Chemotherapy Dosing Schedule Influences Drug Resistance Development in Ovarian Cancer

Raquel De Souza, Payam Zahedi, Rose M. Badame, Christine Allen, and Micheline Piquette-Miller

Abstract

Drug resistance leads to chemotherapy failure and is responsible for the death of a great majority of patients with metastatic, late-stage ovarian cancer. The present study addressed whether changes in the chemotherapy dosing schedule affect the development, further worsening, or circumvention of drug resistance in chemosensitive and chemoresistant ovarian cancer. Severe combined immunodeficient mice bearing HeyA8 and HeyA8-MDR xenografts were treated with docetaxel intermittently (1×/wk or 3×/wk) or continuously for 21 days. Tumor mRNA expression of genes implicated in docetaxel resistance was measured by quantitative real-time-PCR. Analyzed genes included those encoding for the drug efflux transporters *mdr1* and *mrp7* and for molecules that interfere with or overcome the effects of docetaxel, including *β-tubulinIII*, *actinin4*, *stathmin1*, *bcl2*, *rpn2*, *thoredoxin*, and *akt2*. In both models, continuous docetaxel resulted in greater antitumor efficacy than 1×/wk or 3×/wk dosing and did not induce upregulation of any analyzed genes. Once weekly dosing caused upregulation of various drug resistance–related genes, especially in chemoresistant xenografts. More frequent, 3×/wk dosing diminished this effect, although levels of various genes were higher than for continuous chemotherapy. Drug efflux transporter expression was further examined by Western blotting, confirming that intermittent, but not continuous, docetaxel induced significant upregulation. Overall, our results show that the presence and length of treatment-free intervals contribute to the development of drug resistance. Elimination of these intervals by continuous dosing resulted in superior antitumor efficacy and prevented drug resistance induction in chemosensitive and chemoresistant disease. These results encourage the clinical implementation of continuous chemotherapy to overcome and/or prevent drug resistance in newly diagnosed and recurrent, refractory ovarian cancer. *Mol Cancer Ther; 10(7); 1289–99. ©2011 AACR.*

Introduction

The development of drug resistance is the leading cause of chemotherapy failure in the treatment of cancer (1). Patients initially respond well to chemotherapy; however, cancer cells have significant plasticity (2). Multiple chemotherapy cycles have been shown to select tumor cells that are inherently resistant or that have developed resistance over the course of treatment (3, 4) and, eventually, the disease becomes incurable. Ovarian cancer is a prime example of such a scenario, with drug resistance causing 90% of patient deaths with metastatic late-stage disease (4–6). Because ovarian cancer is asymptomatic until late stages, it is essential to identify strategies to prevent and/or overcome drug resistance to maximize chemotherapy efficacy so that better outcomes can be achieved.

Akin to many solid tumors, ovarian cancer treatment involves debulking surgery followed by cycles of chemotherapy administered in an intermittent fashion (7). Systemic exposure to high doses of chemotherapeutics is followed by a 3- to 4-week treatment-free interval to allow for recovery of healthy tissues (7). It has been suggested that short bursts of chemotherapeutics followed by long intervals impose a selective pressure on cancer cells (4, 8). This approach eliminates only vulnerable cells and favors resistant cells that repopulate the tumor at progressively faster rates after each treatment (9, 10), resulting in the development of drug resistance. We have previously developed an injectable, biocompatible polymer–lipid implant (DTX-PoLi$_{gel}$) capable of continuous, localized delivery of the chemotherapeutic drug docetaxel (Supplementary Fig. S1; refs. 11, 12). We have shown that continuous drug exposure results in greater antitumor efficacy than intermittent administration in ovarian cancer xenografts, due to greater tumor cell kill and reduced proliferation and angiogenesis (13). Interestingly, throughout the course of treatment, we observed that intermittently treated tumors displayed equal or less cell

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apoptosis than nontreated controls, suggesting the development of drug resistance and increased survival abilities. Previous work suggests that prolonging drug exposure or shortening treatment-free intervals has the potential to circumvent the development of, or overcome, drug resistance (14). Thus, the present study sought to investigate whether the lack of development of drug resistance is one of the mechanisms responsible for enhanced efficacy observed with continuous chemotherapy. Clinical trials have shown the survival benefits of shortening the length of intervals between chemotherapy treatments from 3 weeks to 1 week in ovarian cancer patients (15–17). In addition, low-dose prolonged exposure of chemotherapeutics, especially those that are cell-cycle specific such as taxanes, has been shown to increase tumor cell kill (13, 18, 19).

Drug resistance can result from both tumor microenvironmental and molecular factors. Issues such as hypoxic regions, irregular blood flow and supply, the extracellular matrix, high density of cells within a tumor, and high interstitial fluid pressure can create pharmacologic sanctuaries or physical barriers through which chemotherapeutics cannot properly diffuse, leading to limited tumor penetration (2, 3). Alternatively, drug delivery to tumor cells can be hindered by upregulation of cell membrane drug efflux transporters. Members of the adenosine triphosphate–binding cassette (ABC) transporter superfamily have been associated with drug resistance by decreasing the intracellular accumulation of hydrophobic chemotherapeutics (20). Of these, the product of the multidrug resistance 1 (*mdr1*) gene, P-glycoprotein (P-gp), is one of the key molecules leading to cancer multidrug resistance (21). Overexpression of *mdr1* has been shown in refractory ovarian cancer tumor cells (22). P-gp is capable of transporting a variety of substrates ranging in size from 300 to 2,000 Da (20). Cancer cells exhibiting upregulation of P-gp become resistant to multiple structurally and mechanistically unrelated chemotherapeutic agents and are classified as multidrug resistant (21). Because intracellular drug accumulation is the basic requirement for chemotherapeutics to exert their effects, the upregulation of drug efflux transporters that hinder the acquisition of proper intracellular drug levels is of key importance in the development of drug resistance (1, 3). In addition, other molecular changes that interfere with the mechanism of action of a drug, such as modifications in the drug target, evasion of apoptosis, and activation of survival pathways, are also important (3, 23).

