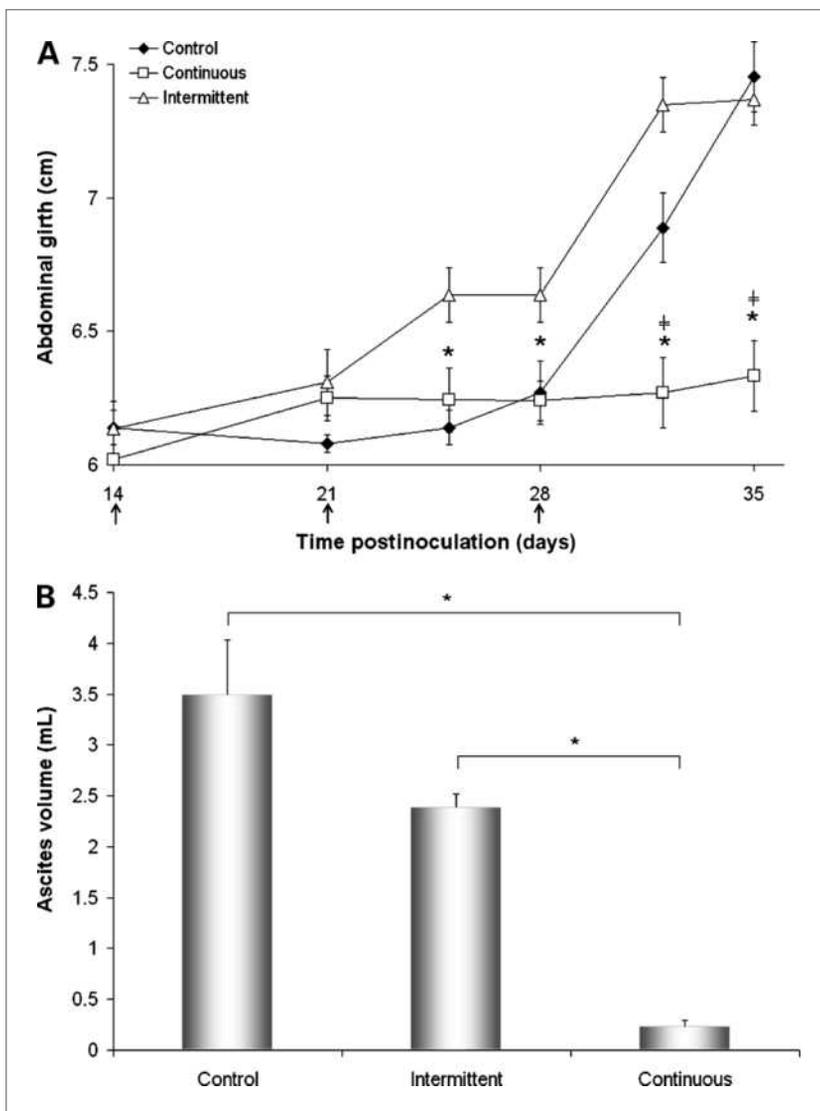


Figure 6. *In vivo* efficacy of continuous and intermittent i.p. DTX in the ID8 syngeneic ovarian cancer model assessed by (A) abdominal growth and (B) postmortem ascites volume. A, continuous therapy: DTX-PoLi_{gel} on day 14 (32 mg/kg); intermittent therapy: weekly Taxotere (8 mg/kg; arrows). *, differences between treatments ($P < 0.05$). ‡, differences between control and intermittent treatment ($P < 0.05$). B, ascites volume on day 35. *, differences between treatments ($P < 0.001$). Columns, mean ($n = 10$ /group); bars, SEM.

treated mice (Fig. 6B). Although there is an apparent decrease in ascites volume due to intermittent therapy when compared with no treatment, this difference is not statistically significant. Mice treated with intermittent DTX and nontreated control mice exhibited anemia during the last treatment cycle, whereas mice treated continuously did not exhibit this condition.

