Bridging the Gap between Cytotoxic and Biologic Therapy with Metronomic Topotecan and Pazopanib in Ovarian Cancer

William M. Merritt[1], Alpa M. Nick[1], Amy R. Carroll[1], Chunhua Lu[1], Koji Matsuo[1], Melissa Dumble[5], Nicholas Jennings[1], ShuYun Zhang[5], Yvonne G. Lin[1], Whitney A. Spannuth[1], Aparna A. Kamat[1], Rebecca L. Stone[1], Mian M.K. Shahzad[4], Robert L. Coleman[1], Rakesh Kumar[5], and Anil K. Sood[1,2,3]

Abstract

This study aimed to investigate the antitumor and antiangiogenic effects utilizing a novel therapy regimen of metronomic topotecan and pazopanib, a multireceptor tyrosine kinase inhibitor. *In vitro* (Western blot) and *in vivo* dose-finding experiments were done following pazopanib therapy in ovarian cancer models. Pazopanib and metronomic (daily) oral topotecan therapy was examined in an orthotopic model of ovarian cancer. Tumor weights, survival, and markers of the tumor microenvironment [angiogenesis (CD31 and pericyte coverage), proliferation (Ki-67), and apoptosis (terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling)] were analyzed by immunostaining following therapy. Pazopanib therapy reduced vascular endothelial growth factor receptor 2 (VEGFR-2) activity *in vitro* and *vivo* in a dose-dependent manner. Compared with control mice, pazopanib reduced tumor weight by 28% to 82% ($P < 0.01$ in the SKOV3ip1 model) and metronomic topotecan reduced tumor weight by 40% to 59% in the HeyA8 ($P = 0.13$) and SKOV3ip1 ($P = 0.07$) models. Combination therapy had the greatest effect with 79% to 84% reduction ($P < 0.01$ for both models). In the SKOV3ip1 and A2780 models, mouse survival was significantly longer ($P < 0.001$ versus controls) with pazopanib and metronomic topotecan therapy. Pazopanib therapy reduced murine endothelial cell migration *in vitro* in a dose-dependent manner following VEGF stimulation and decreased tumor microvessel density and pericyte coverage when given in combination with metronomic topotecan. Tumor cell proliferation decreased in all treatment arms compared with controls ($P < 0.01$ for combination groups) and increased tumor cell apoptosis by 4-fold with combination therapy. Pazopanib therapy in combination with metronomic topotecan therapy showed significant antitumor and antiangiogenic properties in preclinical ovarian cancer models and warrants further investigation as a novel therapeutic regimen in clinical trials. *Mol Cancer Ther; 9(4); 985–95. ©2010 AACR.*

Introduction

Surgical debulking followed by cytotoxic therapy is currently the standard of care for patients with ovarian carcinoma. Unfortunately, despite such aggressive therapy, most patients develop recurrence and die from progressive disease. Over the past decade, numerous novel biological agents have been developed and investigated as the next step towards curing this deadly disease. One of the most attractive targets for biological agents is tumor vasculature or angiogenesis. Folkman first introduced concepts regarding the requirement of angiogenesis for tumor growth in 1971 (1). Since then, a clearer understanding of the process of angiogenesis has emerged, which has contributed to the development of novel therapeutic approaches (2).

Traditional cytotoxic regimens generally employ chemotherapeutic agents that are administered to patients at, or near, the highest dose that can be safely given. The contemporary management for ovarian cancer is no exception, with most patients receiving combination platinum and taxane as primary chemotherapy for advanced-stage disease (3). More than 80% of patients with ovarian cancer will initially respond to this strategy; however, most will eventually develop chemoresistant disease. Moreover, treatment-limiting toxicities acutely and chronically challenge the combination of agents dosed in this manner. An alternative strategy involves

lower, more frequent dosing of the cytotoxic agent, i.e., metronomic therapy (4). The theory underlying this approach relates to less host toxicity than, but similar tumor cell kill as traditional dosing schedules. Several studies have reported success with metronomic therapy in preclinical and clinical models (5–7). Furthermore, metronomic therapy also shows antiangiogenic properties secondary to continued exposure on tumor endothelial cells (8, 9). On the basis of Hif-1α inhibition, we recently reported that metronomic topotecan had similar antitumor activity compared with the traditional maximally tolerated dosing and significantly decreased tumor vascularity in murine models of advanced ovarian cancer (10). We hypothesized that metronomic topotecan would result in greater antitumor activity in combination with other antiangiogenic agents. The antitumor and antivascular benefits of vascular endothelial growth factor (VEGF)-targeted therapy are promising and offer opportunities for further investigation. VEGF and its receptors (VEGFR-1, -2, and -3) are commonly expressed in human cancers and have been identified as optimal angiogenic targets (11). In addition to increased expression, VEGF receptors are also associated with poor prognosis in multiple cancer types, including ovarian cancer (12–15). Based on our earlier results with metronomic topotecan (10), we explored in the current study the antitumor and antiangiogenic efficacy of metronomic topotecan with a multireceptor kinase inhibitor, pazopanib, in ovarian cancer models.

Materials and Methods

Cell lines and reagents

The derivation and source of the human epithelial ovarian cancer cell lines HeyA8, SKOV3ip1, and A2780, and the murine ovarian endothelial cell line (MOEC) have been described previously (16, 17). Cell lines were maintained and propagated in RPMI-1640 medium supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts). All experiments were done using cells grown to 60% to 80% confluence, and all cell lines were routinely tested to confirm the absence of *Mycoplasma*.

Pazopanib (GlaxoSmithKline) was mixed in DMSO (Sigma) for all *in vitro* experiments and 0.5% hydroxypropylmethylcellulose (Sigma) and 0.1% Tween-80 (Sigma) diluted in water for *in vivo* therapy. Topotecan (GlaxoSmithKline) was prepared and stored in 10 mmol/L Tri-HCl (pH 4.0) aliquots prior to being diluted in water for oral therapy *in vivo*.

In vivo orthotopic ovarian cancer model

Female athymic nude mice were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) or Taconic Farms (Hudson, NY) and housed in specific pathogen-free conditions. They were cared for in accordance with the guidelines set forth by the American Association for Accreditation for Laboratory Animal Care and the U.S. Public Health Service's Policy on Human Care and Use of Laboratory Animals, and all studies were approved and supervised by the Institutional Animal Care and Use Committee at either M.D. Anderson Cancer Center or GlaxoSmithKline. The ovarian cancer cell lines were maintained and prepared for *in vivo* i.p. injection as previously described for the orthotopic model by our group (17). For the A2780 survival experiment, 10 million cells were injected i.p. 1 wk prior to initiation of treatment.

In vivo pazopanib and topotecan therapy

To determine the optimal pazopanib dose for future experiments, SKOV3ip1 tumor-bearing mice were treated with either 30 or 100 mg/kg pazopanib daily for 3 d. Mice were sacrificed at 4, 24, 48, and 72 h following 72 h of therapy. Tumors were snap frozen in optimal cutting medium (Miles, Inc.) for immunofluorescence analysis.