Studies described herein address the effects of chemotherapy dosing schedule modifications on development, further worsening, or circumvention of drug resistance in chemosensitive and chemoresistant xenograft models of ovarian cancer, which reflects clinical scenarios of both newly diagnosed and recurrent diseases. This was done by examining changes in the expression of genes that have been implicated in docetaxel or taxane resistance. Tumor cells resistant to docetaxel can exhibit an increased expression of MDR1 and MRP7, the only 2 known ABC transporters capable of transporting this drug (24). The overexpression of *mrp7* has been shown in taxane-resistant patient tumors such as lung cancer (25). Because docetaxel acts by stabilizing microtubules via binding to β-tubulin subunits, the abnormal expression of certain β-tubulin isotypes or proteins such as stathmin1 hinders drug binding to its target (23, 26). The upregulation of genes such as *actinin4*, *akt2*, *thioredoxin*, and *bcl2*, which promote survival or inhibit apoptosis even in the presence of cytotoxic agents, has also been suggested to contribute to taxane or docetaxel resistance (27–30). The expression of these genes was examined in xenografts extracted from severe combined immunodeficient (SCID) mice treated with continuous or intermittent (1×/wk or 3×/wk) docetaxel.

Materials and Methods

Cell lines

The human ovarian cancer cell line HeyA8 and its multidrug-resistant equivalent HeyA8-MDR were purchased from The University of Texas MD Anderson Cancer Center (Houston, TX; no authentication of cell lines was done by the authors). The HeyA8-MDR cell line was established by culturing HeyA8 cells in paclitaxel-containing medium until the cells became resistant to 500 ng/mL paclitaxel (31). Cells were maintained in RPMI medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin (100 units/mL penicillin G and 100 mg/mL streptomycin). Cells were grown in monolayer at 37°C/5% CO_2 and 90% relative humidity.

Xenograft establishment and treatment groups

Animal studies were approved by the University of Toronto Animal Care Committee and adhered to these guidelines and those of the Canadian Animal Care Council. SCID, 6- to 8-week-old, female mice (Charles River) were inoculated i.p. with 1×10^6 HeyA8 or HeyA8-MDR cells suspended in 200 μL sterile PBS (pH 7.6). After 7 days, mice were divided into groups ($n = 6$ per group) for treatment initiation. Continuous therapy consisted of an i.p. injection of DTX-PoLi$_{gel}$ (32 mg/kg), prepared as previously described (11), which releases 8 mg/kg docetaxel weekly. Intermittent therapy consisted of either 1 or 3 i.p. injections of Taxotere (Sanofi Aventis) weekly so that mice in both groups received 8 mg/kg total docetaxel per week. Nontreated controls received a weekly i.p. injection of sterile saline. Mice were monitored daily, and endpoints requiring humane euthanasia included weight loss in excess of 20%, excessive muscle wasting according to the body conditioning scoring system (32), severe abdominal distension, inactivity, and hypothermia. All mice were sacrificed 21 days after treatment initiation, the time at which control mice reached the outlined endpoints. Tumors were weighed for antitumor efficacy evaluation and frozen in liquid

nitrogen for subsequent gene and protein expression analyses.

Quantitative real-time-PCR assessment of drug resistance–related gene mRNA expression

Total mRNA was extracted from tumors using TRIzol reagent (Invitrogen) as per manufacturer's protocol and was quantified using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). After DNase I (Invitrogen) treatment, single-stranded cDNA was synthesized from 2 μg of RNA by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer's instructions. Quantitative real-time-PCR (qRT-PCR) reactions were carried out by incubating 20 ng of cDNA with Power SYBR Green PCR Master Mix (Applied Biosystems) and primers specific for each transcript (Table 1), which were designed using the NCBI Primer Designing Tool. Amplification was done on an Applied Biosystems 7900HT instrument equipped with a 384-well reaction block, using SYBR green chemistry. Samples were run in triplicates. mRNA levels were calculated using a standard curve method, were normalized to those of *cyclophilin*, and are expressed as percentages of nontreated controls.

Western blot analysis of membrane efflux transporter expression

For analysis of membrane transporter expression, membrane protein was extracted by homogenizing 300 mg of tumor tissue in lysis buffer composed of 1× RIPA buffer (Cell Signaling Technology) with 4 μL/mL protease inhibitors (Sigma Aldrich) and 25 μL/mL of 200 mM phenylmethylsulfonylfluoride, which was then centrifuged for 20 minutes at 2,000 × *g*. The resulting pellet was re-homogenized in lysis buffer, followed by another 2,000 × *g* centrifugation for 20 minutes. The total supernatant was centrifuged at 100,000 × *g* for 60 minutes. The membrane protein pellet was washed and resuspended in 0.5× lysis buffer by sonication. Protein samples were quantified using the Bradford assay. Membrane protein (20 μg for P-gp blotting, 10 μg for MRP7 blotting) samples were separated by SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories), which was then blocked with 5% skim milk powder in Tris-buffered saline for 1 hour at room temperature. Membranes were subsequently incubated with C-219 (anti-P-gp; 1:500; Abcam) or M71-3 (anti-MRP7; 1:1,000; Abcam) primary antibodies overnight at 4°C, followed by incubation with anti-mouse horseradish peroxidase–conjugated secondary antibody (1:3,000; Jackson ImmunoResearch Laboratories) and molecular weight markers (1:6,000; Bio-Rad Laboratories). Bands were detected using an ECL Advance Western Blotting Detection kit (GE Healthcare) and visualized using a FluorChem imager (Alpha Innotech). Alpha Ease FC imaging software (Alpha Innotech) was used to quantify bands, which were normalized to AC-15 (anti-β-actin; 1:10,000; Sigma-Aldrich).

Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were done using Statistical Package for the Social Sciences (SPSS; version 16.0). For comparisons between groups, one-way ANOVA was used and multiple

Table 1. Primer sequences used for qRT-PCR

cyclophilin	Forward: 5′-GGAGATGGCACAGGAGGAA-3′
	Reverse: 5′-GCCCGTAGTGCTTCAGCTT-3′
mdr1	Forward: 5′-TGCTCAGACAGGATGTGAGTTG-3′
	Reverse: 5′-AATTACAGCAAGCCTGGAACC-3′
mrp7	Forward: 5′-CATGCAAGCCACGCGGAACG-3′
	Reverse: 5′-AAGCTGGGCTGGTGGAGGGT-3′
β-tubulinIII	Forward: 5′-TGGTGGACCTGGAACCCGGA-3′
	Reverse: 5′-CCGGCCCCACTCTGACCAAA-3′
actinin4	Forward: 5′-GAACGACCGGCAGGGTGAGG-3′
	Reverse: 5′-TCGGTGGTCTCCCGCGACAT-3′
stathmin1	Forward: 5′-GGCCAAAGGAAGCACCAGAGCC-3′
	Reverse: 5′-AGGAGCAAGGCTGGCTTGAGGT-3′
bcl2	Forward: 5′-ACCGGGAGATGTCGCCCCTG-3′
	Reverse: 5′-CAAAGGCATCCCAGCCTCCGT-3′
rpn2	Forward: 5′-AGCCTCGCTGGATCGCCCTT-3′
	Reverse: 5′-GGTACATGCTTTCTTTGCATCTGGCA-3′
thioredoxin	Forward: 5′-GCAGATCGAGAGCAAGACTGCTT-3′
	Reverse: 5′-TTTGCAAGGCCCACACCACG-3′
akt2	Forward: 5′-CACCCGTTCCTCACTGCGCT-3′
	Reverse: 5′-GGGCCCGCTCCTCTGTGAAG-3′

NOTE: Sequences were designed using the NCBI Primer Designing Tool.

comparisons with Bonferroni correction were done upon statistical significance as determined by ANOVA. Significance was assigned at $P < 0.05$.

Results

Impact of treatment-free intervals on the antitumor activity of docetaxel in chemosensitive and chemoresistant ovarian tumors

To determine whether the reduction or elimination of treatment-free intervals improves antitumor efficacy in both chemosensitive and chemoresistant diseases, SCID mice bearing HeyA8 or HeyA8-MDR xenografts were treated with docetaxel 1×/wk, 3×/wk, or continuously for a period of 21 days. The mRNA expression of *mdr1* and *mrp7* in HeyA8-MDR cells is 3.1 and 3.3 times greater than that in HeyA8 cells, respectively (33). In addition, the docetaxel IC_{50} values of HeyA8-MDR cells were shown to be 200 times greater than those in HeyA8 cells (33). The average tumor burden in nontreated mice bearing HeyA8 (1.59 ± 0.11 g) and HeyA8-MDR (1.60 ± 0.15 g) xenografts was significantly greater than in mice treated continuously, 3×/wk, and 1×/wk (HeyA8: 0.10 ± 0.03 g, 0.27 ± 0.04 g, 0.39 ± 0.08 g, respectively; HeyA8-MDR: 0.38 ± 0.03 g, 1.25 ± 0.09 g, 1.05 ± 0.23 g, respectively). In both HeyA8 and HeyA8-MDR models, continuous chemotherapy resulted in greater antitumor efficacy (93.7 ± 2.1% and 76.4 ± 2.0% tumor burden reduction relative to control, respectively) than intermittent therapy (Fig. 1). The presence of treatment-free intervals limited the antitumor efficacy of docetaxel irrespective of interval length, as no significant difference was observed between 1×/wk and 3×/wk treatments in both chemosensitive (75.3 ± 5.3% vs. 73.7 ± 4.0% reduction) and chemoresistant (34.6 ± 14.1% vs. 37.8 ± 3.9% reduction) diseases (Fig. 1).

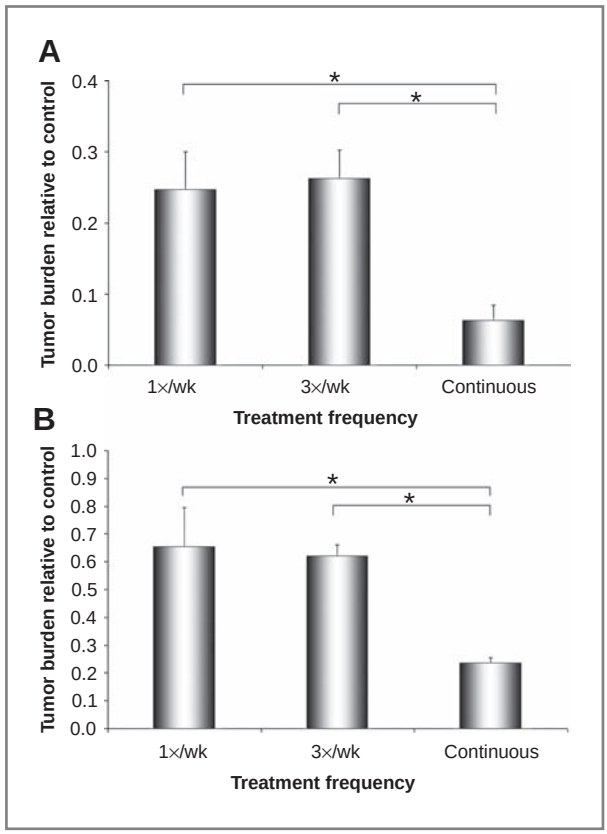

Figure 1. Antitumor efficacy of intermittent (Taxotere 1×/wk or 3×/wk) and continuous (DTX-PoLi$_{gel}$) i.p. docetaxel chemotherapy in HeyA8 (A) and HeyA8-MDR (B) human ovarian cancer xenografts in SCID mice (docetaxel dose in all groups: 8 mg/kg/wk). Data represent mean (SEM) tumor burden on day 21 posttreatment initiation relative to nontreated controls ($n = 6$ per group). *, significant differences between treatment groups ($P < 0.05$). Tumor burden in all treatment groups is significantly lower than in nontreated controls ($P < 0.05$).

