Metronomic Chemotherapy Enhances the Efficacy of Antivascular Therapy in Ovarian Cancer

Aparna A. Kamat,[1] Tae Jin Kim,[1,5] Charles N. Landen, Jr.,[1] Chunhua Lu,[1] Liz Y. Han,[1] Yvonne G. Lin,[1] William M. Merritt,[1] Premal H. Thaker,[1] David M. Gershenson,[1] Farideh Z. Bischoff,[4] John V. Heymach,[2] Robert B. Jaffe,[6] Robert L. Coleman,[1] and Anil K. Sood[1,3]

Departments of [1]Gynecologic Oncology, [2]Thoracic/Head and Neck Medical Oncology, and [3]Cancer Biology, The University of Texas M.D. Anderson Cancer Center; [4]Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, Texas; [5]Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Cheil General Hospital, Sungkyunkwan University School of Medicine, Seoul, Korea; and [6]Center for Reproductive Sciences, Department of Obstetrics Gynecology and Reproductive Sciences, University of California, San Francisco, San Francisco, California

Abstract

Metronomic chemotherapy is the frequent administration of low doses of chemotherapeutic agents targeting tumor-associated endothelial cells. We examined the efficacy of metronomic taxanes alone and in combination with AEE788—a dual epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) inhibitor—in an orthotopic mouse model of ovarian cancer. Growth-modulating effects of metronomic and maximum tolerated dose (MTD) regimens on overall survival were tested *in vivo* using both chemotherapy-sensitive (HeyA8 and SKOV3ip1) and chemotherapy-resistant (HeyA8-MDR) models. Treated tumors were stained for microvessel density (CD31), proliferation index (proliferating cell nuclear antigen), and apoptosis (terminal deoxyribonucleotide transferase–mediated nick-end labeling). The cytotoxic effects of MTD and metronomic dosing were tested with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Effects of metronomic regimens on circulating endothelial precursors (CEP) and tumor-specific cell-free DNA levels were assessed. *In vivo*, metronomic docetaxel resulted in significant reduction of tumor growth in the taxane-sensitive cell lines, whereas metronomic docetaxel plus AEE788 had an additive effect resulting in significant prolongation in survival. Combination therapy was effective even in the taxane-resistant model. Metronomic chemotherapy alone and combined with AEE788 resulted in a decrease in the proliferative index and microvessel density of treated tumors, whereas combination therapy increased the apoptotic index ($P < 0.001$). *In vitro*, metronomic taxanes caused endothelial cell toxicity at 10- to 100-fold lower concentrations compared with MTD dosing. Metronomic regimens inhibited mobilization of CEPs ($P < 0.05$) and led to a decrease in cell-free DNA levels ($P < 0.05$). Our results suggest that metronomic taxane chemotherapy with dual EGFR and VEGFR inhibition is highly efficacious and should be considered for future clinical trials. [Cancer Res 2007;67(1):281–8]

Introduction

Ovarian cancer remains the most common cause of death from a gynecologic malignancy, with over 15,000 deaths per year (1). Thus, better treatment strategies are necessary to improve the outcome of this deadly disease. Following cytoreductive surgery, chemotherapy has been the mainstay of ovarian cancer treatment for many years (2). Traditional dosing of chemotherapy is based on short bursts of the maximum tolerated doses (MTD), which targets proliferating tumor cells (3). In an effort to balance toxicity with efficacy, a conventional dosing schedule calls for episodic administration of a cytotoxic drug at or near the MTD, followed by periods of rest to allow normal tissues to recover. However, growing evidence suggests that metronomic delivery or frequent administration of drugs, such as cyclophosphamide, vinblastine, and taxanes, at doses much lower than the MTD, have antiangiogenic properties and may be more effective (4, 5). The rationale for this approach is that shortening the time between cycles prevents effective recovery of the damaged tumor vasculature (4, 5). This implies that activated tumor vascular endothelial cells may be more sensitive to lower doses of chemotherapeutic drugs compared with normal or cancer cells, when exposed in a frequent or continuous manner (5). Thus, metronomic dosing of standard chemotherapeutic agents, particularly in combination with other antivascular agents, could maximize the growth-limiting effects on the tumor vasculature, providing a means to overcome resistance encountered with cytotoxic agents administered using traditional dosing schedules (6). Additionally, metronomic chemotherapeutic regimens deliver substantially lower cumulative doses of cytotoxic agents, thereby decreasing potential side effects and improving patient tolerance (7).

Angiogenesis plays a critical role in the growth and metastatic spread of tumors (8). VEGF is a key angiogenic factor and its effects are mediated by two high-affinity transmembrane tyrosine kinase receptors [vascular endothelial growth factor (VEGF) receptor (VEGFR)-1 and VEGFR-2] that are expressed on vascular endothelium (9–11). Overexpression of VEGF in tumor cells enhances tumor growth and metastasis in several animal models by stimulating vascularization. Therapeutic targeting of angiogenesis is starting to show promise, as shown by the recent Food and Drug Administration approval of an anti-VEGF antibody, bevacizumab (12). The epidermal growth factor (EGF) and its receptor (EGFR) also play a critical role in the progression of ovarian cancer (13, 14). We have recently shown that inhibition of EGFR and VEGFR phosphorylation using AEE788 (Novartis, East

Requests for reprints: Anil K. Sood, Departments of Gynecologic Oncology and Cancer Biology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 1362, Houston, TX 77030. Phone: 713-745-5266; Fax: 713-792-7586; E-mail: asood@mdanderson.org.

doi:10.1158/0008-5472.CAN-06-3282

Hanover, NJ) has potent efficacy in a murine model of ovarian cancer (15). We hypothesized that metronomic chemotherapy will have at least equal therapeutic efficacy compared with MTD dosing, alone and in combination with dual inhibition of EGFR and VEGFR phosphorylation. To test this hypothesis, metronomic taxanes and AEE788 were tested individually and in combination using an orthotopic mouse model of ovarian cancer.

Materials and Methods

Cell lines and culture. The ovarian cancer cell lines HeyA8 and SKOV3ip1 (15) were maintained in RPMI 1640 supplemented with 15% fetal bovine serum (FBS) and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). The SKOV3ip1 variant was derived from ascites arising in a nude mouse given an i.p. injection of SKOV3 cells (15). Additionally, the HeyA8-MDR cell line, a taxane-resistant line generated by sequential exposure to increasing concentrations of paclitaxel, was a kind gift of Dr. Isaiah J. Fidler (Department of Cancer Biology, U.T.M.D. Anderson Cancer Center, Houston, TX), and maintained in the above medium with 300 μg/mL paclitaxel. Endothelial cells isolated from the mesentery or ovary [mouse mammary epithelial cells (MMEC)] of the immortomouse were a kind gift of Dr. Robert Langley (Department of Cancer Biology, U.T.M.D. Anderson Cancer Center, Houston, TX; ref. 16) and were maintained in DMEM with 10% FBS. MMEC cells have the capacity to proliferate indefinitely by means of SV40 transformation at 33°C. However, when switched to 37°C, SV40 expression is turned off and cells proliferate for only a few more cycles. When using these cells, they were kept at 37°C for 48 h before any experiments were done to allow elimination of SV40 expression. Human umbilical vascular endothelial cell (HUVEC) cells were maintained in DMEM with 10% FBS and 10% basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO). All *in vitro* experiments were conducted at 60% to 80% confluence. For *in vivo* injection, cells were trypsinized and centrifuged at 1,000 rpm for 7 min at 4°C, washed twice, and reconstituted in HBSS (Life Technologies, Carlsbad, CA) at a concentration of 5×10^6/mL (SKOV3ip1 and HeyA8 MDR) or 1.25×10^6/mL (HeyA8) in 200 μL i.p. injections.