Discussion

Intermittent chemotherapy at the MTD, the approach currently used in the treatment of advanced ovarian cancer, has been highly unsuccessful. Studies have shown the benefits of shortening treatment-free periods in between cycles of chemotherapy by administering lower doses more frequently in a regimen termed metronomic therapy. Preclinical models and clinical trials have shown that metronomic administration of cytotoxic agents inhi-

bits tumor growth to a greater extent than intermittent regimens administered at the MTD followed by long treatment-free periods (23, 28–31). For the first time, we have completely eliminated treatment-free periods by administering DTX continuously through an injectable polymer-lipid implant formulation. We show here that continuous DTX is therapeutically beneficial over intermittent administration and is efficacious against SKOV3-luc and ID8 ovarian cancer models *in vitro* and *in vivo*, with a dose-dependent decrease in tumor burden observed with increasing doses. During all animal studies, no signs of drug-related toxicities were observed, including peritonitis. Antitumor efficacy between intermittent and continuous DTX therapy was assessed in mice bearing SKOV3-luc xenografts *in situ* through bioluminescence imaging. Mice in the continuous treatment group experienced a slight tumor growth during the first 3 days of therapy, after which tumor volume remained

stable, whereas tumor growth in intermittently treated and nontreated animals increased gradually. Our results show that continuous DTX administration led to a 7-fold greater tumor inhibition than intermittent DTX.

Frequent low doses of cytotoxic agents are believed to exert their antitumor activity primarily by inhibiting the formation of new vasculature in the tumor microenvironment (31). Angiogenesis promotes the recovery of tumors following cytotoxic attacks, and it provides adequate nutritional and oxygen supply essential for tumor proliferation and metastasis (32). In fact, the angiogenic potential of a tumor is directly correlated with poor prognosis. Metronomic taxane administration has been shown to inhibit neoangiogenesis while normalizing preexisting vessels to reduce leakiness, and DTX administered in this manner has shown particularly high potency (33, 34). Similarly, our CD31 labeling results indicate that little or no neoangiogenesis occurred in tumors treated continuously, whereas intermittent treatment led to a steady increase in CD31 labeling that was at par with levels in nontreated controls. This may have been due not only to the intermittent MTD regimen but also due to the vehicle of Taxotere, polysorbate 80, which has been shown to nullify the antiangiogenic activity of DTX (35).

Our results show that continuous DTX therapy affects the tumor microenvironment by targeting vascular endothelial cells, as metronomic DTX does, and also targets tumor cells by inducing high levels of apoptosis and maintaining proliferation low. These could be direct effects on tumor cells, collateral effects caused by the lack of angiogenesis progression, which deprives tumors of proper nutrition and oxygenation, or a combination of both. Overall, DTX administered continuously leads to greater SKOV3-luc cell death both *in vitro* and *in vivo* than intermittent DTX. *In vivo*, we have shown that continuous administration of DTX induces considerably higher levels of apoptosis on days 21 and 24 than high intermittent doses, as shown by caspase-3 activity. Interestingly, tumors of nontreated mice showed a greater apoptotic index than intermittently treated tumors, which may suggest the development of DTX resistance during intermittent therapy, a possibility that will be addressed in future studies. Apoptosis is beneficial for tumor volume reduction and aids drug penetration into tumors by decreasing tumor cell density through cell shrinkage, which expands the tumor interstitium (36). In fact, taxane penetration into tumors is dependent on the duration of drug exposure (37). As with caspase-3, TUNEL labeling was higher in continuously treated tumors on days 21 and 24 than in intermittently treated tumors, although TUNEL labeling was higher than caspase-3 labeling due to intermittent treatment. Studies have shown that DNA fragmentation, the phenomenon detected by TUNEL labeling, is not always specific to apoptosis and happens at early stages of necrosis (38). Therefore, because caspase-3 activity levels are very low in intermittently treated tumors, the slightly higher labeling by TUNEL may indicate necrosis. In support of this,

cytotoxic drugs have been shown to induce cancer cell apoptosis if administered at low doses but induce necrosis at high doses (36, 39).