Expression of drug resistance–related genes in chemosensitive (HeyA8) tumors following intermittent and continuous docetaxel chemotherapy

To assess the consequences of treatment-free intervals on the development of drug resistance in treatment-naive tumors, the mRNA expression of key docetaxel resistance genes was measured by qRT-PCR in HeyA8 xenografts extracted from mice treated 1×/wk, 3×/wk, or continuously. Intermittent, 1×/wk docetaxel treatment led to significant upregulation of *mdr1* (593.0 ± 170.1% of control), whereas there was no difference in tumors treated 3×/wk, continuously, or no-treatment controls (Fig. 2A). Intermittent treatment 1×/wk also led to upregulation of *mrp7*, *actinin4*, and *bcl2* relative to controls (168.6 ± 32.8%, 255.7 ± 49.8%, and 224.1 ± 15.2%, respectively), and expression levels of *mrp7*, β-*tubulinIII*, *actinin4*, *stathmin1*, and *bcl2* were higher than in tumors treated continuously. As compared with controls, administration of docetaxel 3×/wk induced only upregulation of *bcl2* (182.7 ± 33.1%), although expression levels of *stathmin1* were higher than in tumors treated continuously. Interestingly, mRNA levels resulting from 3×/wk docetaxel treatment were not significantly lower than the levels in tumors treated 1×/wk, with the exception of *actinin4*. Continuous docetaxel did not induce upregulation of any analyzed genes; in fact, a downregulation of *mrp7*, *β-tubulinIII*, and *stathmin1* was observed (63.2 ± 6.8%, 65.6 ± 7.0%, and 54.3 ± 10.6% of control, respectively; Fig. 2B). No change in *rpn2*, *thioredoxin*, and *akt2* resulted from any of the 3 dosing schedules.

Expression of drug resistance–related genes in chemoresistant (HeyA8-MDR) tumors following intermittent and continuous docetaxel chemotherapy

The mRNA expression of docetaxel resistance genes was measured by qRT-PCR in HeyA8-MDR xenografts from mice treated 1×/wk, 3×/wk, or continuously to determine whether the presence and length of treatment-free intervals further aggravate drug resistance in already chemoresistant disease. A remarkable increase of 3,069.9

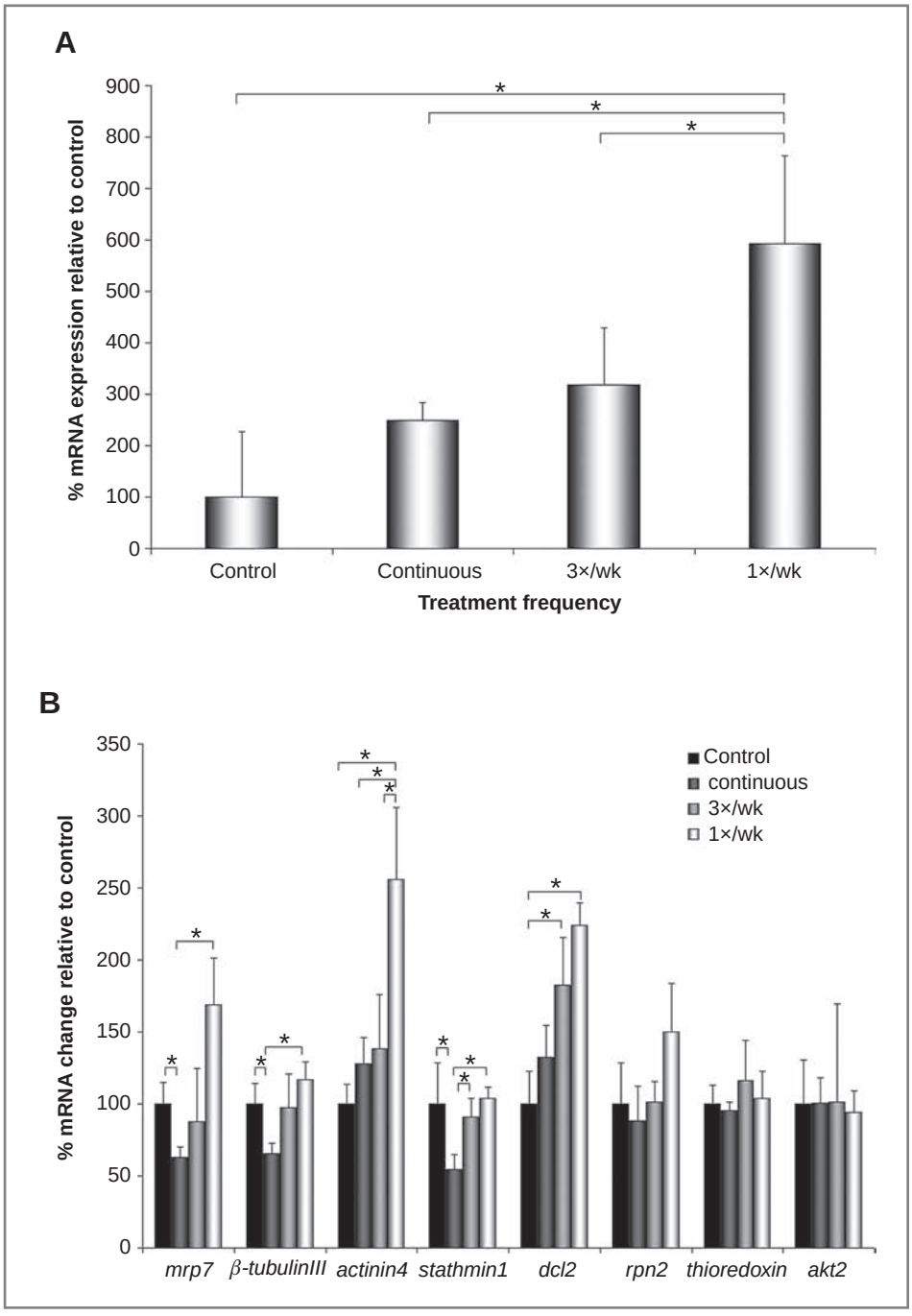

Figure 2. Effect of chemotherapy dosing schedule on mRNA expression of *mdr1* (A) and other genes (B) implicated in docetaxel resistance in HeyA8 xenografts as measured by qRT-PCR. mRNA concentrations were normalized to *cyclophilin*. Data represent mean (SEM) normalized mRNA concentrations as percentages of control values ($n = 4$–6 per group). *, significant differences between treatment groups ($P < 0.05$).