Cell viability assay. To determine the sensitivity of both ovarian cancer cells and human and murine endothelial cells to paclitaxel and docetaxel, 2,000 cells were plated in each well of a 96-well plate, and experimental conditions were set in triplicate. To assess viability with MTD and metronomic paclitaxel and docetaxel, serum-containing growth medium with varying concentrations of the paclitaxel (0.001–500 nmol/L) or docetaxel (0.001–100 nmol/L) were used. For metronomic treatment, medium containing the drug was changed daily. The number of viable cells was assessed 5 days after drug exposure for HeyA8 and SKOV3ip1 and 9 days for HUVEC and MMEC. To assess viability, 50 μL of 0.15% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) were added to each well. After incubation for 2 h at 37°C, the medium/MTT was removed, and cells were reconstituted in 100 μL DMSO (Sigma). After shaking, the absorbance at 570 nm was recorded using a FALCON microplate reader (Becton Dickinson Labware, Franklin Lakes, NJ). The IC_{50} was determined by calculating the mean absorbance at 570 nm [(max absorbance − min absorbance) / 2 + min absorbance] and finding the paclitaxel and docetaxel concentration at which this absorbance reading intersected with the dose-response curve.

Orthotopic *in vivo* model and tissue collection. Female athymic nude mice were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and housed in specific pathogen-free conditions. They were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the USPHS Policy on Human Care and Use of Laboratory Animals, and all studies were approved and supervised by the M.D. Anderson Cancer Center Institutional Animal Care and Use Committee. To determine the optimal metronomic dose of docetaxel, therapy with different doses was initiated 1 week after tumor cell injection. Mice were treated thrice a week with i.p. injections of the following agents: (*a*) PBS, (*b*) docetaxel (0.5 mg/kg), (*c*) docetaxel (1 mg/kg), (*d*) docetaxel (2.5 mg/kg), or (*e*) docetaxel (5 mg/kg). The 5 mg/kg thrice weekly dose was chosen as the highest dose so that the cumulative metronomic dose would be equal to previously reported MTD for docetaxel (15–20 mg/kg; ref. 17).

For long-term experiments to assess tumor growth, therapy began 1 week after tumor cell injection. Therapy consisted of six groups: (*a*) PBS, (*b*) MTD docetaxel (15 mg/kg every 2 weeks; ref. 17), (*c*) metronomic docetaxel (0.5 mg/kg thrice weekly), (*d*) AEE788 (50 mg/kg p.o. thrice weekly; ref. 15), (*e*) MTD docetaxel plus AEE788, or (*e*) metronomic docetaxel plus AEE788. Mice were monitored for adverse effects, and tumors were harvested after 3 to 4 weeks of therapy. If animals in any group began to seem moribund and required sacrifice, all animals in the experiment were sacrificed together. Mouse weight, tumor weight, and distribution of tumor were recorded. Tissue specimens were fixed in formalin for paraffin embedding and frozen in optimum cutting temperature medium for frozen slide preparation. Additional survival experiments were also done, which were initiated either 1 week or 17 days after tumor cell injection. Mice were treated as described above and individually killed when moribund (unable to move or reach food). The date of death was recorded as the day a mouse was sacrificed.

For assessment of biomarkers, mice with established tumors (17 days after tumor cell injection) were treated with the following regimens: (*a*) MTD docetaxel, (*b*) metronomic docetaxel, (*c*) MTD docetaxel plus AEE788, and (*d*) metronomic docetaxel plus AEE788 for 7 days.

Collection of blood and extraction of plasma. For collection of blood, mice were anesthetized using nembutal (0.1 mL/g of body weight) by i.p. injection. Approximately 1 mL of blood was collected by retro-orbital puncture in tubes containing EDTA as an anticoagulant. Blood was kept at 4°C on ice until 150 μL of blood were analyzed by four-color flow cytometry to quantify circulating endothelial cells (CEC). Next, the remaining blood was subjected to two consecutive centrifugations at $1,200 \times g$ for 10 min each at room temperature to extract the plasma and remove the cellular component. Plasma aliquots were stored at −80°C until further use.

Immunohistochemistry for proliferating cell nuclear antigen, CD31, and terminal deoxyribonucleotide transferase–mediated nick-end labeling. For immunohistochemical analysis, paraffin-embedded tissues were sectioned (5-μm-thick) and used to detect expression of proliferating cell nuclear antigen (PCNA) and terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL). Frozen sections were used for detecting CD31. Generally, formalin-fixed, paraffin-embedded sections were deparaffinized by sequential washing with xylene, 100% ethanol, 95% ethanol, 80% ethanol, and PBS. After antigen retrieval, endogenous peroxide was blocked with 3% H_2O_2 in methanol for 5 min. After PBS washes, slides were blocked with 5% normal horse serum and 1% normal goat serum in PBS for 15 min at room temperature, followed by incubation with primary antibody in blocking solution overnight at 4°C. Next, the appropriate secondary antibody conjugated to horseradish peroxidase (HRP) in blocking solution was added for 1 h at room temperature. HRP was detected with 3,3′-diaminobenzidine (DAB; Phoenix Biotechnologies, Huntsville, AL) substrate for 5 min, washed, and counterstained with Gil no. 3 hematoxylin (Sigma). Primary antibodies used included anti-CD31 (platelet/endothelial cell adhesion molecule 1, rat IgG; PharMingen, San Diego, CA) and anti-PCNA (PC-10, mouse IgG; DAKO, Carpinteria, CA), using the appropriate secondary HRP-conjugated antibody; followed by development with DAB (no biotin/streptavidin system was needed for these antigens). Antigen retrieval for studies on paraffin-embedded slides was done by microwave heating for 5 min in 0.1 mol/L citrate buffer (pH 6.0; for PCNA). After endogenous peroxidase block, these slides were incubated with 0.13 μg/mL mouse IgG Fc blocker (The Jackson Laboratory, Bar Harbor, ME) for 2 h before primary antibody incubation. Immunohistochemistry for CD31 was done on freshly cut frozen tissue. These slides were fixed in cold acetone for 10 min and did not require antigen retrieval. For TUNEL staining, after deparaffinizing, samples were treated with proteinase K (1:500 dilution) and rinsed with distilled water. One set of slides was treated with DNase 1:50 dilution as a positive control. Samples were incubated with terminal 1:400 and biotin-16-dUTP 1:200 in terminal deoxynucleotidyl transferase buffer at 37°C for 1 h and then incubated with 2% bovine serum albumin/normal horse serum in double-distilled water. Samples were incubated with peroxidase streptavidin 1:400 in house detection diluent at

37°C for 40 min. A positive reaction was indicated by a reddish-brown precipitate in the nucleus. Images were captured with the use of a three-chip camera (Sony Corporation of America, Montvale, NJ) and Optimas Image Analysis software (Bioscan, Edmond, WA).