For the HeyA8 and SKOV3ip1 therapy models, the mice were randomly separated and assigned to treatment groups (*n* = 10/group) approximately 1 wk after cell line inoculation. Treatment groups were control (vehicle only), metronomic topotecan (0.5 mg/kg, oral, daily, dose established in ref. 10), pazopanib (100 mg/kg, oral, every other day), and topotecan plus pazopanib (same doses used in single-treatment arms). Therapy was continued until mice became moribund in any of the treatment groups. All treatment groups were then sacrificed by cervical dislocation, and tumor and mouse weights were recorded. Representative tumor specimens were collected from each treatment group and stored for further immunohistochemical analyses.

To determine the survival benefit of metronomic topotecan and pazopanib, experiments were carried out using the SKOV3ip1 and A2780 cell line models. Mice were inoculated i.p. with either SKOV3ip1 or A2780 cells as in the therapy models. In the SKOV3ip1 model, the mice (*n* = 10/group) were treated with similar regimens as in the previous therapy experiment. In the A2780 model, the mice (*n* = 10/group) were treated with the following regimens: vehicle 1 (hydroxypropylmethylcellulose/Tween), vehicle 2 (tartaric acid), pazopanib 100 mg/kg (oral, twice daily), maximum tolerated dose (MTD) topotecan 5 mg/kg (i.v., daily × 5 d every 21 d), MTD topotecan 10 mg/kg (i.v., every 7 d × 3 doses), metronomic topotecan 1 mg/kg (oral, daily), pazopanib plus topotecan 5 mg/kg, pazopanib plus topotecan 10 mg/kg, or pazopanib plus metronomic topotecan 1 mg/kg. Mice were sacrificed (recorded as date of death) when they appeared significantly moribund as determined by investigator and animal care veterinarians.

Western blot assay for VEGFR-2/phospho-VEGFR-2

To determine whether pazopanib or topotecan inhibited phosphorylation of VEGFR-2 in ovarian cancer cells, SKOV3ip1 and HeyA8 cells were serum starved for 24 h and then treated with either pazopanib (0.001–100 μmol/L) for approximately 3 to 4 h or topotecan (6.25–100 nmol/L) for 12 h. In the pazopanib assay,

VEGF-A (10 ng/mL; Invitrogen) was added 1 h prior to collecting cell lysates. In both assays, cell lysates were collected in radioimmunoprecipitation assay lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton, 0.5% deoxycholate plus 25 μg/mL leupeptin, 10 μg/mL aprotinin, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate), centrifuged at 13,500 rpm for 15 min at 4°C, and protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad). Protein bands were separated by 10% SDS-PAGE and transferred to nitrocellulose paper, blocked with 5% milk for 2 h at room temperature, incubated with either rabbit anti-human VEGFR-2 antibody Tyr (Santa Cruz Biotechnology) or rabbit anti-human phospho-VEGFR 2/3 (Calbiochem) overnight at 4°C followed by incubation with appropriate secondary anti-rabbit horseradish peroxidase (HRP)-conjugated antibody for 2 h at room temperature. Blots were developed with the ECL Western Blotting Detection Kit (GE Healthcare). Actin was used for loading control, and all experiments were repeated in duplicate. Densitometry was done to analyze phosphorylation levels.

Migration assay

To determine the effects of inhibiting VEGFR-2 activity on murine endothelial cell or ovarian cancer cell migration, 1×10^5 viable MOECs or SKOV3ip1 cells (determined by trypan blue exclusion) were resuspended in serum-free media and plated onto a 0.1% gelatin-coated membrane matrix using a membrane culture system (MICS) as previously described (17). Prior to placement into the MICS system, cells were maintained in serum-free media for approximately 12 h and then treated with pazopanib (0.001, 1, and 10 μmol/L) for 4 to 5 h followed by addition of VEGF-A (10 ng/mL; Invitrogen) 1 h prior to placement into MICS top wells. The bottom wells were filled with serum-free media and VEGF-A (Invitrogen). Chambers were incubated for 8 h at 37°C. At completion, cells in the bottom chambers were removed and quantified (5 random fields/well) as a percentage of migrated cells/1×10^5 cells plated as previously described (17). Experiments were done in duplicate.

Immunohistochemistry

Immunofluorescent staining of VEGFR-2 was done on 4-μm-thick frozen orthotopic ovarian cancer specimens. After hydration in cold acetone for 10 min, endogenous peroxidases were blocked with 3% hydrogen peroxide followed by primary antibody incubation, phospho-VEGFR2/3 (Calbiochem) overnight at 4°C. The following day, slides were washed and incubated with appropriate antirabbit secondary antibody. Images were taken using a Zeiss Axioplan2 microscope (Carl Zeiss, Inc.) with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics) and captured with ImagePro Plus software v5.1 (Media-Cybernetics). Immunohistochemical analyses of CD31 (primary antibody: rat monoclonal anti-mouse, 1:800 dilution, BD Bioscience, Pharmingen) and terminal deoxy-

nucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) were conducted on orthotopic specimens as described previously (17). Ki-67 staining was done on paraffin-embedded slides. Slides were washed and hydrated with sequential washes in ethanol, blocked with 5% normal horse/1% normal goat serum in PBS. Following overnight incubation with Ki-67 rabbit polyclonal anti-human antibodies (1:200 dilution; BioCare Medical) at 4°C, the slides were washed with PBS, incubated with appropriate goat anti-rabbit HRP secondary antibody at 4°C for 4 h, and then antibody detection was done following incubation with 3,3′-diaminobenzidine substrate for 10 min. For pericyte coverage analysis, dual immunohistochemical staining for CD31 and α smooth muscle actin (αSMA) was done on paraffin sections. Following antigen retrieval with citrate buffer, sections were incubated with αSMA antibody (Abcam) overnight at 4°C and then with HRP-conjugated IgG (Jackson ImmunoResearch) for 1 h at room temperature. Slides were then stained with 3,3′-diaminobenzidine, washed, and incubated with CD31 antibody (BD Bioscience) overnight at 4°C, followed by incubation with rat probe and HRP polymer (BioCare). Lastly, the slides were stained with Ferangi Blue Chromogen (BioCare). Quantification of microvessel density (CD31), tumor cell proliferation (Ki-67), apoptosis (TUNEL), and perictye coverage was done on slides from each treatment group, as previously described (17, 18). Images were taken with a Nikon Microphot-FXA microscope (Nikon Instruments) and a Leica DFC-320 camera (Leica Microsystems) and captured with ImagePro Plus v5.1.

Statistical analysis

For animal experiments, 10 mice were assigned per treatment group. This sample size gave 80% power to detect a 50% reduction in tumor weight at a 5% level of statistical significance. Mouse and tumor weights and the number of tumor nodules for each group were compared using Student's t-test (for comparisons of two groups) and ANOVA (for multiple group comparisons). Normality of weight distributions was tested by Kolmogrov-Smirnov test. Kaplan-Meier survival curves and log-rank test were used to determine relationships between therapy regimens and mouse survival. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using SPSS version 12 for Windows statistical software (SPSS, Inc.).