± 922.4% in *mdr1* expression resulted from 1×/wk docetaxel treatment relative to control (Fig. 3A). Intermittent, 1×/wk chemotherapy also induced upregulation of *mrp7*, β-*tubulinIII*, *actinin4*, *rpn2*, *thioredoxin*, and *akt2* (190.5 ± 28.9%, 161.2 ± 22.7%, 148.0 ± 10.3%, 209.8 ± 44.7%, 155.9 ± 28.4%, and 175.6 ± 26.8% of control, respectively). In the case of all genes analyzed, mRNA levels were higher after 1×/wk treatment than after continuous treatment, with the exception of *bcl2*, for which levels did not change irrespective of treatment. A more frequent, 3×/wk treatment schedule also led to upregulation of *mdr1*, with levels 1,575.3 ± 94.3% greater than those in nontreated controls. This dosing schedule resulted in upregulation of β-*tubulinIII* and *rpn2* relative to control (137.5 ± 10.6% and 167.7 ± 17.5%, respectively), which were also higher than levels in continuously treated tumors. Similarly to results obtained from HeyA8 xenografts, mRNA levels resulting from 3×/wk

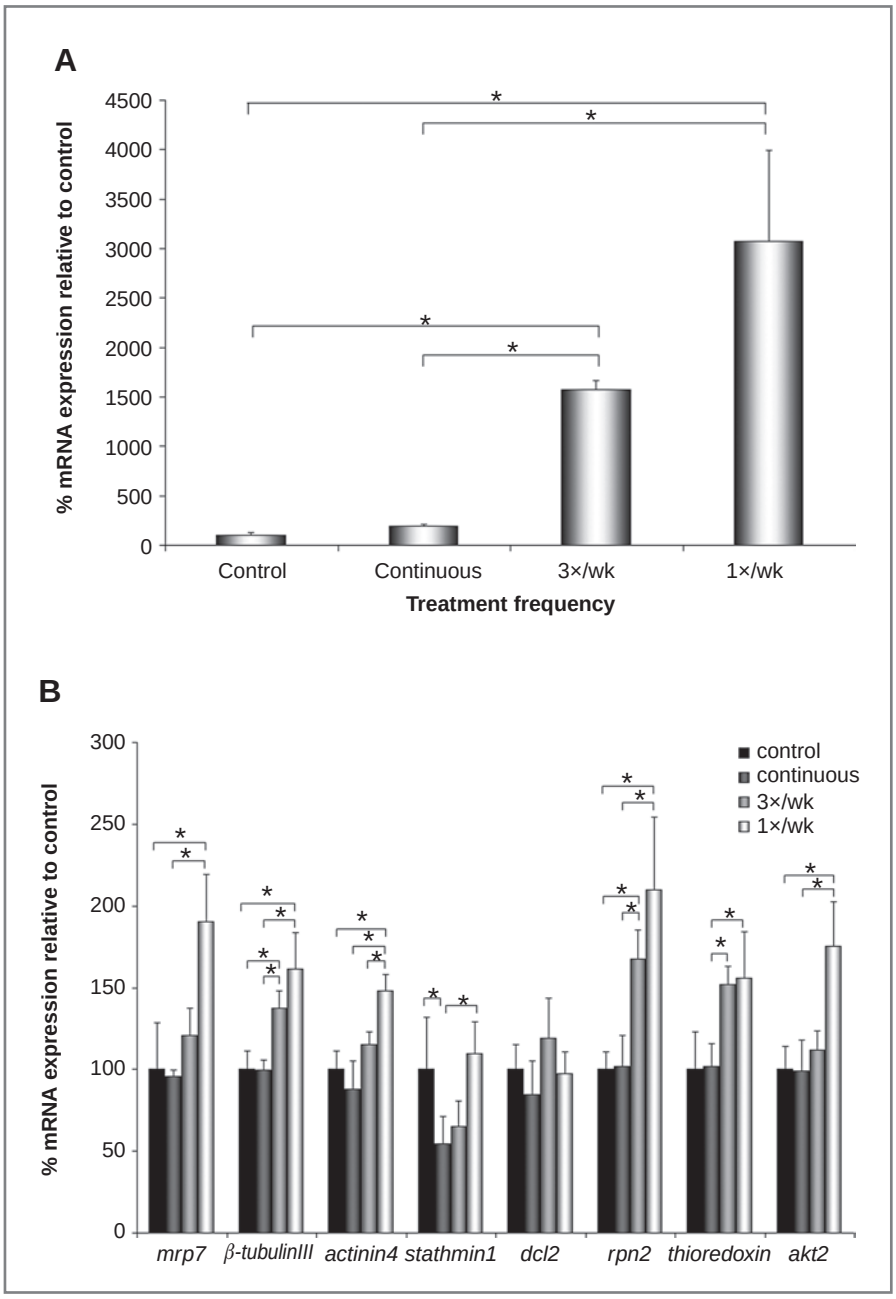

Figure 3. Effect of chemotherapy dosing schedule on mRNA expression of *mdr1* (A) and other genes (B) implicated in docetaxel resistance in HeyA8-MDR xenografts as measured by qRT-PCR. mRNA concentrations were normalized to *cyclophilin*. Data represent mean (SEM) normalized mRNA concentrations as percentages of control values ($n = 4$–6 per group). *, significant differences between treatment groups ($P < 0.05$).

docetaxel treatment were not significantly lower than levels in tumors treated 1×/wk, with the exception of *actinin4*. Relative to nontreated controls, mRNA levels of all genes in tumors of continuously treated mice did not increase whereas *stathmin1* levels actually decreased (54.3 ± 17.1% of control; Fig. 3B).