Staining for PCNA, CD31, and TUNEL was conducted on tumors collected at the conclusion of 4-week therapy trials. For quantification of PCNA or TUNEL expression, the number of PCNA- or TUNEL-positive and total tumor cells were counted in 10 random fields at ×100 magnification. To quantify microvessel density, microvessel-like structures consisting of endothelial cells that were stained with the anti-CD31 antibody were counted in similar fields.

Flow cytometry analysis of CECs. CECs in peripheral blood were evaluated using three- or four-color flow cytometry as previously described (18, 19). Red cell lysis was done using FACSLyse solution (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. The following directly conjugated antibodies were used to detect CECs and circulating endothelial precursors (CEP) in murine peripheral blood: anti-mouse CD45-PerCP, Flk-1-PE (mouse VEGFR-2), CD31-APC (platelet/endothelial adhesion molecule-1), and CD117-FITC (c-kit receptor; all from BD Biosciences). Flow cytometry was done using a FACSCalibur flow cytometer and acquired data were analyzed with FLOWJO flow cytometry analysis software (Treestar, Ashland, OR). Analysis gates were designed to remove any residual platelets and cellular debris. Between 50,000 and 100,000 events were typically counted for each animal. MS-1 cells, a transformed murine endothelial cell line, was added to the blood samples as a positive control.

For comparison of CECs, the number of mature CECs or CEPs detected for each mouse were expressed as a percentage of peripheral blood mononuclear cells detected for that mouse. Mean CEC and CEP values per microliter of blood for each treatment group were then calculated and compared with control mice.

Plasma DNA extraction and quantitative real-time PCR. Plasma DNA was extracted from 200 μL of plasma using the QIAamp Blood Mini kit (Qiagen) in accordance with the manufacturer's instructions. Carrier RNA (5 μL) was added to each plasma sample before DNA extraction. DNA was eluted in 25 μL AE buffer and stored at −20°C until further use. Quantification of tumor-specific plasma DNA was done as previously described (20). A standard curve consisting of known concentrations of human genomic DNA was created to quantify the number of genome equivalents (GE) per milliliter in each of the unknown samples. The assay was linear at least over 5 orders of magnitude. Primers directed toward human *β-actin* gene with no cross-reaction with murine sequences were used (Applied Biosystems, Foster City, CA). The DNA concentration, expressed in copies or GE/mL, was calculated with the following equation (20, 21):

$$C = Q \times (V_{\mathrm{DNA}}/V_{\mathrm{PCR}}) \times (1/V_{\mathrm{ext}})$$

where C is the target concentration in plasma (GE/mL), Q is the target quantity (copies) by the ABI Prism 7700 Sequence Detection System (Applied Biosystems), V_{DNA} is the total volume of DNA extraction (50 μL), V_{PCR} is the volume of DNA used per PCR reaction (5 μL), and V_{ext} is the volume of plasma used to extract DNA (200 μL). All reactions were done in triplicate and values represent the mean of these determinations.

Statistics. Continuous variables were compared with the Student's t test (between two groups) or ANOVA (for all groups) if normally distributed, and the Mann-Whitney rank-sum test or Kruskal-Wallis test (for all groups) if nonparametric. Pairwise differences in normally distributed variables in our treatment groups were compared by the Tukey-Kramer statistic for multiple comparisons. A Bonferoni adjustment to α (default value 0.05) was made based on the number of pairwise comparisons within a treatment experiment using the formula: $a(\alpha) = 0.05/k$, where k = number of comparisons against control. For *in vivo* therapy experiments, 10 mice in each group were used, as directed by a power analysis to detect a 50% reduction in tumor size (β-error 0.2). Survival curves were plotted by the method of Kaplan and Meier and tested for survival differences with the log-rank statistic. A $P < 0.05$ on two-tailed testing was considered significant.

Results

Determining the optimal metronomic dose for docetaxel. Due to a paucity of data regarding the optimal *in vivo* metronomic dose for docetaxel, we first did dose-finding experiments. Female nude mice (n = 10 in each group) injected with HeyA8 cells i.p. were treated with either vehicle or docetaxel at doses ranging from 0.5 to 5 mg/kg i.p. thrice weekly, starting at 1 week after tumor cell injection. All metronomic doses of docetaxel were highly effective in reducing tumor growth (Fig. 1). The lowest dose of metronomic docetaxel (0.5 mg/kg) resulted in an 87% reduction in mean tumor weight compared with controls ($P < 0.05$). Doses lower than 0.5 mg/kg were not effective (data not shown). Therefore, we selected 0.5 mg/kg docetaxel thrice weekly as the optimal metronomic dose, which was used for all subsequent experiments.

Long-term therapy with metronomic docetaxel. To determine the therapeutic efficacy of metronomic docetaxel alone and in combination with AEE788, we initiated therapy 1 week after tumor cell injection according to the following six groups (10 mice per group): (*a*) PBS, thrice weekly; (*b*) MTD docetaxel 15 mg/kg, every 2 weeks; (*c*) metronomic docetaxel 0.5 mg/kg thrice weekly; (*d*) AEE788 50 mg/kg by p.o. gavage thrice weekly; (*e*) MTD docetaxel plus AEE788; and (*f*) metronomic docetaxel plus AEE788. In the HeyA8 model, both MTD and metronomic docetaxel monotherapy led to a 65% and 76% reduction in tumor growth, respectively, compared with PBS treatment ($P < 0.05$; Fig. 2*A* and *B*). Similar results were observed with AEE788 alone. Combination therapy of MTD docetaxel with AEE788 resulted in an 89% reduction in tumor growth compared with PBS alone

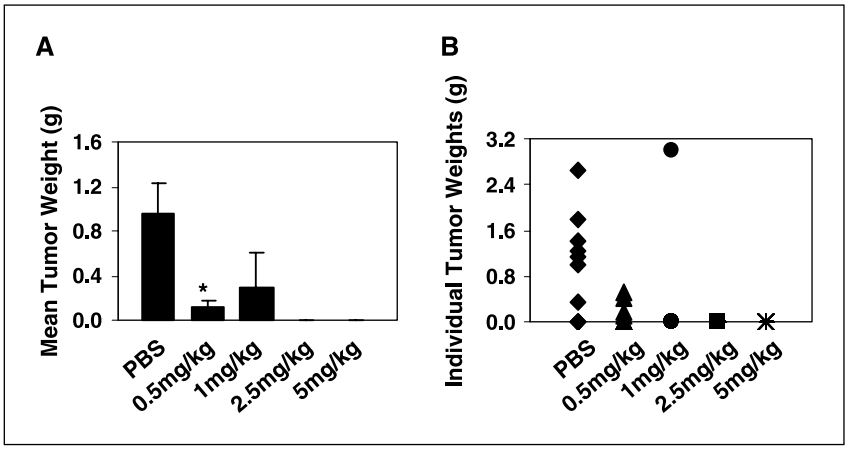

Figure 1. Determination of the optimal metronomic dose of docetaxel. Mice bearing HeyA8 ovarian tumors in the peritoneal cavity were treated with either PBS or metronomic docetaxel (0.5, 1, 2.5, and 5 mg/kg) thrice weekly. *A,* mean tumor weights (g). *B,* distribution of the individual tumor weights (g) in different groups. *, $P < 0.05$.