Results

In vitro and *in vivo* effects of pazopanib therapy on VEGFR

Prior to initiating *in vivo* therapy experiments, we tested the effects of pazopanib and topotecan on VEGFR-2 expression and phosphorylation in ovarian cancer cells. *In vitro,* SKOV3ip1 and HeyA8 cells were treated with

a range of pazopanib concentrations followed by stimulation with VEGF. The total VEGFR-2 expression was not affected by pazopanib therapy; however, VEGFR-2 phosphorylation decreased at low doses but this was more apparent at higher doses, ≥ 1 μmol/L doses in the SKOV3ip1 cell line (Fig. 1A). In HeyA8 cells, pazopanib therapy also decreased VEGFR-2 phosphorylation, but at higher doses compared with the SKOV3ip1 cells (data not shown). Topotecan treatment alone had no effect on VEGFR-2 phosphorylation in SKOV3ip1 and HeyA8 cells (Supplementary Fig. S1). *In vivo*, tumor-bearing mice were treated with two different doses of oral pazopanib. Mice were sacrificed at different time points, and harvested tumors were examined for VEGFR-2 phosphorylation. As early as 4 hours after treatment, a noticeable decrease in VEGFR-2 phosphorylation on tumor cells was observed in mice treated with pazopanib at 100 mg/kg compared with controls (Fig. 1B). Modulation of phosphorylated VEGFR-2 expression was still evident up to 48 hours following treatment until returning to baseline at 72 hours. The lower pazopanib dose (30 mg/kg) showed no observable effect on VEGFR-2 phosphorylation (data not shown).

Effect of metronomic topotecan and antiangiogenesis therapy in an orthotopic ovarian cancer model

To determine the effects of pazopanib on ovarian cancer growth, we did therapy experiments with oral pazopanib therapy alone and in combination with metronomic topotecan in the HeyA8 and SKOV3ip1 orthotopic murine cancer models. Study arms (n = 10 mice/arm) included control (vehicle only, 100 μL oral gavage daily), pazopanib (100 mg/kg diluted in 100 μL oral gavage every other day), topotecan (0.5 mg/kg diluted in 100 μL oral gavage daily), and combination (pazopanib and topotecan, same doses as single-agent therapy). Single-agent pazopanib therapy reduced mean tumor weight by 28% (versus controls, $P = 0.22$) and 82% ($P = 0.007$) in the HeyA8 and SKOV3ip1 models, respectively (Fig. 2). Consistent with our previous report, metronomic topotecan therapy reduced mean tumor weights by 40% (HeyA8, $P = 0.13$) and 59% (SKOV3ip1, $P = 0.07$)
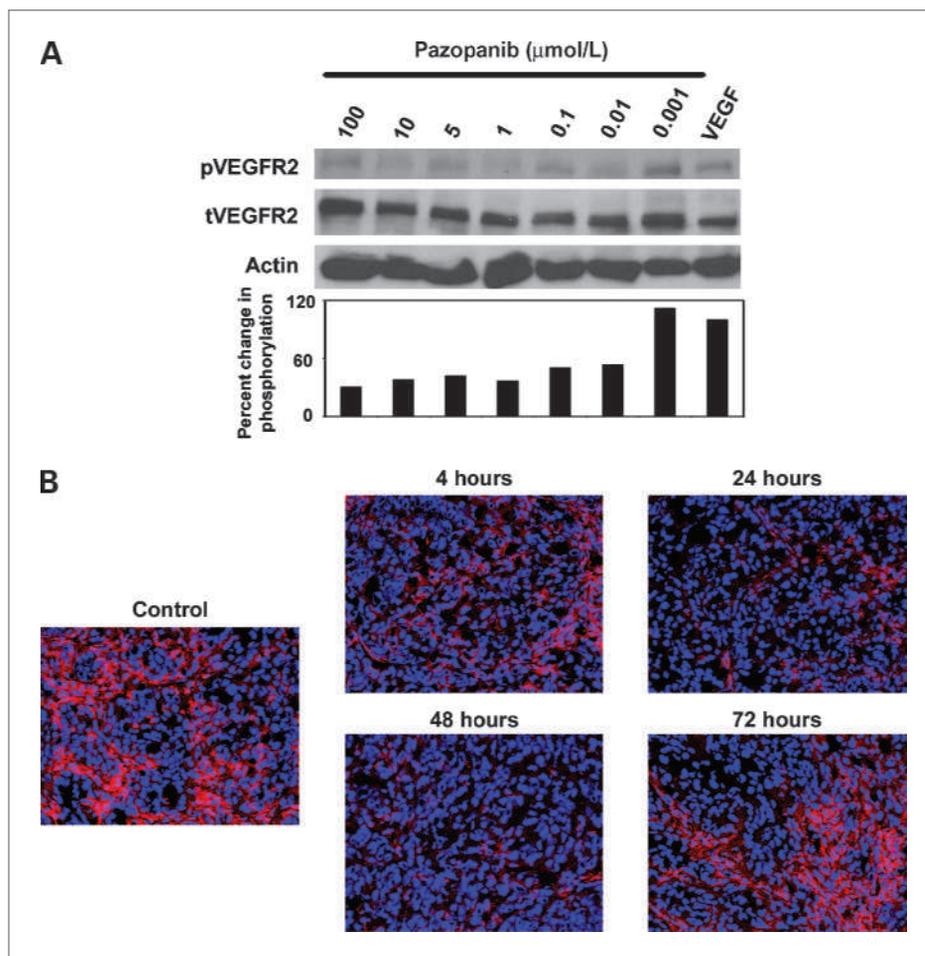

Figure 1. Inhibition of VEGFR-2 phosphorylation in ovarian cancer cells. A, Western blot analysis of SKOV3ip1 cells treated with pazopanib for 3 to 4 h followed by VEGF-A stimulation (10 ng/mL). Lysates were probed for total (tVEGFR-2) and phosphorylated (pVEGFR-2) VEGFR-2. Actin was used as loading control. Densitometry analysis (bottom) showing percent decrease in pVEGFR-2 relative to cells stimulated by VEGF only. B, SKOV3ip1 tumor-bearing mice were treated with pazopanib (100 mg/kg, daily × 3 d), sacrificed at respective time points. Photomicrographs (original magnification ×100) illustrate tumor cells (blue) and phosphorylated VEGFR-2 (red).