Transporter expression in HeyA8 and HeyA8-MDR tumors following intermittent and continuous docetaxel chemotherapy

Because the ability of a drug to reach its intracellular target is crucial in determining the fate of cancer cells, the role of drug efflux transporters is of key importance in the development of drug resistance (1, 3). For this reason, the protein levels of P-gp and MRP7 in HeyA8 and HeyA8-MDR tumors were assessed by Western blotting after treatment with continuous or 1×/wk intermittent docetaxel treatment to determine whether protein levels correlate with *mdr1* and *mrp7* mRNA levels. Intermittent, 1×/wk treatment led to upregulation of P-gp in both HeyA8 and HeyA8-MDR tumors (357.7 ± 96.1% and 635.9 ± 246.4% of control, respectively), whereas no induction resulted from continuous therapy (Fig. 4). A similar trend was seen with MRP7, as upregulation was

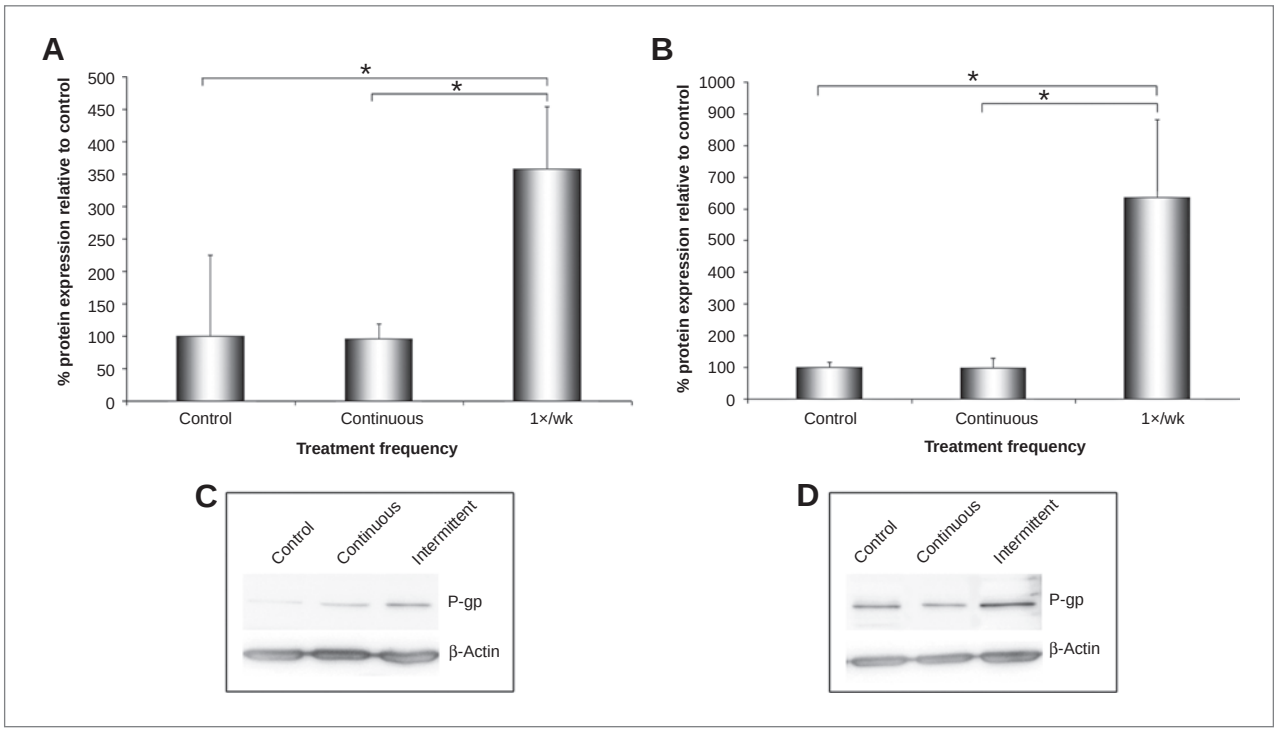

Figure 4. Effect of continuous and intermittent (1×/wk) chemotherapy on P-gp transporter expression in HeyA8 (A and C) and HeyA8-MDR (B and D) tumors as assessed by Western blotting. Protein concentrations were normalized to β-actin. Data represent mean (SEM) normalized P-gp protein concentrations as percentages of control values ($n = 4$–6 per group). *, significant differences between groups ($P < 0.05$).

seen upon intermittent, 1×/wk chemotherapy in HeyA8 and HeyA8-MDR tumors (346.4 ± 100.7% and 265.3 ± 33.9% of control, respectively), and no change was observed upon continuous treatment (Fig. 5).

Discussion

Ovarian cancer is the number one killer of all gynecologic malignancies (34). Current therapeutic approaches have a high failure rate, leading to a low 5-year survival rate of 25% to 35% (35). The development of drug resistance alone is responsible for the great majority of ovarian cancer deaths (6). For this reason, much effort has focused on overcoming drug resistance to achieve better therapeutic outcomes. One strategy is combination chemotherapy with agents that have different mechanisms of action and are structurally distinct; however, tumor cells respond to this approach by becoming multidrug resistant (3). Because P-gp, the product of *mdr1*, has been implicated in multidrug resistance of various cancers (21), another possible approach to overcome resistance is to use P-pg inhibitors to increase intracellular accumulation of chemotherapeutics that are substrates to this transporter (36). Although these agents have shown much promise *in vitro*, nonspecific toxicities due to interference with clearance or metabolism of chemotherapeutics in healthy tissues have thus far limited their clinical utility (36). In this study, it was shown that drug resistance can be prevented and circumvented simply by modifying the schedule of chemotherapy administration.

Previous studies have shown that shortening the treatment-free intervals between chemotherapy treatments can enhance therapeutic efficacy (15–17). We have previously shown that the complete elimination of these intervals through a continuous chemotherapy approach leads to a substantial improvement in ovarian cancer tumor suppression over intermittent administration (13, 37). Similarly, the present study highlights that continuous docetaxel therapy leads to significantly greater antitumor efficacy than intermittent administration in HeyA8 xenografts. The elimination of treatment-free intervals can circumvent drug-resistant disease, as tumor burden in mice bearing HeyA8-MDR xenografts, which has an IC_{50} value for docetaxel 200 times greater than HeyA8 (33), was about 2.5-fold lower than that in mice treated intermittently. This supports previous evidence that a change in treatment schedule can improve efficacy in taxane-refractory disease (14, 38). Surprisingly, shortening treatment-free intervals by administering docetaxel 3×/wk did not improve antitumor efficacy when compared with a 1×/wk treatment schedule in both chemosensitive and chemoresistant tumor models, indicating that complete elimination of these intervals between doses is necessary for perceptible efficacy improvement.