($P < 0.01$). However, metronomic docetaxel with AEE788 resulted in the greatest efficacy with 96% tumor growth inhibition ($P < 0.001$). Similar results were observed in the SKOV3ip1 model, with the greatest efficacy noted in the metronomic docetaxel plus AEE788 group ($P < 0.001$; Fig. 2*C* and *D*). In both experiments, there was no significant difference in the average mouse weights among the various treatment groups (data not shown).

Despite surgery and chemotherapy, most patients with advanced ovarian cancer eventually develop resistance to conventional chemotherapy. To study the potential efficacy of metronomic dosing in experimental models of chemotherapy resistance, we used the taxane-resistant HeyA8-MDR cell line. As expected, there was no effect on tumor growth with MTD docetaxel in the HeyA8-MDR model. Interestingly, metronomic docetaxel monotherapy resulted in a 57% reduction of tumor growth compared with the PBS group ($P < 0.05$; Fig. 2*E* and *F*). Furthermore, the combination of either MTD ($P < 0.01$) or metronomic docetaxel ($P < 0.001$) with AEE788 was superior to the control arm. Similar effects on tumor inhibition were observed with metronomic paclitaxel therapy (Fig. 2*G* and *H*). In addition, the combination of metronomic paclitaxel and AEE788 was superior to both PBS and metronomic docetaxel alone ($P = 0.02$), with an 80% reduction in tumor growth.

Effect of long-term metronomic therapy on survival in ovarian cancer. Based on these encouraging results with regard to inhibition of *in vivo* tumor growth using metronomic regimens, we next examined the effects of these regimens on survival using the HeyA8 model. Treatment with MTD ($P = 0.03$) and metronomic docetaxel ($P = 0.002$) both significantly prolonged survival, whereas AEE788 alone did not have a significant effect ($P = 0.09$). The most significant effect on survival time was in the combination arm with metronomic docetaxel and AEE788, where survival was increased by at least 60 days ($P < 0.0001$; Fig. 3*A*). After 100 days, the remaining mice were sacrificed. Overall, the difference in survival among the various treatment arms was highly significant (log-rank test, $P < 0.0001$; Fig. 3*A*). There was no significant difference in mean body weight of the mice in the various treatment groups (data not shown), suggesting that treatment was well tolerated.

Patients with recurrent or chemorefractory disease frequently have large tumors at the initiation of therapy. To examine the efficacy of metronomic therapy in the presence of established

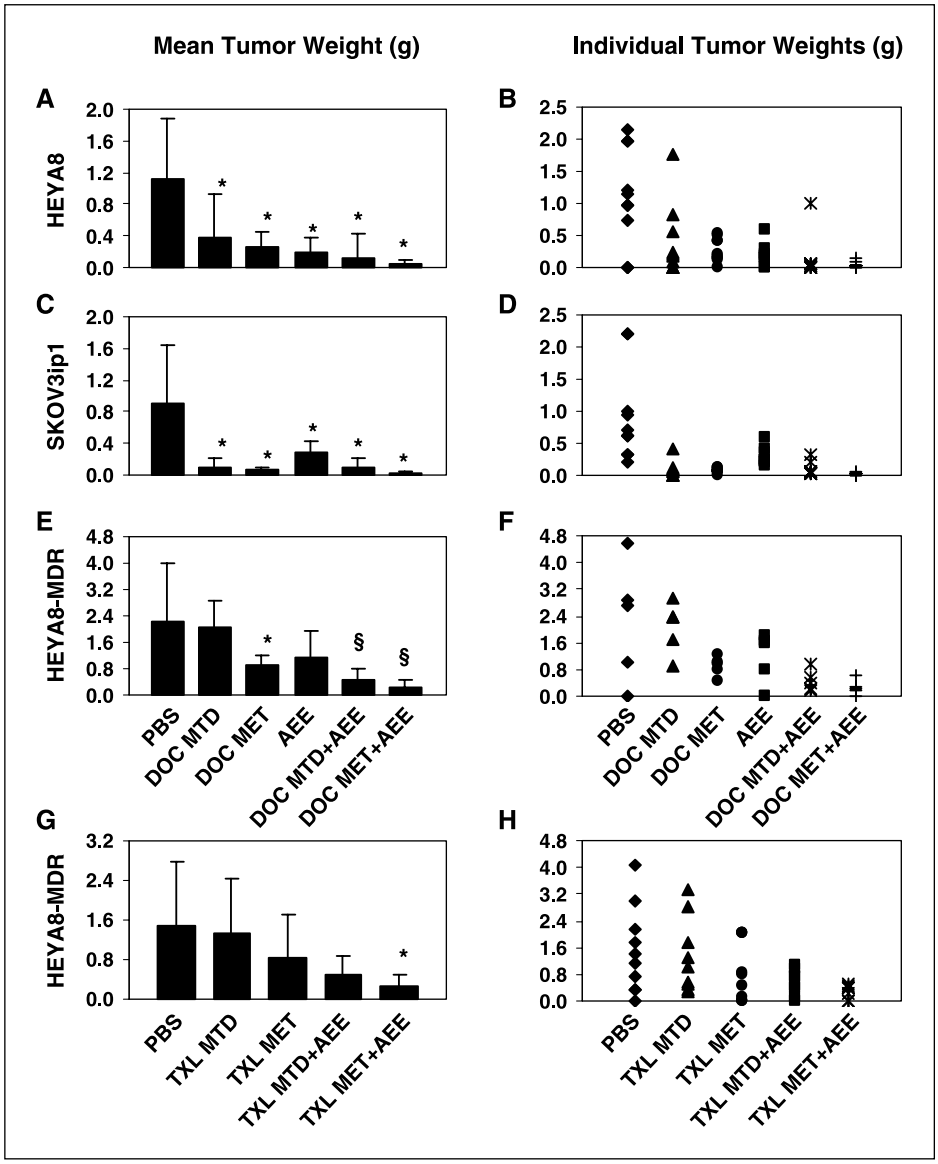

Figure 2. Tumor growth inhibition with MTD and metronomic docetaxel alone and in combination with AEE788. Mice were injected with HeyA8 (*A* and *B*), SKOV3ip1 (*C* and *D*), or HeyA8-MDR (*E* and *F*), and were treated with the MTD and metronomic docetaxel alone and in addition to AEE788 (*AEE*). In the HeyA8-MDR model, another trial with MTD and metronomic paclitaxel (*TXL*) was done (*G* and *H*). When control mice were moribund (4–5 wks after cell injection), animals in all groups were sacrificed, tumors were excised, and tumor weights were recorded. *Columns,* mean weights; *points,* individual weights; *bars,* SD. *, $P < 0.05$ compared with vehicle controls (*A*); *, $P < 0.001$ compared with vehicle controls (*C*); *, $P < 0.05$; §, $P < 0.01$ compared with vehicle controls (*E*); *, $P = 0.02$ compared with vehicle controls (*G*).