Figure 2. Effect of pazopanib and metronomic topotecan therapy in HeyA8 (A) and SKOV3ip1 (B) ovarian cancer models. Mice (n = 10/group) were randomized to one of the following treatment regimens: vehicle (oral, daily), metronomic topotecan (0.5 mg/kg, oral, daily), pazopanib (100 mg/kg, oral, every other day), or metronomic topotecan plus pazopanib (same doses as single-agent regimen). Left, mean tumor weights; right, tumor weight distribution. Error bars, SE. *, $P < 0.05$ compared with the control group.
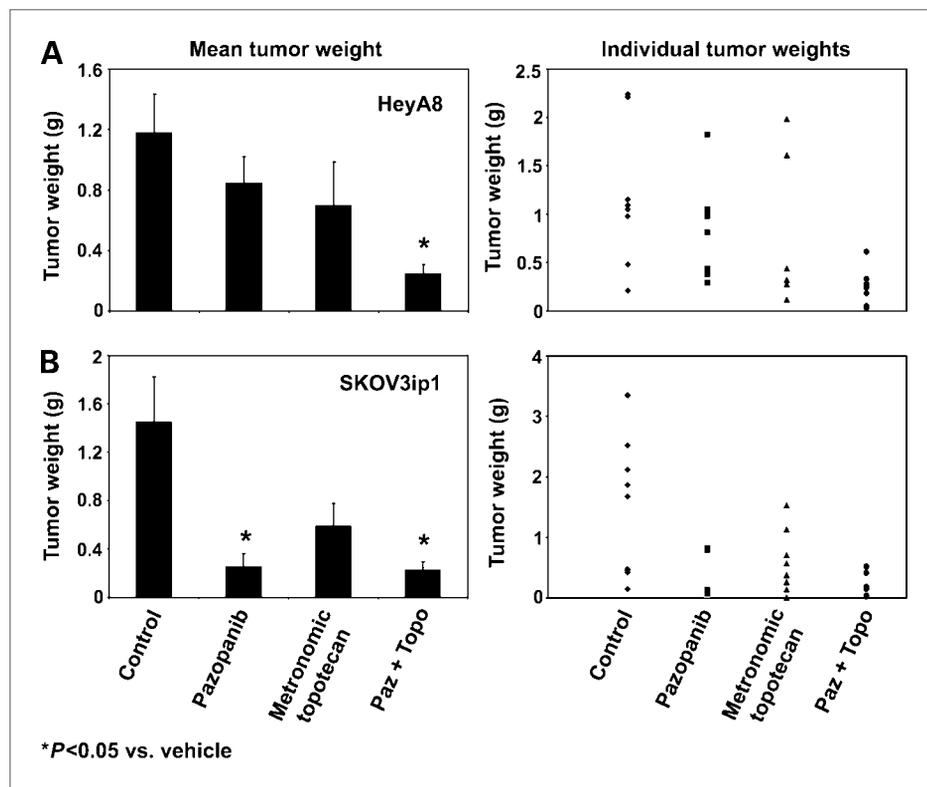
compared with mice treated with vehicle only. The greatest reduction in tumor weight was observed in mice treated with combination therapy. When compared with controls, a 79% to 84% ($P < 0.01$ for both models) reduction was noted with pazopanib and metronomic topotecan. In comparison with single-agent therapy in the HeyA8 model, combination therapy inhibited tumor growth by 70% (versus pazopanib, $P < 0.01$) and 64% (versus metronomic topotecan, $P = 0.22$). In the SKOV3ip1 model, single-agent pazopanib therapy exhibited similar antitumor activity as combination therapy (12% difference in mean tumor weight, P = NS). No differences in eating/drinking habits, mouse activity, and mean mouse weights were noted between treatment groups in both models, suggesting minimal toxicity from administered agents.

On the basis of encouraging efficacy data, we next examined the effects of metronomic topotecan and antiangiogenesis treatment on animal survival. For these experiments, mice inoculated with either SKOV3ip1 or A2780 cells were treated with regimens as described above until the mice became moribund, as determined by primary investigators or animal staff. In the SKOV3ip1 model, single-agent pazopanib and metronomic topotecan therapy prolonged mouse survival compared with controls; however, the greatest effect was observed in the combination therapy arm ($P < 0.001$ versus all other groups; Fig. 3A). To validate these findings, a separate survival experiment was carried out using the A2780 model in a separate laboratory. The design included an expanded treatment regimen of single-agent pazopanib, MTD or metronomic dosing as well as combination therapy with both agents. As illustrated in the Kaplan-Meier curve (Fig. 3B), single-agent metronomic topotecan therapy was similar to single-agent pazopanib. MTD dosing was superior to metronomic topotecan; however, when metronomic topotecan was combined with pazopanib therapy, survival was noticeably superior to other groups ($P < 0.001$ versus pazopanib alone; $P = 0.09$ versus MTD topotecan 10 mg/kg plus pazopanib arm).

Antiangiogenic properties of pazopanib therapy

Based on the prior evidence that pazopanib therapy targets tumor vasculogenesis (19), we first examined whether the pazopanib therapy directly affected murine endothelial cells *in vitro* (Fig. 4A). Following pazopanib treatment, migration of MOECs was analyzed after stimulation with VEGF. VEGF alone increased endothelial cell migration (90%) compared with untreated cells. With the addition of pazopanib therapy, migration of MOECs decreased in a dose-dependent manner with concentrations ranging from 0.001 μmol/L to 10 μmol/L (28–61% reduction versus VEGF stimulated cells; $P < 0.05$ for 1 and 10 μmol/L doses).

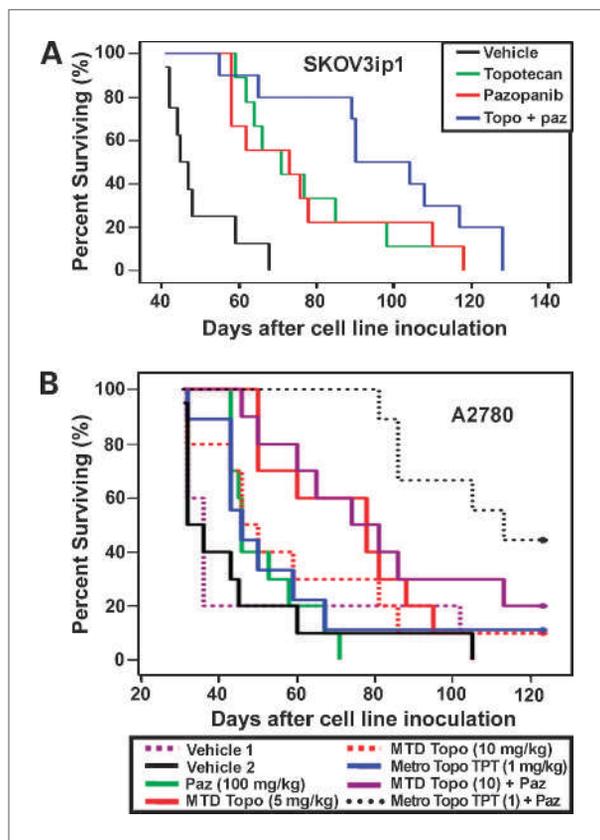With the known antiangiogenic activity of both pazopanib and metronomic topotecan, we analyzed the

Figure 3. Kaplan-Meier survival curve illustrating effects of pazopanib (paz) and metronomic (metro) topotecan (topo) therapy. A, SKOV3ip1 model comparing: vehicle, metronomic topotecan (0.5 mg/kg, daily), pazopanib (100 mg/kg every other day), combination (metronomic topotecan and pazopanib). $P < 0.01$ combination arm versus all other groups. B, A2780 model comparing: vehicle 1, pazopanib (100 mg/kg daily), maximum tolerated dose (MTD) topotecan 5 mg/kg (daily × 5 d every 21 d), MTD topotecan 10 mg/kg (every 7 d × 3 doses), metronomic topotecan (1 mg/kg daily), MTD topo 10 mg/kg + paz, metro topo 1 mg/kg + paz, vehicle 2. $P = 0.002$ and $P < 0.001$ for MTD topo 10 + paz and metro topo 1 + paz versus paz alone, respectively. $P = 0.09$ for MTD topo 10 + paz versus metro topo 1 + paz.