In addition to the benefits of long drug exposure provided by our treatment regimen, cytotoxic agents more effectively target tumor cells if the treatment-free period is reduced or, in this case, eliminated altogether (11). In chemotherapy and radiotherapy, local tumor control and survival are significantly improved when the total dose is given in a short period of time, thereby decreasing the length of treatment-free periods (11, 13, 40, 41). Cytotoxic doses are achieved immediately upon administration; however, levels decrease rapidly within a few hours, allowing surviving cells to reenter the cell cycle, proliferate, and repopulate the tumor (12, 42). The rate of tumor cell proliferation has been shown to accelerate upon each treatment cycle (11, 12, 41–43). Fittingly, we show that intermittent DTX results in greater tumor proliferation than continuous DTX and nontreated controls, suggesting accelerated proliferation as a result of intermittent dosing. Closer examination *in vitro* revealed that at every treatment cycle, the number of colony-forming SKOV3-luc cells decreases immediately upon intermittent dosing, followed by a sharp increase that is only slowed by another intermittent dose. Continuous DTX completely eliminates the clonogenic ability of SKOV3-luc cells within the first treatment cycle.

Interestingly, *in vitro* studies of cell death and proliferation in ID8 cells revealed no differences between intermittent and continuous DTX exposure. This is likely due to the intrinsic differences between ID8 and SKOV3-luc cells. ID8 cells are derived from the mouse ovary epithelium and were immortalized *in vitro*. Thus, these cells were not exposed to any treatment. SKOV3 cells are derived from a previously treated patient, and they lack p53 activity and are, therefore, more chemoresistant (11). The IC₅₀ of DTX in ID8 cells is over 100 times lower than in SKOV3 cells. Our results suggest that the benefits of continuous chemotherapy in affecting tumor cells directly may be more applicable to more chemoresistant tumors. On the other hand, the effects of the two different treatment regimens on the tumor microenvironment must be considered. *In vivo*, continuous DTX resulted in substantially greater therapeutic benefit over intermittent DTX in mice bearing ID8 xenografts. Because our *in vitro* results indicate no differences in the biology of tumor cells treated with the two approaches, we suggest that differences in efficacy observed *in vivo* are a result of changes to the microenvironment. The ID8 xenograft model is mostly characterized by a large accumulation of ascites fluid. The permeability of vasculature contributes greatly to the formation of ascites fluid, which facilitates the dissemination of malignant cells throughout the peritoneal cavity. The vascular endothelial growth factor plays a major role in increasing the permeability of existing vasculature and has been proven essential for the ascites formation process in ovarian cancer (32). *In vitro* studies have shown the ability of taxanes

to decrease vascular endothelial growth factor expression (43). Our results show that continuous DTX deterred ascites fluid accumulation, whereas intermittent DTX showed no effect in the ID8 xenograft model. It is plausible that although continuous and intermittent DTX induce the same effects on ID8 cells, as shown *in vitro*, the effect on angiogenesis differs greatly. In addition, continuous DTX targets vascular endothelial growth factor to an extent not possible in intermittent therapy, thereby significantly decreasing ascites fluid accumulation. This theory will be addressed in future studies.

In summary, we have shown for the first time that continuous DTX therapy is superiorly efficacious to intermittent therapy in ovarian cancer at the same cumulative DTX dose. In less chemosensitive disease, such as SKOV3-luc xenografts, greater antitumor efficacy by continuous chemotherapy is a result of changes to the tumor cells themselves, leading to slower proliferation and greater cell death, and changes to the tumor microenvironment by inhibiting neoangiogenesis. In more chemosensitive disease, such as ID8 xenografts, continuous and intermittent DTX equally increase tumor cell death and decrease proliferation, although it is likely that continuous DTX affects angiogenesis, since it inhibits ascites fluid accumulation and results in greater efficacy. Future studies will examine the effects of continuous DTX therapy on neoangiogenesis. Because intermittent DTX induced less apoptosis than observed in nontreated tumors, we will also investigate the development of DTX resistance as a result of intermittent and continuous ther-

apy. Overall, our results provide a conceptual framework for eliminating treatment-free periods and increasing tumor drug exposure through continuous chemotherapy in the treatment of ovarian cancer. Clinically, this can be achieved by administering a delivery system such as the one described herein during debulking surgery. Although the present formulation of DTX-PoLi_{gel} releases its drug load over a period of 1 month, material ratios could be modified to further prolong drug release over extended periods.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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