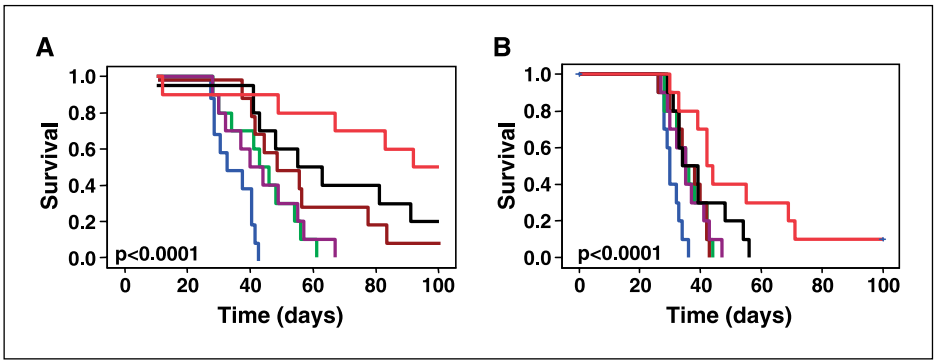

Figure 3. Survival of mice treated with MTD and metronomic docetaxel alone and in combination with AEE788. Nude mice (n = 10 per group) were injected with HeyA8 cells and subsequently randomized into the different treatment regimens. Treatment was continued until mice were individually moribund, and days of life were recorded. Treatment was initiated 7 d (*A*) or 17 d (*B*) after tumor cell injection after development of clinically detectable tumors (by palpation). Survival data were compared for significance with the log-rank statistic. Treatment groups are PBS (*blue line*); MTD docetaxel 15 mg/kg every 2 wks (*green line*); metronomic docetaxel 0.5 mg/kg thrice a week (*brown line*); AEE788 50 mg/kg p.o. thrice weekly (*purple line*); MTD docetaxel plus AEE788 (*black line*); or metronomic docetaxel plus AEE788 (*red line*).

tumors, we conducted a survival experiment with the HeyA8 model where therapy was initiated 17 days after injection (palpable tumors of 0.5–0.75 cm^3). Combination therapy with metronomic docetaxel and AEE788 significantly prolonged survival even in this model (Fig. 3*B*; $P < 0.0001$).

Effect of metronomic chemotherapy on tumor cell and endothelial cell survival *in vitro*. To test whether metronomic administration of taxanes has differential effects on tumor cell and endothelial cell survival compared with MTD dosing, we analyzed both tumor cells (HeyA8 and SKOV3ip1) and endothelial cells (HUVEC and MMEC) *in vitro* using MTT assays. The IC_{50} levels of MTD and metronomically administered paclitaxel and docetaxel are shown in Table 1. Although IC_{50} levels of MTD (4–5 nmol/L) and metronomic taxanes (2–3 nmol/L) were not significantly different in the tumor cells, metronomic treatment with both drugs resulted in endothelial cell toxicity at substantially lower (10- to 100-fold) levels (IC_{50} 0.05–0.1 nmol/L) compared with MTD dosing (IC_{50} 1.6–5 nmol/L) *in vitro*.

Effect of metronomic chemotherapy therapy on proliferation, microvessel density, and apoptosis *in vivo*. To determine the effect of therapy on proliferative index, tumors were subjected to immunohistochemistry for PCNA. Both MTD and metronomic docetaxel alone each significantly reduced proliferation ($P < 0.001$ for both; Fig. 4). Tumors treated with a combination of AEE788 plus MTD docetaxel ($P < 0.001$) or metronomic docetaxel (proliferation rate 18.8%; $P < 0.001$) had a lower proliferative index compared with controls or chemotherapy-alone groups.

Given the limited effect of metronomic chemotherapy on tumor cell viability *in vitro*, a likely explanation for the efficacy of metronomic dosing on tumor growth is that these effects are secondary to effects on the tumor-associated vasculature (7). To test this hypothesis, microvessel density was counted for each experimental group (Fig. 4). The microvessel density of MTD-treated tumors was similar to controls, but the microvessel density was significantly lower in tumors from the metronomic group (44% reduction compared with PBS; $P < 0.001$). However, the combination of AEE788 with metronomic docetaxel had an even more profound effect on tumor vasculature (73% reduction compared with controls, $P < 0.001$, and 40% reduction over metronomic docetaxel alone, $P < 0.01$; Fig. 4). Thus, the combination of metronomic therapy with AEE788 had an additive effect on microvessel density as well as the proliferative index of tumor cells.

Because the combination of metronomic docetaxel with AEE788 resulted in significantly lower microvessel density, we next analyzed the effects of these regimens on the rate of tumor cell apoptosis by the TUNEL method. There was a moderate increase in apoptosis in the monotherapy groups, but a significant increase in tumor cell apoptosis was noted in both of the combination groups (both $P < 0.01$; Fig. 4). These results indicate that the addition of AEE788 to MTD and metronomic docetaxel has an additive effect on treatment-induced apoptosis in tumor cells.

Assessment of CECs and cell-free DNA levels. Antiangiogenic agents have been shown to have differential effects on the levels of CECs in the circulation (22). Therefore, we sought to determine the levels of two subpopulations of CECs, namely CEP cells and mature CECs, following short-term treatment of mice with metronomic docetaxel alone and in combination with AEE788, as potential surrogate biomarkers of response. Mice (n = 8 per group) bearing HeyA8 tumors were treated (starting at day 17) for 1 week with either PBS, metronomic docetaxel alone, or in combination with AEE788; levels of CECs were assessed using flow cytometry on murine peripheral blood with a modified protocol as previously described (22). There was no significant difference in the levels of mature CECs in the various treatment groups (Fig. 5*A*). Treatment with AEE788 resulted in a 69% reduction in CEPs compared with controls ($P = 0.06$). Interestingly, the combination of AEE788 with

Table 1. Effect of MTD and metronomic administration of paclitaxel and docetaxel on the viability of tumor and endothelial cells *in vitro*

Cell lines	Paclitaxel IC_{50} (nmol/L)		Docetaxel IC_{50} (nmol/L)	
	MTD	Metronomic	MTD	Metronomic
Ovarian cancer cell lines				
HeyA8	4–5	2.5–3	3–5	1.8–2
SKOV3ip1	4–4.2	2–2.2	3–4	0.9–1.4
Endothelial cell lines				
HUVEC	3–5	0.03–0.48	2.5–2.7	0.05–0.06
MMEC	2.5–3	0.65–0.75	1.6	0.1