tumors from both treatment experiments for micro-vessel density (MVD) and pericyte coverage. In the HeyA8 model, pazopanib and metronomic topotecan alone reduced MVD by 24% ($P = 0.07$) and 29% ($P = 0.01$), respectively (Fig. 4B). In the SKOV3ip1 tumors, both single-agent therapies significantly reduced MVD (pazopanib at 46% reduction versus controls, $P = 0.03$; topotecan at 34% reduction, $P = 0.04$; Fig. 4B and C). The greatest decrease in MVD paralleled the effects observed with tumor weights using combination therapy in the HeyA8 (66% decrease, $P < 0.001$) and SKOV3ip1 (50%, $P = 0.002$) models. The effects of therapy on vessel maturation were examined by assessing the extent of pericyte coverage (Fig. 4D). No differences were observed in tumors between the control group and mice treated with metronomic topotecan. However, single-agent pazopanib and combination therapy led to a sig-

nificant decrease in pericyte coverage compared with controls ($P < 0.05$).

Alterations in tumor microenvironment with pazopanib and topotecan therapy

To determine the effects of pazopanib and metro-nomic therapy on other critical components of the tu-mor microenvironment, we examined tumor cell migration, proliferation, and apoptosis. Following treat-ment with pazopanib, SKOV3ip1 cell migration de-creased by 68% ($P < 0.01$ compared with controls; Supplementary Fig. S2). Examination of tumors from the *in vivo* therapy experiments showed that single-agent pazopanib therapy reduced tumor cell prolifera-tion in the SKOV3ip1 ($P < 0.001$; Fig. 5A) and HeyA8 ($P = 0.18$; Fig. 5A and B) models compared with con-trols. Metronomic topotecan therapy was effective in both SKOV3ip1 (14% reduction versus controls, $P = 0.085$) and HeyA8 (19%, $P = 0.35$) models. Combination therapy exhibited the greatest effect over controls with 22% to 42% reduction ($P < 0.01$).

Tumor cell apoptosis was examined in tumors from the HeyA8 therapy experiment by TUNEL immunofluores-cence analysis (Fig. 5C). Single-agent therapy increased tumor cell apoptosis by 1.8-fold ($P = 0.06$) and 1.9-fold ($P = 0.05$) in the pazopanib and topotecan arms, respec-tively. Combination therapy significantly increased apo-ptosis (4.1-fold versus controls, $P = 0.001$) and remained superior when compared with other single-agent thera-pies ($P < 0.05$ for both regimens).

Discussion

The key findings of this study are that pazopanib therapy effectively inhibits VEGFR-2 activity, and results in significant reduction in tumor growth in combination with metronomic topotecan. Furthermore, combination therapy led to prolonged mouse survival compared with controls and single-agent regimens. These effects likely reflect the antiangiogenic properties of both agents.

Tumor angiogenesis is led by an increase in proangio-genic factors, predominantly driven by VEGF and its receptors (20). The predominant ligand for VEGFRs, VEGF-A, is reported to provide a paracrine loop between tumor cells and endothelial cells expressing high VEGFR-2 levels (20). VEGFR-2 stimulation leads to vasculogenesis in embryonic development and increased endothelial cell migration, proliferation, and tubule formation in tumor vasculature (21–23). Moreover, high expression of VEGFRs in tumor and endothelial cells supports the basis of anti-VEGFR-targeted cancer therapy (15, 24–26). Recently, we reported that inhibition of host and tumor VEGFR-2 activity decreased tumor growth and angiogenesis in a murine model of advanced ovarian car-cinoma (15). Others have reported similar findings with VEGFR-2 blockade in ovarian carcinoma (27, 28). These and other reports led us to investigate the efficacy of

targeting VEGFR-2 utilizing a small molecular tyrosine kinase inhibitor, pazopanib, in orthotopic ovarian cancer models.

The combination of biological agents and cytotoxic therapy is a unique and more recently proposed method for treating cancer patients. In the current study, we chose to test the effects of metronomic therapy with oral topotecan in combination with pazopanib. Pazopanib is a potent angiogenic small molecule inhibitor of the tyro-

sine kinases VEGRF-1, VEGRF-2, VEGRF-3, platelet-derived growth factor receptor (PDGFR), and c-kit (19, 29, 30). We and others have shown that pazopanib therapy reduces endothelial cell proliferation and migration (19). The effects of VEGFR-2 inhibition in ovarian tumor cells has also been reported. For example, Spannuth et al. reported that ovarian cancer cell migration was reduced using a monoclonal antibody targeting VEGFR-2 (15). Here, we show similar findings following pazopanib

Figure 4. Antiangiogenic effects of pazopanib therapy. A, murine endothelial cell migration *in vitro* following pazopanib therapy with VEGF stimulation. Bars, SD. *, $P < 0.05$ versus VEGF-treated cells. B, microvessel density analyses of HeyA8 (left) and SKOV3ip1 (right) tumors following pazopanib (paz) and metronomic topotecan (topo) therapy. C, representative photographs of CD31 staining in SKOV3ip1 tumors (original magnification ×100). D, pericyte coverage analysis of SKOV3ip1 tumors. Bars, SE. *, $P < 0.05$ versus control. δ, $P < 0.05$ versus all other groups.