It has been suggested that frequent administration of low-dose chemotherapeutics, such as the metronomic

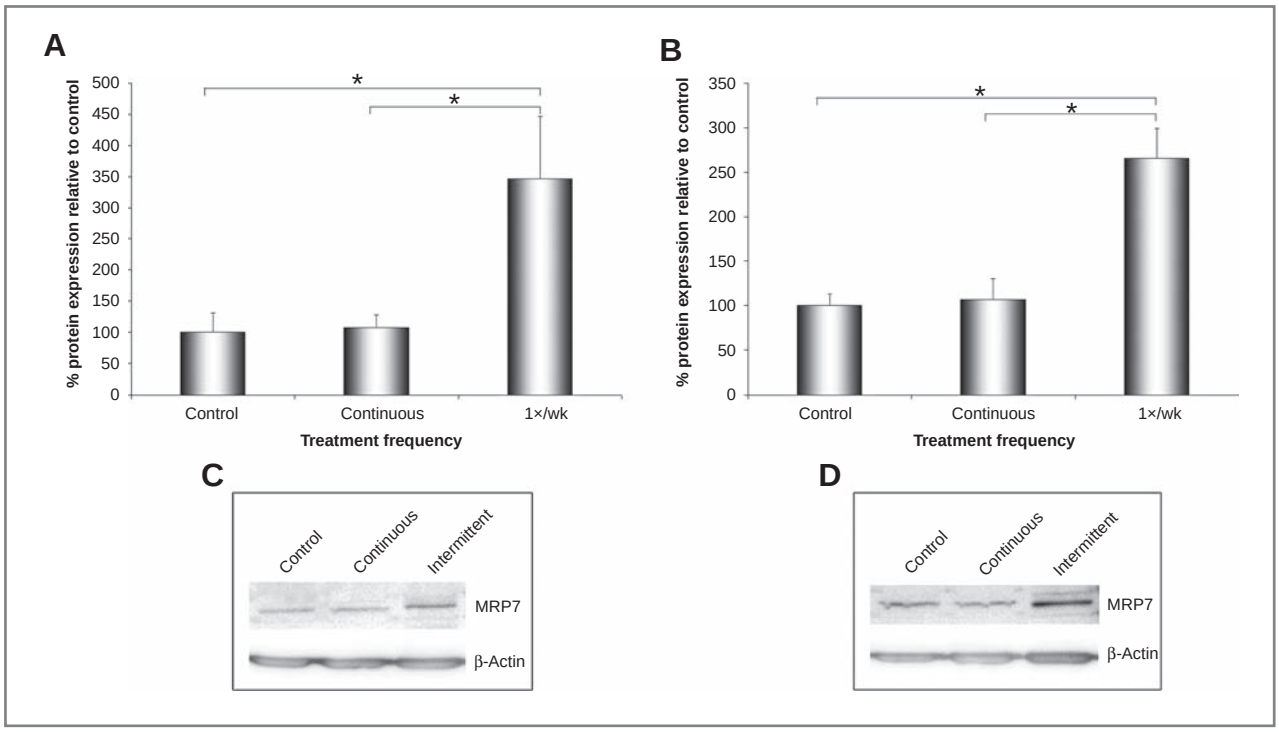

Figure 5. Effect of continuous and intermittent (1×/wk) chemotherapy on MRP7 transporter expression in HeyA8 (A and C) and HeyA8-MDR (B and D) tumors as assessed by Western blotting. Protein concentrations were normalized to β-actin. Data represent mean (SEM) normalized MRP7 protein concentrations as percentages of control values ($n = 4$–6 per group). *, significant differences between groups ($P < 0.05$).

chemotherapy approach, targets mainly endothelial cells rather than cancer cells for an antiangiogenic effect (39). Although we have shown that continuous chemotherapy does greatly decrease tumor angiogenesis (13), significant effects on tumor cells have also been shown, including inhibition of tumor cell repopulation (13, 40). Therefore, a focus on the development of drug resistance upon more frequent chemotherapy is warranted not only in endothelial cells, but especially in tumor cells, which are highly heterogeneous and mutagenic (2). The present study addressed this issue by examining the development of drug resistance upon an intermittent, 1×/wk docetaxel administration schedule, a more frequent, 3×/wk schedule as used in preclinical metronomic chemotherapy studies (41), and a continuous schedule with no treatment-free intervals.

Overall, our results show substantial advantages of continuous chemotherapy in preventing the development of drug resistance in treatment-naive disease and inhibiting an increase in resistance in multidrug-resistant, refractory disease. After 21 days of continuous docetaxel therapy in both HeyA8 and HeyA8-MDR xenografts, no increase in mRNA expression was detected in any of the 9 genes analyzed relative to nontreated controls, including *mdr1*, which has been implicated in multidrug resistance of various solid tumors. On the other hand, intermittent 1×/wk treatment resulted in an almost 6-fold upregulation in *mdr1* mRNA expression in HeyA8 xenografts. This is consistent with previous work that shows upregulation of *mdr1* upon intermittent therapy of ovarian cancer xenografts with paclitaxel (8). This effect was further pronounced in HeyA8-MDR tumors, with a 30-fold increase in *mdr1* mRNA expression relative to control. This highlights the perils of intermittent chemotherapy in recurrent, refractory disease. This treatment strategy shows poor efficacy in recurrent disease (42); however, a greater concern may be the striking increase in *mdr1*, which renders tumor cells resistant to several other chemotherapeutics, significantly hindering good prognosis. Intermittent therapy also led to an increase in *rpn2* in HeyA8-MDR tumors, which contributes to P-gp–mediated docetaxel resistance, as it stabilizes the transporter in the cellular membrane via glycosylation (43). Knockdown of *rpn2* by siRNA leads to reduced P-gp glycosylation, greater docetaxel retention within cancer cells, and higher drug sensitivity (43). The *mrp7* gene, upregulated in both HeyA8 and HeyA8-MDR tumors, encodes for the only other known ABC transporter, in addition to P-gp, capable of taxane transport (24). MRP7 has the lowest degree of structural resemblance to other MRPs, and its overexpression conferred strongest resistance to docetaxel in a screen of various other anticancer agents (24). Achieving adequate intracellular drug levels is the most important requirement for therapeutic efficacy; thus, hindering this by overexpression of efflux transporters contributes greatly to drug resistance of cancer cells (1, 3). On both mRNA and protein levels, intermittent 1×/wk chemotherapy led to an upregulation

of P-gp and MRP7 efflux transporters whereas continuous chemotherapy had no effect.