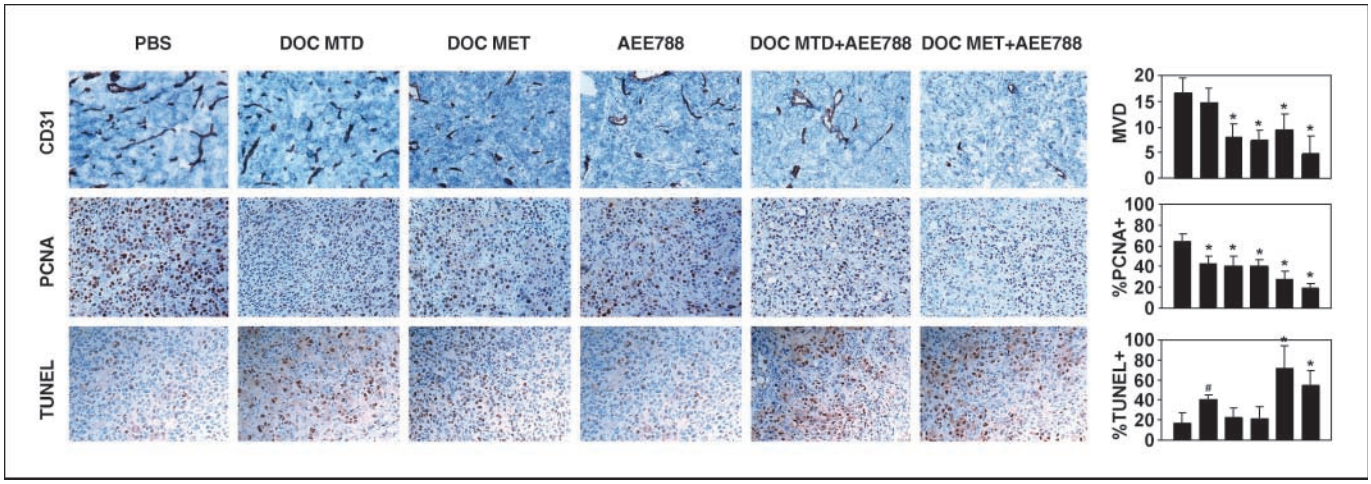

Figure 4. Proliferation index, microvessel density (*MVD*), and apoptotic index in tumors treated with MTD and metronomic (*MET*) docetaxel (*DOC*) alone and in combination with AEE788. HeyA8 tumors collected at the conclusion of therapy experiments seen in Fig. 2*A* and *B* were stained for PCNA, CD31, and TUNEL. Representative sections (final magnification, ×100) are shown for the various treatment groups. A graphic representation of the average number of CD31-positive vessels per field, mean percentage of PCNA-positive cells, and mean percentage of TUNEL-positive cells are shown in the adjoining graphs (columns in graphs correspond to the columns of images). Five fields per slide, and at least five slides per group, were examined, and compared with Student's *t* test and ANOVA. #, $P < 0.05$; *, $P < 0.01$.

metronomic docetaxel led to a 76% reduction in CEP levels ($P = 0.03$). Thus, treatment with AEE788 alone and in combination with metronomic docetaxel inhibited the mobilization of CEPs, which could, in part, contribute to their antiangiogenic effects.

Recent studies suggest that circulating cell-free DNA levels may be a useful biomarker of therapeutic response (23, 24). To evaluate the usefulness of plasma cell-free DNA as a biomarker of response, we quantified the levels of tumor-derived plasma DNA levels by real-time PCR using β-actin primers directed specifically to human sequences. The mean tumor-derived cell-free DNA levels in controls was 356.6 GE/mL. Short-term treatment with metronomic docetaxel and AEE788 resulted in a 47% and 31% reduction in cell-free DNA levels, but this was not statistically significant (Fig. 5*B*). Combination therapy with metronomic docetaxel and AEE788 led to a 73% reduction in cell-free DNA levels ($P = 0.04$). Furthermore, there was a close correlation between tumor weights and cell-free DNA levels ($r = 0.8$, $P < 0.01$; Fig. 5*C*).

Discussion

These data show that metronomic scheduling of taxane-based chemotherapy enhances the efficacy of antivascular agents such as AEE788. These effects were seen at remarkably low cumulative doses of docetaxel compared with MTD doses and were well tolerated. The combination of metronomic chemotherapy with AEE788 significantly prolonged survival. More importantly, this combination regimen had efficacy even in models with established tumors as well as in the chemotherapy-resistant model.

The evolution toward metronomic administration of chemotherapeutic drugs is based on several factors. First, high-dose chemotherapy is not very effective and is associated with high toxicity (25). In addition, dose-dense chemotherapy, in which one or more chemotherapeutic agent is administered at more frequent intervals, has shown efficacy in randomized phase III clinical trials (7, 26, 27). Metronomic chemotherapy, a variant of dose-dense therapy, in which the cumulative dose is significantly less than MTD-based

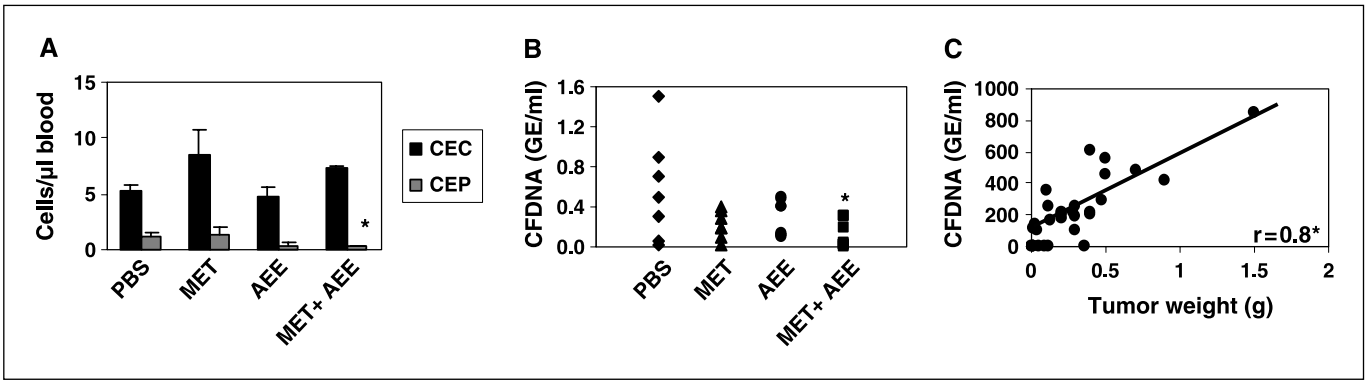

Figure 5. Assessment of CECs and tumor-derived cell-free DNA levels as surrogate biomarkers of response to metronomic chemotherapy. Nude mice with established tumors (17 d after HeyA8 tumor cell injection) were treated with the following regimens: PBS, metronomic docetaxel, AEE788, and metronomic docetaxel plus AEE788 for 7 d. Peripheral blood was collected by retro-orbital puncture and used for analysis of mature CECs and CEPs with four-color flow cytometry, and plasma was used for quantification of cell-free DNA levels by real-time PCR. *A,* levels of mature CECs (*black columns*) and CEPs (*gray columns*) in the various treatment groups. *, $P = 0.03$ for CEPs compared with vehicle controls. *B,* distribution of individual tumor-specific cell-free DNA levels in the various treatment groups. *, $P = 0.04$ compared with vehicle controls. *C,* scatter plot showing correlation of cell-free DNA levels with tumor weight; correlation coefficient was 0.8, *$P < 0.01$, Pearson's correlation test.