Figure 5. Tumor cell proliferation and apoptosis analyses following *in vivo* therapy. A, Ki-67 staining in HeyA8 (left) and SKOV3ip1 (right) tumors following pazopanib and metronomic topotecan. B, representative photographs of Ki-67 staining in SKOV3ip1 tumors (original magnification ×100). Bars, SE. C, TUNEL analysis of tumor cell apoptosis in HeyA8 tumors. Bars, SD. *, $P < 0.05$ versus control. δ, $P < 0.05$ versus all other groups.
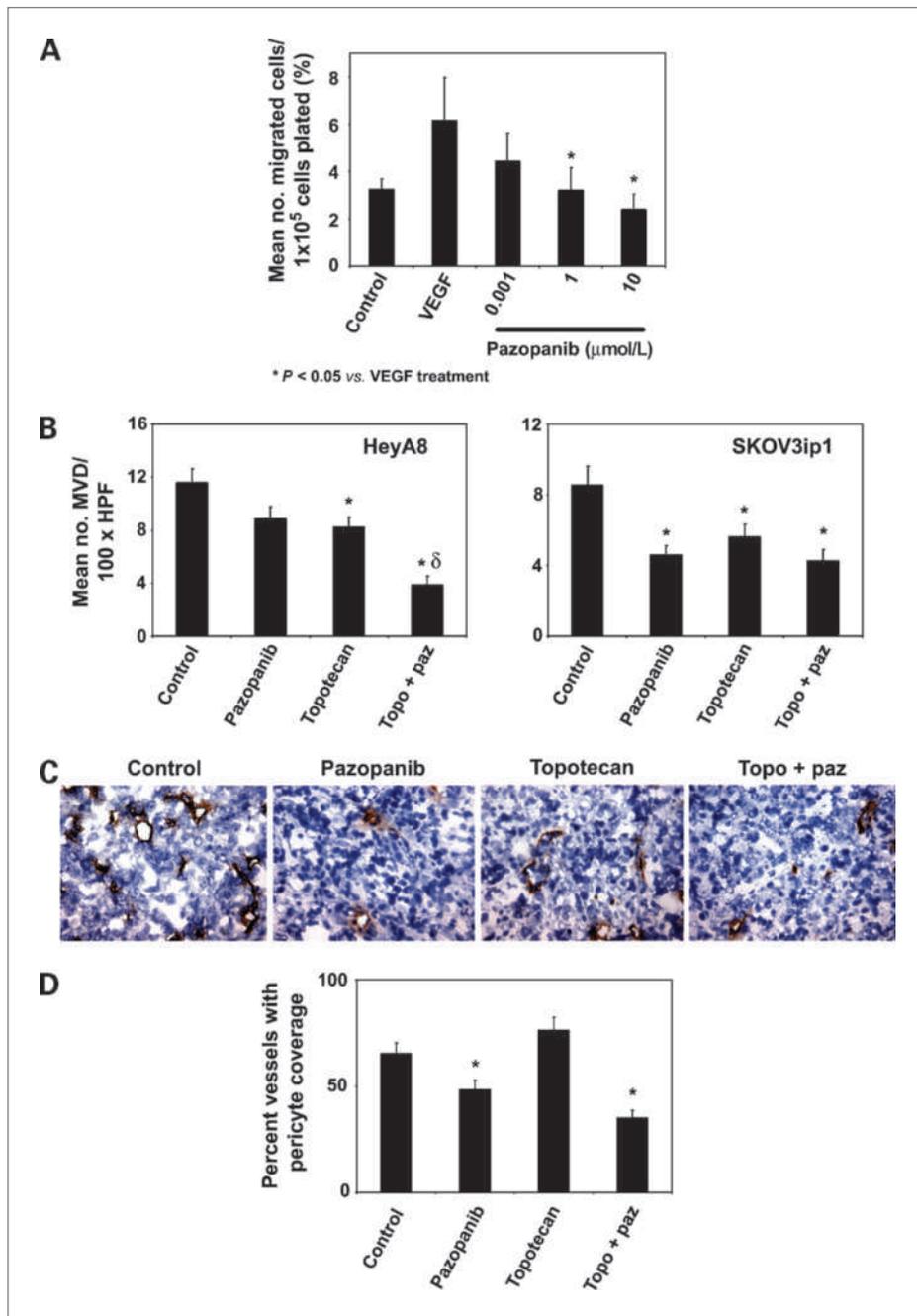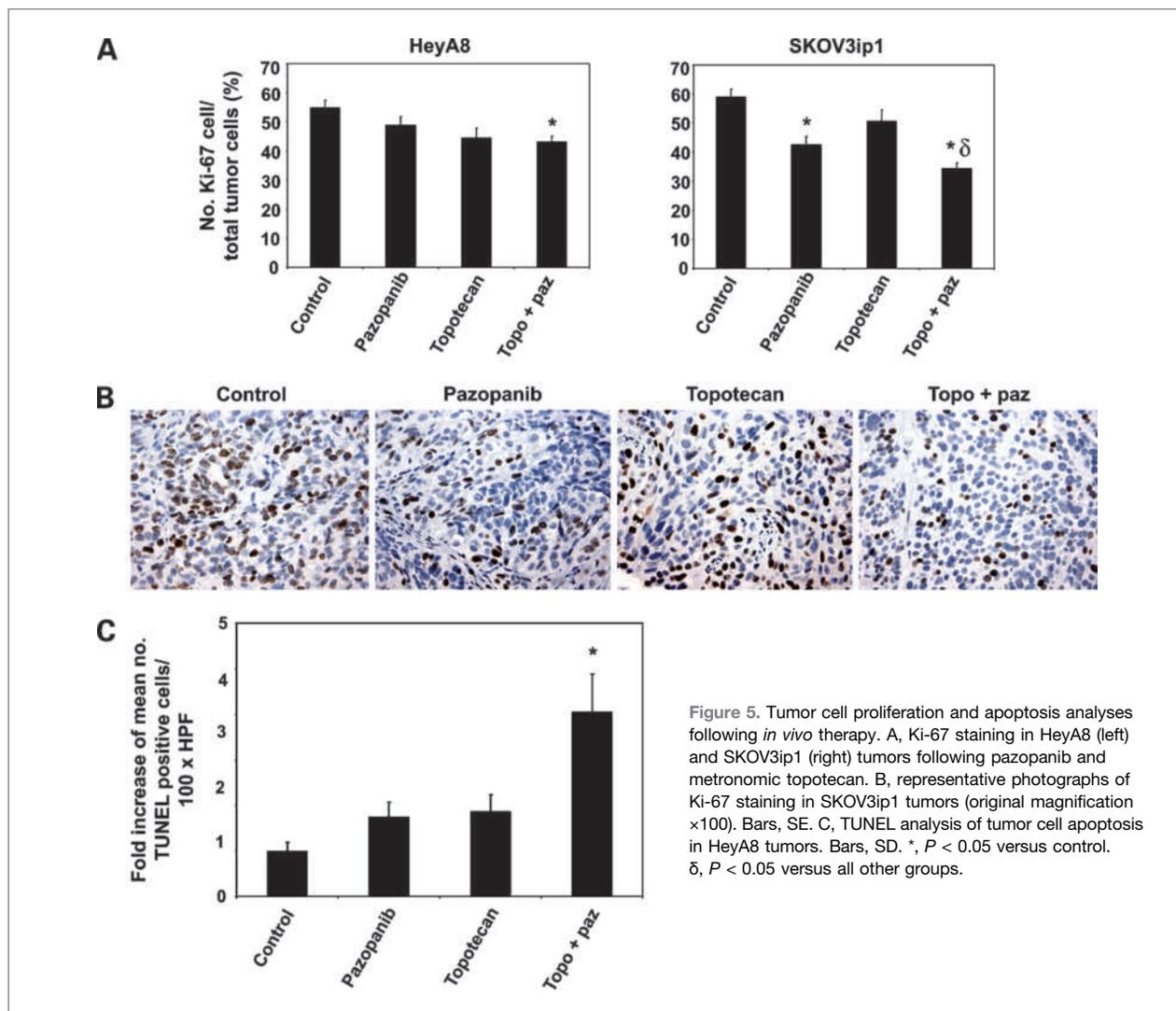
therapy. VEFGR-2 phosphorylation in the SKOV3ip1 and HeyA8 cell lines was reduced following treatment with pazopanib; however, there were differences between these cell lines with regard to the dose required to reduce phosphorylation. These findings would suggest that pazopanib not only affects endothelial cells, but may also have direct effects on tumor cells. Pazopanib has also been shown effective in previous phase I, II, and III clinical trials (29, 31, 32). For example, in a large study of 225 patients with advanced or metastatic renal cell carcinoma taking single-agent pazopanib (800 mg daily), 27% experienced a partial response whereas approximately 46% were found to have stable disease at first analysis (33). These results were further confirmed in the recently reported phase III study in advanced renal cell carcinoma, where patients treated with pazopanib had a median progression-free survival of 9.2 months compared with 4.2 months for the placebo arm (32). In another smaller phase II trial of single-agent pazopanib (800 mg daily) in patients with advance ovarian, fallopian tube, or peritoneal carcinomas, 25% of the patients showed stable disease at >100 days after starting therapy (34). All of these studies report acceptable tolerability with minimal side effects from therapy. Together, these data suggest the feasibility of combining pazopanib with metronomic cytotoxic therapy.

