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Bridging the gap between cytotoxic and biologic therapy with metronomic topotecan and pazopanib in ovarian cancer

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Abstract

To investigate the anti-tumor and anti-angiogenic effects utilizing a novel therapy regimen of metronomic topotecan and pazopanib, a multi-receptor tyrosine kinase inhibitor. *In vitro* (Western blot) and *in vivo* dose-finding experiments were performed 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 tumor microenvironment (angiogenesis [CD31 and pericyte coverage], proliferation [Ki-67], and apoptosis [TUNEL]) were analyzed by immunostaining following therapy. Pazopanib therapy reduced VEGFR-2 activity *in vitro* and *vivo* in a dose-dependent manner. Compared to control mice, pazopanib reduced tumor weight by 28-82% ($p < 0.01$ in the SKOV3ip1 model) and metronomic topotecan reduced tumor weight by 40-59% in the HeyA8 ($p = 0.13$) and SKOV3ip1 ($p = 0.07$) models. Combination therapy had greatest effect with 79-84% reduction ($p < 0.01$ for both models). In the SKOV3ip1 and A2780 models, mouse survival was significantly longer ($p < 0.001$ vs. 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 to 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 demonstrated significant anti-tumor and anti-angiogenic properties in preclinical ovarian cancer models and warrants further investigation as a novel therapeutic regimen in clinical trials.

Keywords

VEGFR-2; ovarian carcinoma; pazopanib; angiogenesis

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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 targeting tumor vasculature or angiogenesis. Folkman first introduced concepts regarding 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, challenging the combination of agents dosed in this manner is treatment-limiting toxicities, acutely and chronically. 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, 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 demonstrates anti-angiogenic properties secondary to continued exposure on tumor endothelial cells (8,9). On the basis of Hif-1 α inhibition, we recently reported that metronomic topotecan had similar anti-tumor activity compared to the traditional maximally tolerated dosing and significantly decreased tumor vascularity in murine models of advanced ovarian cancer (10). We hypothesized that metronomic topotecan will result in greater anti-tumor activity in combination with other anti-angiogenic agents. The anti-tumor and anti-vascular benefits of VEGF-targeted therapy are promising and offer opportunities for further investigation. Vascular endothelial growth factor (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 anti-tumor and anti-angiogenic efficacy of metronomic topotecan with a multi-receptor kinase inhibitor, pazopanib, in ovarian cancer models.

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 (FBS) and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). All experiments were performed using cells grown to 60%–80% confluence, and all cell lines were routinely tested to confirm absence of *Mycoplasma*.

Pazopanib (GlaxoSmithKline, Philadelphia, PA) was mixed in DMSO (Sigma, St. Louis, MO) 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 10mM 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 *Policy on Human Care and Use of Laboratory Animals*, and all studies were approved and supervised by the Institutional Animal Care and Use Committees at either M.D. Anderson Cancer Center or GlaxoSmithKline. The ovarian cancer cell lines were maintained and prepared for *in vivo* intraperitoneal injection as previously described for the orthotopic model by our group (17). For the A2780 survival experiment, 10 million cells were injected intraperitoneally one week 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 days. Mice were sacrificed at 4, 24, 48, and 72 hours following 72 h of therapy. Tumors were snap frozen in optimal cutting medium (OCT; Miles, Inc., Elkhart, IN) for immunofluorescence analysis.

For HeyA8 and SKOV3ip1 therapy models, mice were randomly separated and assigned to treatment groups (n=10/group) approximately 1 week 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 performed using the SKOV3ip1 and A2780 cell line models. Mice were inoculated intraperitoneally with either SKOV3ip1 or A2780 cells as in the therapy models. In the SKOV3ip1 model, mice (n=10/group) were treated with similar regimens as in the previous therapy experiment. In the A2780 model, mice (n=10/group) were treated with the following regimens: vehicle 1 (HPMC/Tween), vehicle 2 (tartaric acid), pazopanib 100 mg/kg (oral, twice daily), maximum tolerated dose (MTD) topotecan 5 mg/kg (IV, daily $\times$ 5 d every 21 d), MTD topotecan 10 mg/kg (IV, every 7 d $\times$ 3 doses), metronomic topotecan 1 mg/kg (oral, daily), pazopanib + topotecan 5 mg/kg, pazopanib + topotecan 10 mg/kg, or pazopanib + 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 μ M) for approximately 3-4 h or topotecan (6.25-100 nM) for 12 h. In the pazopanib assay, VEGF-A (10 ng/ml; Invitrogen, Carlsbad, CA) was added for 1 h prior to collecting cell lysates. In both assays, cell lysates were collected in RIPA lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton, 0.5% deoxycholate plus 25 μ g/mL leupeptin, 10 μ g/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 determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). Protein bands were separated by 10% SDS-PAGE and transferred to nitrocellulose paper, blocked with 5% milk for 2 h at room temperature (RT), incubated with either rabbit anti-human VEGFR-2 antibody Tyr (Santa Cruz Biotechnology, Santa Cruz, CA)