chemotherapy, has several potential advantages, including lower toxicity and adverse side effects (7, 28). More importantly, it seems that despite the lower cumulative doses administered, metronomic chemotherapy is superior to MTD-based regimens for inhibiting tumor growth in preclinical models (29, 30). Our findings show that metronomic docetaxel at one fifth the cumulative dose of MTD-based regimens had significant effects on therapeutic response and survival. Clinically, weekly taxane chemotherapy has resulted in a high rate of objective responses even in chemotherapy-resistant cancers (31, 32). Our data support these findings in that metronomic paclitaxel and docetaxel monotherapy resulted in growth inhibition in the taxane-resistant model.

An attractive application of metronomic chemotherapy is the ability to combine these regimens with biological agents, in particular antiangiogenic drugs. Certain chemotherapeutic agents, such as vinblastine, cyclophosphamide, and taxanes, have anti-angiogenic properties at 1/10 to 1/20 the MTD in combination with an anti-VEGF-R2 antibody (5, 7, 33). Such combinations are particularly appealing because high local concentrations of VEGF in the tumor environment can promote multidrug resistance in tumor endothelium (6, 7, 34). Hurwitz et al. (12) reported that bevacizumab (humanized monoclonal antibody against VEGF) combined with standard chemotherapy regimens significantly improved survival for patients with advanced-stage metastatic colorectal carcinoma. These benefits may extend to the combination of antiangiogenic agents with metronomic regimens of cytotoxic agents. Theoretically, these combinations would have a more tolerable toxicity profile and could potentially be administered for prolonged periods. In our study, metronomic docetaxel plus AEE788 led to a substantial tumor reduction over metronomic chemotherapy alone, as well as a significant prolongation in survival of mice with established tumors. Thus, metronomic chemotherapy with dual inhibition of both VEGFR and EGFR signaling with AEE788 targets both tumor cells and tumor-associated endothelial cells and results in a profound effect on tumor growth inhibition.

Most cytotoxic chemotherapy agents cause disruption of cell division but also damage normally dividing cells of rapidly regenerating tissues, leading to the well-known side effects of MTD-based regimens. Although chemotherapeutic agents also exert their cytotoxic effect on endothelial cells, the long drug-free periods between treatments provide time for endothelial cells to repopulate, thereby reducing the antiangiogenic effects of these drugs. There is increasing evidence, mostly *in vitro*, which indicates that endothelial cells are sensitive to very low doses of various chemotherapeutic drugs (35, 36). Our findings also suggest that endothelial cells are highly sensitive to paclitaxel and docetaxel at 10 to 100 times lower cytotoxic doses compared with tumor cells when administered metronomically. Our *in vitro* findings do not support a significant effect of metronomic dosing on tumor cell survival and proliferation compared with MTD regimens. However, we found a significant decrease in the percentage of proliferating tumor cells *in vivo* in the group treated with metronomic docetaxel with AEE788. Additionally, whereas metronomic docetaxel alone did not result in enhanced tumor cell apoptosis, the combination of metronomic docetaxel with AEE788 had a significant effect on the apoptotic index of treated tumors. These effects are most likely secondary to the antivascular effects of the metronomic combination group. Recent studies also suggest thrombospondin 1, a known inhibitor of angiogenesis, as a potential mediator of the effects of metronomic chemotherapy (37).

The clinical testing of antiangiogenic regimens is hampered by the lack of surrogate biomarkers for identifying patients most likely to benefit from these agents (38). Such biomarkers are essential as conventional imaging techniques to assess response may not accurately reflect the biological effectiveness of these drugs. Metronomic regimens have an inhibitory effect on the mobilization and viability of bone marrow–derived CEP cells (19, 39). It is thought that these cells are mobilized from the bone marrow in response to growth factors such as VEGF, then migrate to sites of ongoing angiogenesis and differentiate into mature endothelial cells (40). Bertolini et al. (19) showed that MTD-cyclophosphamide regimens caused a sharp decline in CEPs following therapy, but these cells quickly increased during the drug-free period. In contrast, with metronomic cyclophosphamide, there was a sustained suppression of CEPs along with a decrease in viability. In the current study, metronomic regimens did not have any effect on the levels of mature CECs, derived primarily from mature vasculature (40, 41), but the combination of metronomic docetaxel with AEE788 caused a significant reduction of CEPs, suggesting that CEP levels could be used to monitor therapeutic response. Because combination of metronomic therapy with AEE788 results in tumor cell apoptosis, we also tested the use of tumor-derived cell-free DNA as a potential biomarker of both tumor burden and response to therapy. Although both AEE788 and metronomic docetaxel resulted in a decrease in levels of cell-free DNA, the maximal reduction was seen in the combination group. The presence of tumor-specific alterations in plasma DNA has been reported in several studies. Point mutations in *KRAS* oncogene have been detected in the plasma DNA of patients with colorectal and pancreatic cancer, even preceding clinical diagnosis of pancreatic cancer by 5 to 14 months (42, 43). Wei et al. (24) did quantitative analysis of tumor-specific EBV DNA in the plasma of nasopharyngeal carcinoma patients and found that surgical resection of the tumor was associated with a significant decrease in EBV DNA copy numbers. Furthermore, absence of EBV DNA in plasma reflected complete surgical resection in these patients (24). Our data indicate that tumor-derived cell-free DNA levels correlate with tumor weight and could be a surrogate measure for tumor burden in clinical trials.

In summary, we have shown the efficacy of metronomic docetaxel, at the optimal metronomic dose, to inhibit tumor growth and prolong survival using an orthotopic ovarian cancer model. Furthermore, metronomic docetaxel significantly enhanced the growth-inhibitory effects of AEE788. These effects were evident both in mice with established tumors as well as those with taxane-resistant disease. These effects seem to be mediated in part by antivascular, antiproliferative, and apoptotic effects of the metronomic regimens alone and in combination with AEE788. Both assessment of CEPs and tumor-derived cell-free DNA levels hold promise as noninvasive biomarkers of therapeutic response. Our data suggest that the combination of metronomic docetaxel with dual inhibition of VEGFR and EGFR is highly efficacious and should be considered for future clinical trials.

Acknowledgments

Received 9/5/2006; revised 10/26/2006; accepted 10/31/2006.