In addition to the advantage of low toxicity with sustained "tumor cell kill," metronomic dosing of cytotoxic agents has shown significant antiangiogenic properties partially secondary to increased endothelial cell apoptosis and decreased resistance (8, 9, 35). For example, Kamat and colleagues showed that metronomic taxane therapy alone and in combination with AEE788, a dual epidermal growth factor receptor and VEGFR, led to

significant tumor growth inhibition and decreased tumor angiogenesis in an orthotopic murine ovarian cancer model (6). Recently, we showed the antitumor efficacy of oral metronomic topotecan in ovarian cancer (10). Moreover, metronomic topotecan therapy led to downstream target modulation of key proangiogenic factors, VEGF and Hif-1α, resulting in decreased angiogenesis and tumor growth *in vivo* (10); however, it does not seem to affect VEGFR-2 phosphorylation. Here, we report that pazopanib therapy resulted in antitumor and antiangiogenic activity in preclinical models of ovarian cancer. Moreover, when combined with topotecan, there was substantial tumor growth inhibition and improvement in survival of tumor-bearing mice. Previously, we showed that metronomic and MTD dosing were equally effective in reducing tumor growth; however, metronomic therapy showed superior antiangiogenic activity (10). Interestingly, metronomic therapy seemed more efficacious compared with MTD therapy when combined with pazopanib. Although a clear benefit with the combination therapy was observed in all models, differences in response to both single-agent and combination therapy were observed. In support of these data, Kerbel and colleagues report equivalent efficacy using metronomic topotecan in combination with pazopanib in an ovarian cancer model (36). In a recent study, Rapisarda and colleagues also showed significant antitumor and antivascular activity with combination therapy using an anti-VEGF antibody, bevacizumab, with metronomic topotecan therapy (37). Indeed, most VEGFR-targeted small molecule inhibitors and antibodies result in increased systemic VEGF, which is considered in part to be a homeostatic response to overcome receptor inhibition by increased ligand production. This is most likely mediated by increased hypoxia due to inhibition of vascular permeability and angiogenesis by VEGFR inhibitors (38). Our findings of decreased pericyte coverage, thereby making the endothelium vulnerable to antiangiogenic therapy, may further increase the extent of hypoxia in the tumor microenvironment. The decrease in pericyte coverage is expected given the anti-PDGFR effects of pazopanib, and has been noted previously with targeting this pathway (39). Together, these findings favor the selection of combining a Hif-1α inhibitor in combination with an antiangiogenic agent targeting VEGFR activity, such as pazopanib, given it's mechanism of action.

There are limited reports of metronomic therapy with biological antiangiogenic agents. Klement and colleagues reported that low-dose metronomic vinblastine therapy with a VEGFR-2 inhibitor (DC101) decreased tumor vessel formation and complete resolution of large neuroblastoma tumors *in vivo* (40). In a recent clinical trial, bevacizumab and metronomic cyclophosphamide resulted in a 24% overall response rate and survival of 17 months in patients with recurrent ovarian carcinoma (5). The findings in the current study provide evidence that metronomic therapy with pazopanib may provide a unique therapeutic strategy for cancer patients, offering potent antitumor activity with less treatment-delaying toxicity.

The addition of biologically targeted agents and novel cytotoxic regimens, such as metronomic therapy, begs the question of whether preclinical and clinical studies are accurately addressing tumor/host response. This issue may directly impose limitations in accurately assessing clinical and preclinical studies as presented here. The "gold standard" of addressing tumor response has been tumor measurement, usually by imaging studies, and direct assessment of tumor weights and spread is common practice in preclinical studies. Here, we present a novel therapeutic regimen with a VEGFR inhibitor and metronomic topotecan therapy. Based on tumor weight alone, one of the two models showed significant tumor growth inhibition when analyzing combination therapy compared with other arms, but antiangiogenic activity was significantly decreased in both models. Here, we showed antiangiogenic activity using traditional measurements of immunohistochemistry although we recognize there are limitations and alternatives to this method (41). Moreover, immunostaining analyses showed downstream effects on the tumor microenvironment, but these methods may not be as accessible in patients receiving biological agents. Serkova and colleagues recently published a review discussing radiologic assessment of treatment response in patients receiving novel cancer therapies (42). They stressed the importance of exploring newer protocols that can assess common end points of targeted therapy, such as, angiogenesis, cell density, proliferation, apoptosis, hypoxia, tumor perfusion, and glucose uptake. Although not included in the current study, we are currently investigating modalities that may assist in addressing these end points of tumor response in important pathways of cancer biology. These differences may allow a better evaluation of treatment response with not only antiangiogenic, but also other novel biological agents in preclinical and clinical studies in the future.