or rabbit anti-human phospho-VEGFR 2/3 (Calbiochem, San Diego, CA) overnight at 4°C followed by incubation with appropriate secondary anti-rabbit horseradish peroxidase-conjugated antibody for 2 h at RT. 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 performed 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 re-suspended in serum-free media (SFM) 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 SFM for approximately 12 h and then treated with pazopanib (0.001, 1, and 10 μ M) for 4-5 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 SFM and VEGF-A (Invitrogen). Chambers were incubated for 8 h at 37°C. At completion, cells in 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 performed in duplicate.

Immunohistochemistry

Immunofluorescent staining of VEGFR-2 was performed on 4- μ m-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, incubated with appropriate anti-rabbit secondary antibody. Images were taken using a Zeiss Axioplan2 microscope (Carl Zeiss, Inc., Thornwood, NY) with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Hamamatsu City, Japan) and captured with ImagePro Plus software v5.1 (MediaCybernetics, Bethesda, MD). Immunohistochemical analyses of CD31 (primary antibody: rat monoclonal anti-mouse, 1:800 dilution, BD Bioscience, Pharmingen, San Jose, CA) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) were conducted on orthotopic specimens as described previously (17). Ki-67 staining was performed 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, Concord, CA) at 4°C, 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 performed following incubation with 3,3'-diaminobenzidine substrate for 10 min. For pericyte coverage analysis, dual immunohistochemical staining for CD31 and alpha smooth muscle actin (α SMA) was performed on paraffin sections. Following antigen retrieval with citrate buffer, sections were incubated with α SMA antibody (Abcam, Cambridge, MA) overnight at 4°C and then with horseradish peroxidase (HRP)-conjugated IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Slides were then stained with DAB, washed, and incubated with CD31 antibody (BD Bioscience) overnight at 4°C, followed by incubation with rat probe and HRP polymer (BioCare, Concord, CA). Lastly, slides were stained with Ferangi Blue Chromogen (BioCare, Concord, CA). Quantification of microvessel density (CD31), tumor cell proliferation (Ki-67), apoptosis (TUNEL) and pericyte coverage was performed on slides from each treatment group, as previously described (17,18). Images were taken with a Nikon Microphot-FXA microscope (Nikon Instruments, Melville, NY) and Leica DFC-320 camera (Leica Microsystems, Bannockburn, IL) 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 analysis of variance (for multiple group comparisons). Normality of weight distributions was tested by Kolmogorov-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* values less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 12 for Windows statistical software (SPSS, Inc., Chicago, IL).

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 more apparent at higher doses $\geq 1 \mu\text{M}$ doses in the SKOV3ip1 cell line (Figure 1A). In HeyA8 cells, pazopanib therapy also decreased VEGFR-2 phosphorylation, but at higher doses compared to the SKOV3ip1 cells (data not shown). Topotecan treatment alone had no effects on VEGFR-2 phosphorylation in SKOV3ip1 and HeyA8 cells (Supplementary Figure 1). *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 h after treatment, a noticeable decrease in VEGFR-2 phosphorylation on tumor cells was observed in mice treated with pazopanib at 100 mg/kg compared to controls (Figure 1B). Modulation of phosphorylated VEGFR-2 expression was still evident up to 48 h following treatment until returning to baseline at 72 h. The lower pazopanib dose (30 mg/kg) demonstrated no observable effect on VEGFR-2 phosphorylation (data not shown).

Effect of metronomic topotecan and anti-angiogenesis therapy in an orthotopic ovarian cancer model