Intermittent 1×/wk docetaxel administration also resulted in increased mRNA expression of other genes implicated in docetaxel and taxane resistance. Docetaxel acts by binding to β-tubulin subunits to promote microtubule polymerization, stabilizing them so that depolymerization cannot occur, leading to cell-cycle arrest and subsequent cell death (23). Upregulation of the β-*tubulinIII* isoform, as observed clinically in taxane-resistant ovarian cancer, causes docetaxel resistance by inhibiting taxane-induced microtubule assembly (23). The action of docetaxel on microtubules is also hindered by *stathmin1*, a gene found overexpressed in ovarian tumors (44). *Stathmin1* stimulates microtubule depolymerization and interferes with taxane binding to β-tubulin subunits (26). This process is also obstructed by *thioredoxin* by interfering with the redox regulation of tubulin cysteine residues, which microtubule assembly depends on (29). It has been shown that *thioredoxin* expression increases in tumors after docetaxel treatment (29). In chemoresistant (HeyA8-MDR) tumors, we found that intermittent 1×/wk therapy leads to an upregulation of β-*tubulinIII*, *stathmin1*, and *thioredoxin* relative to controls and/or continuous chemotherapy and an upregulation of β-*tubulinIII* and *stathmin1* was observed in chemosensitive (HeyA8) tumors.

Resistance can also result from increased activity of survival pathways. The serine/threonine kinase Akt2 is a member of the protein kinase AKT/PKB family that is found overexpressed in 40% of epithelial ovarian cancers and the one that most influences cell survival and proliferation in this type of cancer (28). In one study, downregulation of *akt2* by siRNA was sufficient to increase docetaxel sensitivity in cancer cells (45). *akt2* promotes docetaxel resistance either by inactivating or inhibiting the transcription of proapoptotic factors or by increasing transcription of prosurvival genes upon chemotherapeutic exposure (46). The overexpression of *actinin4* has also been shown to increase chemoresistance in ovarian cancer (27). This molecule influences the phosphorylation and activation of *akt2*, indirectly contributing to docetaxel resistance in this manner (27). The antiapoptotic protein Bcl-2 becomes deactivated upon phosphorylation by docetaxel; thus, an overexpression of Bcl-2 would lead to the prevention of apoptosis and drug resistance (47). Relative to controls and/or continuous chemotherapy, we found that intermittent 1×/wk therapy leads to upregulation of *actinin4* and *akt2* in chemoresistant (HeyA8-MDR) tumors and to upregulation of *actinin4* and *bcl2* in chemosensitive (HeyA8) tumors.

Although a decrease in the length of treatment-free intervals did not affect antitumor efficacy, increasing the treatment frequency to 3×/wk slightly reduced the development of drug resistance as compared with 1×/wk. In both chemosensitive HeyA8 and chemoresistant HeyA8-MDR xenografts, levels of docetaxel resistance–related genes upon 3×/wk treatment were between levels resulting from continuous and 1×/wk docetaxel treatment. Nevertheless, drug resistance still ensued from 3×/wk treatment. This was especially the case in HeyA8-MDR xenografts, in which *mdr1* expression was almost 16-fold greater than that in nontreated controls. An increased expression in other genes including β-*tubulinIII*, *rpn2*, and *thioredoxin* was also found in these tumors.

The present and previous works from our group have shown promising potential for the clinical application of continuous chemotherapy. The main concern of continuous drug exposure is toxicity to healthy tissues. However, we have shown a lack of systemic and local toxicity in mice treated in this manner (11). We have previously shown that continuous docetaxel treatment did not cause myelosuppression in mice (48, 49), which is the most significant toxicity caused by docetaxel (50), as counts of red blood cells, platelets, and leukocytes were within normal baseline values (data not shown). Neutropenia, the main adverse event associated with myelosuppression, also did not occur in healthy mice upon continuous docetaxel treatment. While neutrophils were 50% lower than normal baseline values upon intermittent docetaxel treatment, continuous treatment did not cause a decrease in neutrophils, which remained within the normal range (data not shown; refs. 48, 49). This can likely be explained by the fact that very low doses of docetaxel are present at any given time due to continuous drug release, rather than immediate exposure to very high doses, as is the case with intermittent chemotherapy. Although further studies must be conducted, our results to date indicate that continuous chemotherapy does not cause the expected toxicities. The benefits of this treatment strategy compounded with the observed lack of toxicity make the clinical implementation of continuous chemotherapy a realistic goal.

In summary, our results show that the presence and length of treatment-free intervals greatly contribute to the development of drug resistance. Intermittent 1×/wk docetaxel administration led to an upregulation of various genes implicated in docetaxel resistance. Particularly high upregulation was observed for *mdr1* (P-gp), which leads to multidrug resistance and, ultimately, treatment failure (21). This effect was more pronounced in HeyA8-MDR xenografts, which reflect recurrent, refractory disease. Reducing the interval length between each chemotherapy administration diminished the development of drug resistance particularly in HeyA8 tumors, although upregulation of certain docetaxel resistance–related genes still occurred, mainly in HeyA8-MDR tumors, in which *mdr1* was remarkably upregulated. Although the development of drug resistance was reduced upon more frequent administration, docetaxel treatment 1×/wk or 3×/wk yielded the same level of antitumor efficacy in both HeyA8 and HeyA8-MDR xenografts. The complete elimination of treatment-free

intervals resulted in substantially greater antitumor efficacy in both chemosensitive and chemoresistant xenografts and did not induce upregulation of any of the examined genes related to docetaxel resistance. Because the development of drug resistance is a key cause of chemotherapy failure, the results presented herein support the clinical implementation of a continuous chemotherapy approach to prevent the development or increase in drug resistance in solid tumors such as ovarian cancer, ultimately leading to improved therapeutic outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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