In summary, we have shown that oral pazopanib therapy modulated VEGFR-2 activity in tumor and endothelial cells in ovarian cancer models. Furthermore, a novel therapeutic regimen utilizing oral metronomic topotecan and pazopanib significantly reduced tumor growth and angiogenesis, while prolonging animal survival. Changes in the tumor microenvironment with combination therapy were shown by decreased tumor cell proliferation and increased tumor cell apoptosis *in vivo*. Decreased endothelial cell migration by pazopanib and modulation of key angiogenic regulators by metronomic topotecan therapy suggest this regimen is highly active against tumor vasculature and warrants evaluation in future clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–6.
2. Spannuth WA, Sood AK, Coleman RL. Angiogenesis as a strategic target for ovarian cancer therapy. Nat Clin Pract Oncol 2008;5:194–204.
3. McGuire WP, Hoskins WJ, Brady MF, et al. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III, stage IV. ovarian cancer. N Engl J Med 1996;334:1–6.
4. Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. Nat Rev Cancer 2004;4:423–36.
5. Garcia AA, Hirte H, Fleming G, et al. Phase II clinical trial of bevacizumab and low-dose metronomic oral cyclophosphamide in recurrent ovarian cancer: a trial of the California, Chicago, and Princess Margaret Hospital phase II consortia. J Clin Oncol 2008;26:76–82.
6. Kamat AA, Kim TJ, Landen CN, Jr., et al. Metronomic chemotherapy enhances the efficacy of antivascular therapy in ovarian cancer. Cancer Res 2007;67:281–8.
7. 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.
8. 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.
9. Tonini G, Schiavon G, Silletta M, Vincenzi B, Santini D. Antiangiogenic properties of metronomic chemotherapy in breast cancer. Future Oncol 2007;3:183–90.
10. Merritt WM, Danes CG, Shahzad MM, et al. Anti-angiogenic properties of metronomic topotecan in ovarian carcinoma. Cancer Biol Ther 2009;8:1596–603.
11. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003;9:669–76.
12. Yamamoto S, Konishi I, Mandai M, et al. Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels. Br J Cancer 1997;76:1221–7.
13. Cooper BC, Ritchie JM, Broghammer CL, et al. Preoperative serum vascular endothelial growth factor levels: significance in ovarian cancer. Clin Cancer Res 2002;8:3193–7.
14. Ishigami SI, Arii S, Furutani M, et al. Predictive value of vascular endothelial growth factor (VEGF) in metastasis and prognosis of human colorectal cancer. Br J Cancer 1998;78:1379–84.
15. Spannuth WA, Nick AM, Jennings NB, et al. Functional significance of VEGFR-2 on ovarian cancer cells. Int J Cancer 2009;124:1045–53.
16. Merritt WM, Lin YG, Han LY, et al. Dicer, Drosha, and outcomes in patients with ovarian cancer. N Engl J Med 2008;359:2641–50.
17. Merritt WM, Lin YG, Spannuth WA, et al. Effect of interleukin-8 gene silencing with liposome-encapsulated small interfering RNA on ovarian cancer cell growth. J Natl Cancer Inst 2008;100:359–72.
18. Lu C, Kamat AA, Lin YG, et al. Dual targeting of endothelial cells and pericytes in antivascular therapy for ovarian carcinoma. Clin Cancer Res 2007;13:4209–17.
19. Kumar R, Knick VB, Rudolph SK, et al. Pharmacokinetic-pharmacodynamic correlation from mouse to human with pazopanib, a multikinase angiogenesis inhibitor with potent antitumor and antiangiogenic activity. Mol Cancer Ther 2007;6:2012–21.
20. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997;18:4–25.
21. Shibuya M. Vascular endothelial growth factor (VEGF)-Receptor2: its biological functions, major signaling pathway, and specific ligand VEGF-E. Endothelium 2006;13:63–9.
22. Shibuya M. Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. J Biochem Mol Biol 2006;39:469–78.
23. Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp Cell Res 2006;312:549–60.
24. Chen H, Ye D, Xie X, Chen B, Lu W. VEGF, VEGFRs expressions and activated STATs in ovarian epithelial carcinoma. Gynecol Oncol 2004;94:630–5.
25. Plate KH, Breier G, Millauer B, Ullrich A, Risau W. Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. Cancer Res 1993;53:5822–7.
26. Wu Y, Hooper AT, Zhong Z, et al. The vascular endothelial growth factor receptor (VEGFR-1) supports growth and survival of human breast carcinoma. Int J Cancer 2006;119:1519–29.
27. Garofalo A, Naumova E, Manenti L, et al. The combination of the tyrosine kinase receptor inhibitor SU6668 with paclitaxel affects ascites formation and tumor spread in ovarian carcinoma xenografts growing orthotopically. Clin Cancer Res 2003;9:3476–85.
28. Sallinen H, Anttila M, Narvainen J, et al. Antiangiogenic gene therapy with soluble VEGFR-1, -2, and -3 reduces the growth of solid human ovarian carcinoma in mice. Mol Ther 2009;17:278–84.
29. Sloan B, Scheinfeld NS. Pazopanib, a VEGF receptor tyrosine kinase inhibitor for cancer therapy. Curr Opin Investig Drugs 2008;9:1324–35.
30. Podar K, Tonon G, Sattler M, et al. The small-molecule VEGF receptor inhibitor pazopanib (GW786034B) targets both tumor and endothelial cells in multiple myeloma. Proc Natl Acad Sci U S A 2006;103:19478–83.
31. Sleijfer S, Ray-Coquard I, Papai Z, et al. Pazopanib, a multikinase angiogenesis inhibitor, in patients with relapsed or refractory advanced soft tissue sarcoma: a phase II study from the European Organisation for Research and Treatment of Cancer-Soft Tissue and Bone Sarcoma Group (EORTC Study 62043). J Clin Oncol 2009;27:3126–32.
32. Sternberg CN, Szczylik C, Lee E, et al. A randomized, double-blind phase III study of pazopanib in treatment-naive and cytokine-pretreated patients with advanced renal cell carcinoma (RCC). J Clin Oncol 2009;27, Abst. 5021.
33. Hutson TE, Davis ID, Machiels JP, et al. Pazopanib (GW-786034) is active in metastatic renal cell carcinoma (RCC): interim results of a Phase II randomized discontinuation trial (RDT). J Clin Oncol (Meeting Abstracts) 2007;25:5031.
34. Friedlander M, Hancock KC, Benigno B, et al. Pazopanib (GW-786034) is active in women with advanced epithelial ovarian, fallopian tube, and peritoneal cancers: results of a phase II study. Ann Oncol 2008;19:viii211–6.
35. Drevs J, Fakler J, Eisele S, et al. Antiangiogenic potency of various chemotherapeutic drugs for metronomic chemotherapy. Anticancer Res 2004;24:1759–63.

36. Hashimoto K, Man S, Xu P, et al. Potent preclinical impact of metronomic low-dose oral topotecan combined with the antiangiogenic drug pazopanib for the treatment of ovarian cancer. Mol Cancer Ther 2010;9:996–1006.

37. Rapisarda A, Hollingshead M, Uranchimeg B, et al. Increased antitumor activity of bevacizumab in combination with hypoxia inducible factor-1 inhibition. Mol Cancer Ther 2009;8:1867–77.

38. Loges S, Mazzone M, Hohensinner P, Carmeliet P. Silencing or fueling metastasis with VEGF inhibitors: antiangiogenesis revisited. Cancer Cell 2009;15:167–70.

39. Lu C, Thaker PH, Lin YG, et al. Impact of vessel maturation on antiangiogenic therapy in ovarian cancer. Am J Obstet Gynecol 2008;198:477, e1–9; discussion e9–10.

40. 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.

41. Merritt WM, Sood AK. Markers of angiogenesis in ovarian cancer. Dis Markers 2007;23:419–31.

42. Serkova NJ, Garg K, Bradshaw-Pierce EL. Oncologic imaging endpoints for the assessment of therapy response. Recent Pat Anticancer Drug Discov 2009;4:36–53.

Molecular Cancer Therapeutics

Bridging the Gap between Cytotoxic and Biologic Therapy with Metronomic Topotecan and Pazopanib in Ovarian Cancer

William M. Merritt, Alpa M. Nick, Amy R. Carroll, et al.

Updated version	Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-09-0967
Supplementary Material	Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2010/04/05/1535-7163.MCT-09-0967.DC1

Cited articles	This article cites 41 articles, 14 of which you can access for free at: http://mct.aacrjournals.org/content/9/4/985.full#ref-list-1
Citing articles	This article has been cited by 8 HighWire-hosted articles. Access the articles at: http://mct.aacrjournals.org/content/9/4/985.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/9/4/985. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.