To determine the effects of pazopanib on ovarian cancer growth, we performed 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% (vs. controls, $p=0.22$) and 82% ($p=0.007$) in the HeyA8 and SKOV3ip1 models, respectively (Figure 2). Consistent with our previous report, metronomic topotecan therapy reduced mean tumor weights by 40% (HeyA8, $p=0.13$) and 59% (SKOV3ip1, $p=0.07$) compared to mice treated with vehicle only. The greatest reduction in tumor weight was observed in mice treated with combination therapy. When compared to controls, a 79-84% ($p<0.01$ for both models) reduction was noted with pazopanib and metronomic topotecan. In comparison to single agent therapy in the HeyA8 model, combination therapy inhibited tumor growth by 70% (vs. pazopanib, $p<0.01$) and 64% (vs. metronomic topotecan, $p=0.22$). In the SKOV3ip1 model, single agent pazopanib therapy exhibited similar anti-tumor activity as combination therapy (12% difference in mean tumor weight, $p=\text{NS}$). 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 anti-angiogenesis treatment on animal survival. For these experiments, mice inoculated with either SKOV3ip1 or A2780 cells were treated with regimens as described above until 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 to controls; however, the greatest effect was observed in the combination therapy arm ($p < 0.001$ vs. all other groups; Figure 3A). To validate these findings, a separate survival experiment was performed 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 (Figure 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 ($p < 0.001$ vs. pazopanib alone; $p = 0.09$ vs. MTD topotecan 10 mg/kg + pazopanib arm).

Anti-angiogenic 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* (Figure 4A). Following pazopanib treatment, migration of MOECs was analyzed after stimulation with VEGF. VEGF alone increased endothelial cell migration (90%) compared to untreated cells. With addition of pazopanib therapy, migration of MOECs decreased in a dose-dependent manner with concentrations ranging from 0.001 μ M - 10 μ M (28 - 61% reduction vs. VEGF stimulated cells; $p < 0.05$ for 1 and 10 μ M doses).

With the known anti-angiogenic activity of both pazopanib and metronomic topotecan, we analyzed the tumors from both treatment experiments for microvessel 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 (Figure 4B). In the SKOV3ip1 tumors, both single agent therapies significantly reduced MVD (pazopanib 46% reduction vs. controls, $p = 0.03$; topotecan 34% reduction, $p = 0.04$; Figure 4B & 4C). 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 (Figure 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 significant decrease in pericyte coverage compared to controls ($p < 0.05$).

Alterations in tumor microenvironment with pazopanib and topotecan therapy

To determine the effects of pazopanib and metronomic therapy on other critical components of the tumor microenvironment, we examined tumor cell migration, proliferation and apoptosis. Following treatment with pazopanib, SKOV3ip1 cell migration decreased by 68% ($p < 0.01$ compared to controls; Supplemental Figure 2). Examination of tumors from the *in vivo* therapy experiments demonstrated that single agent pazopanib therapy reduced tumor cell proliferation in the SKOV3ip1 ($p < 0.001$; Figure 5A) and HeyA8 ($p = 0.18$; Figure 5A & 5B) models compared to controls. Metronomic topotecan therapy was effective in both SKOV3ip1 (14% reduction vs. controls; $p = 0.085$) and HeyA8 (19%, $p = 0.35$) models. Combination therapy exhibited the greatest effect over controls with 22-42% reduction ($p < 0.01$).

Tumor cell apoptosis was examined in tumors from the HeyA8 therapy experiment by TUNEL immunofluorescence analysis (Figure 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, respectively.

Combination therapy significantly increased apoptosis (4.1 fold vs. controls, $p=0.001$) and remained superior when compared to other single agent therapies ($p<0.05$ 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 to controls and single agent regimens. These effects likely reflect anti-angiogenic properties of both agents.

Tumor angiogenesis is led by an increase in pro-angiogenic 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 carcinoma (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 biologic 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 tyrosine kinases VEGFR-1, -2, -3, 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 therapy. VEGFR-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 affects not only 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 (RCC) taking single agent pazopanib (800 mg daily), 27% experienced a partial response while 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 RCC, where patients treated with pazopanib had a median progression free survival (PFS) of 9.2 months compared to 4.2 months for the placebo arm (32). In another smaller phase II trial of single agent pazopanib (800 mg daily) in patients with advanced ovarian, fallopian tube, or peritoneal carcinomas, 25% of the patients demonstrated stable disease at more than 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 demonstrated significant anti-angiogenic properties partially secondary to increased endothelial cell apoptosis and decreased resistance (8,9,35). For example, Kamat and colleagues demonstrated 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 demonstrated the anti-tumor efficacy of oral metronomic topotecan in ovarian cancer (10). Moreover, metronomic topotecan therapy led to downstream target modulation of key pro-angiogenic factors, VEGF and Hif-1 α , resulting in decreased angiogenesis and tumor growth *in vivo* (10); however, it does not appear to affect VEGFR-2 phosphorylation. Here, we report that pazopanib therapy resulted in anti-tumor and anti-angiogenic 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 have demonstrated that metronomic and MTD dosing were equally effective in reducing tumor growth; however, metronomic therapy demonstrated superior anti-angiogenic activity (10). Interestingly, metronomic therapy appeared more efficacious compared to 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 (Hashimoto et al. - accompanying article). In a recent study, Rapisarda and colleagues also demonstrated significant anti-tumor and anti-vascular activity with combination therapy using an anti-VEGF antibody, bevacizumab, with metronomic topotecan therapy (36). 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 (37). Our findings of decreased pericyte coverage and thereby making the endothelium vulnerable to anti-angiogenic 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 have been noted previously with targeting this pathway (38). Together, these findings favor the selection of combining a Hif-1 α inhibitor in combination with an anti-angiogenic agent targeting VEGFR activity, such as pazopanib, given its mechanism of action.