Grant support: Bettyann Asche-Murray Fellowship Award at the University of Texas M.D. Anderson Cancer Center (A.A. Kamat), the University of Texas M.D. Anderson Cancer Center Specialized Programs of Research Excellence in ovarian cancer grant 2P50 CA083639-06A1 (A.K. Sood and R.B. Jaffe), and the Marcus Foundation (A.K. Sood).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics. CA Cancer J Clin 2006;56:106–30.
2. Lister-Sharp D, McDonagh MS, Khan KS, Kleijnen J. A rapid and systematic review of the effectiveness and cost-effectiveness of the taxanes used in the treatment of advanced breast and ovarian cancer. Health Technol Assess 2000;4:1–113.
3. Berek J, Hacker N. Practical gynecologic oncology. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2000.
4. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest 2000; 105:1045–7.
5. Browder T, Butterfield CE, Kraling BM, et al. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 2000;60:1878–86.
6. Kerbel RS. Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anticancer therapeutic agents. Bioessays 1991;13:31–6.
7. Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. Nat Rev Cancer 2004;4:423–36.
8. Risau W. Mechanisms of angiogenesis. Nature 1997; 386:671–4.
9. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000;6:389–95.
10. Bergers G, Banjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;3:401–10.
11. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003;9:669–76.
12. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350:2335–42.
13. Morishige K, Kurachi H, Amemiya K, et al. Evidence for the involvement of transforming growth factor α and epidermal growth factor receptor autocrine growth mechanism in primary human ovarian cancers *in vitro*. Cancer Res 1991;51:5322–8.
14. Apte SM, Bucana CD, Killion JJ, Gershenson DM, Fidler IJ. Expression of platelet-derived growth factor and activated receptor in clinical specimens of epithelial ovarian cancer and ovarian carcinoma cell lines. Gynecol Oncol 2004;93:78–86.
15. Thaker PH, Yazici S, Nilsson MB, et al. Antivascular therapy for orthotopic human ovarian carcinoma through blockade of the vascular endothelial growth factor and epidermal growth factor receptors. Clin Cancer Res 2005;11:4923–32.
16. Langley RR, Ramirez KM, Tsan RZ, Van Arsdall M, Nilsson MB, Fidler IJ. Tissue-specific microvascular endothelial cell lines from H-2K(b)-tsA58 mice for studies of angiogenesis and metastasis. Cancer Res 2003;63:2971–6.
17. Dykes DJ, Bissery MC, Harrison SD, Jr., Waud WR. Response of human tumor xenografts in athymic nude mice to docetaxel (RP 56976, Taxotere). Invest New Drugs 1995;13:1–11.
18. Schuch G, Heymach JV, Nomi M, et al. Endostatin inhibits the vascular endothelial growth factor-induced mobilization of endothelial progenitor cells. Cancer Res 2003;63:8345–50.
19. Bertolini F, Paul S, Mancuso P, et al. Maximum tolerated dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. Cancer Res 2003;63:4342–6.
20. Lo YM, Tein MS, Lau TK, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62:768–75.
21. Boddy JL, Gal S, Malone PR, Harris AL, Wainscoat JS. Prospective study of quantification of plasma DNA levels in the diagnosis of malignant versus benign prostate disease. Clin Cancer Res 2005;11:1394–9.
22. Beaudry P, Force J, Naumov GN, et al. Differential effects of vascular endothelial growth factor receptor-2 inhibitor ZD6474 on circulating endothelial progenitors and mature circulating endothelial cells: implications for use as a surrogate marker of antiangiogenic activity. Clin Cancer Res 2005;11:3514–22.
23. Fiegl H, Millinger S, Mueller-Holzner E, et al. Circulating tumor-specific DNA: a marker for monitoring efficacy of adjuvant therapy in cancer patients. Cancer Res 2005;65:1141–5.
24. Wei WI, Yuen AP, Ng RW, Kwong DL, Sham JS. Quantitative analysis of plasma cell-free Epstein-Barr virus DNA in nasopharyngeal carcinoma after salvage nasopharyngectomy: a prospective study. Head Neck 2004;26:878–83.
25. Nieto Y. The verdict is not yet in. Analysis of the randomized trials of high-dose chemotherapy for breast cancer. Haematologica 2003;88:201–11.
26. Tuma RS. Dosing study seen as victory for clinical trials, mathematical methods. J Natl Cancer Inst 2003; 95:254–5.
27. Citron ML, Berry DA, Cirrincione C, et al. Randomized trial of dose-dense versus conventionally scheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node-positive primary breast cancer: first report of Intergroup Trial C9741/Cancer and Leukemia Group B Trial 9741. J Clin Oncol 2003;21:1431–9.
28. Klement G, Baruchel S, Rak J, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest 2000;105:R15–24.
29. Man S, Bocci G, Francia G, et al. Antitumor and antiangiogenic effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. Cancer Res 2002;62:2731–5.
30. Bello L, Carrabba G, Guissani C, et al. Low dose chemotherapy combined with an antiangiogenic drug reduces human glioma growth *in vivo*. Cancer Res 2001; 61:7501–6.
31. Markman M, Hall J, Spitz D, et al. Phase II trial of weekly single-agent paclitaxel in platinum/paclitaxel-refractory ovarian cancer. J Clin Oncol 2002;20:2365–9.
32. Terauchi F, Hirano T, Taoka H, et al. Weekly docetaxel for patients with platinum/paclitaxel/irinotecan-resistant relapsed ovarian cancer: a phase I study. Int J Clin Oncol 2003;8:348–51.
33. Sweeney CJ, Miller KD, Sissons SE, et al. The antiangiogenic property of docetaxel is synergistic with a recombinant humanized monoclonal antibody against vascular endothelial growth factor or 2-methoxyestradiol but antagonized by endothelial growth factors. Cancer Res 2001;61:3369–72.
34. Castilla MA, Caramelo C, Gazapo RM, et al. Role of vascular endothelial growth factor (VEGF) in endothelial cell protection against cytotoxic agents. Life Sci 2000;67:1003–13.
35. Belotti D, Vergani V, Drudis T, et al. The microtubule-affecting drug paclitaxel has antiangiogenic activity. Clin Cancer Res 1996;2:1843–9.
36. Bocci G, Nicolaou KC, Kerbel R. Protracted low-dose effects on human endothelial cell proliferation and survival *in vitro* reveal a selective antiangiogenic window for various chemotherapeutic drugs. Cancer Res 2002;62:6938–43.
37. Bocci G, Francia G, Man S, Lawler J, Kerbel RS. Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose chemotherapy. Proc Natl Acad Sci U S A 2003;100:12917–22.
38. Davis DW, McConkey DJ, Abbruzzese JL, Herbst RS. Surrogate markers in antiangiogenesis clinical trials. Br J Cancer 2003;89:8–14.
39. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–7.
40. Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434–8.
41. Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999;18:3964–72.
42. Sorenson GD. Detection of mutated KRAS2 sequences as tumor markers in plasma/serum of patients with gastrointestinal cancer. Clin Cancer Res 2000;6:2129–37.
43. Anker P, Mulcahy H, Chen Xq, Stroun M. Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients. Cancer Metastasis Rev 1999;18:65–73.