There are limited reports of metronomic therapy with biologic anti-angiogenic 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* (39). In a recent clinical trial, bevacizumab and metronomic cyclophosphamide resulted in a 24% overall response rate and survival of 17 months in patient 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 patient offering potent anti-tumor activity with less treatment-delaying toxicity.

The addition of biologically targeted agents and novel cytotoxic regimens, such as metronomic therapy, beg the question of whether pre-clinical and clinical studies are accurately addressing tumor/host response. This issue may directly impose limitations in accurately assessing clinical and pre-clinical 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 pre-clinical 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 to other arms, however, anti-angiogenic activity was significantly decreased in both models. Here, we demonstrated anti-angiogenic activity using traditional measurements of immunohistochemistry though we recognize there are limitations and alternatives to this method (40). Moreover, immunostaining analyses demonstrated downstream effects on the tumor microenvironment, but these methods may not be as accessible in patients receiving biologic agents. Serkova and colleagues recently published a

review discussing radiologic assessment of treatment response in patients receiving novel cancer therapies (41). They stressed the importance of exploring newer protocols that can assess common endpoints 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 endpoints of tumor response in important pathways of cancer biology. These differences may allow a better evaluation of treatment response with not only anti-angiogenic, but other novel biologic agents in pre-clinical and clinical studies in the future.

In summary, we have demonstrated 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 demonstrated 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 suggests this regimen is highly active against tumor vasculature and warrants evaluation in future clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Inhibition of VEGFR-2 phosphorylation in ovarian cancer cells. **A**) Western blot analysis of SKOV3ip1 cells treated with pazopanib for 3-4 hours followed by VEGF-A stimulation (10 ng/ml). Lysates were probed for total (tVEGFR-2) and phosphorylated (pVEGFR-2) VEGFR-2. Actin used as loading control. Densitometry analysis (below) demonstrating percent decrease in pVEGFR-2 relative to VEGF only stimulated cells. **B**) SKOV3ip1 tumor bearing mice were treated with pazopanib (100 mg/kg; daily $\times$ 3 days), sacrificed at respective time points. Photomicrographs (original magnification $\times$ 100) illustrate tumor cells (blue) and phosphorylated VEGFR-2 (red).

Figure 2.

Effect of pazopanib and metronomic topotecan therapy in **A)** HeyA8 and **B)** SKOV3ip1 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). (Mean tumor weights (left panels) and tumor weight distribution (right panels) are shown. Error bars represent SEM. *p<0.05 compared to the control group.

Figure 3.

Kaplan-Meier survival curve illustrating effects of pazopanib and metronomic topotecan 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 vs. all other groups. **B)** A2780 model comparing: vehicle 1, pazopanib (100 mg/kg daily), maximum tolerated dose (MTD) topotecan 5 mg/kg (daily $\times$ 5d every 21 days), MTD topotecan 10 mg/kg (every 7d $\times$ 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 vs. paz alone, respectively. $p = 0.09$ MTD topo 10 + paz vs. metro topo 1 + paz.

Figure 4.

Anti-angiogenic effects of pazopanib therapy. **A)** Murine endothelial cell migration *in vitro* following pazopanib therapy with VEGF stimulation. Bars represent SD. * p<0.05 vs. VEGF treated cells **B)** Microvessel density analyses of HeyA8 (left) and SKOV3ip1 (right) tumors following pazopanib and metronomic topotecan therapy **C)** Representative photographs of CD31 staining in SKOV3ip1 tumors (original magnification 100 x) **D)** Pericyte coverage analysis of SKOV3ip1 tumors. Bars represent SEM. * p<0.05 vs. control. δ p<0.05 vs. 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 x). Bars represent SEM **C)** TUNEL analysis of tumor cell apoptosis in HeyA8 tumors. Bars represent standard deviation. * $p < 0.05$ vs. control. $\delta p < 0.05$ vs. all other groups