Cancer Therapy: Preclinical

Metronomic Oral Topotecan with Pazopanib Is an Active Antiangiogenic Regimen in Mouse Models of Aggressive Pediatric Solid Tumor

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Abstract

Purpose: Low dose metronomic (LDM) chemotherapy, combined with VEGF signaling pathway inhibitors, is a highly effective strategy to coordinately inhibit angiogenesis and tumor growth in many adult preclinical cancer models. We have tested the efficacies of daily oral LDM topotecan alone and in combination with pazopanib, a VEGF receptor inhibitor, in three pediatric extracranial solid tumor mouse models.

Experimental Design: In vitro dose–response study of topotecan and pazopanib was conducted on several neuroblastoma, osteosarcoma, and rhabdomyosarcoma cell lines. In vivo antitumor efficacies of the LDM topotecan and pazopanib as single agents and in combination were tested on 4 subcutaneous xenograft models and on 2 neuroblastoma metastatic models. Circulating angiogenic factors such as circulating endothelial cells (CEC), circulating endothelial progenitor cells (CEP), and microvessel densities were used as surrogate biomarker markers of antiangiogenic activity.

Results: In vitro, topotecan caused a dose-dependent decrease in viabilities of all cell lines, while pazopanib did not. In vivo, combination of topotecan + pazopanib (TP + PZ) showed significant antitumor activity and significant enhancement in survival compared with the respective single agents in all models. Reductions in viable CEP and/or CEC levels and tumor microvessel density were correlated with tumor response and therefore confirmed the antiangiogenic activity of the regimens. Pharmacokinetic studies of both drugs did not reveal any drug–drug interaction.

Conclusion: Metronomic administration of TP + PZ showed a statistically significant antitumor activity compared with respective single agents in pediatric tumor mouse models and represent a valid option as a maintenance therapy in aggressive pediatric solid tumors. Clin Cancer Res; 17(17); 5656–67. ©2011 AACR.

Introduction

Neuroblastoma and sarcoma are the most common extracranial childhood solid tumors. Although the 5-year overall survival rates in childhood cancers have reached 80%, the survival rates of a subtype of these aggressive cancers are still below 70% (1). New strategies targeting alternate mechanisms of resistance and minimal residual disease are needed to improve the outcome of these aggressive cancers. Discovery of new maintenance treatment regimens targeting residual disease represent an exciting opportunity to improve survival rate. Most of these recurrent patients would have received dose intensity chemotherapy as an induction or consolidation therapy, therefore a minimally toxic regimen targeting alternate targets should be used in this context.

Angiogenesis is a potential therapeutic target for several types of cancers including pediatric cancers (2). Low dose metronomic (LDM) chemotherapy, which acts in part by targeting the endothelial cells of the neovasculature of tumors and blocking mobilization of endothelial progenitor cells (CEP) from the bone marrow, represents an attractive and effective antiangiogenic strategy (3–5). Drawbacks to using pulse topotecan include resistance of tumor cells and the crossresistance with topoisomerase-II inhibitors in neuroblastoma cell lines (6, 7). LDM regimens of topotecan, which may target endothelial cells and tumor cells by other mechanisms, can possibly overcome some of these limitations.

Despite its advantages, relapses may occur in patients who have initially responded to single agent LDM chemotherapy. VEGF, a survival factor for endothelial cells,
Metronomic Topotecan and Pazopanib in Pediatric Solid Tumors

Translational Relevance

Despite advancements in treatments for pediatric cancers, high mortality is still prevalent in aggressive and advanced stage diseases. Therefore, alternate strategies are needed to improve childhood cancer outcome. The impact of active and abnormal angiogenesis on tumor growth and drug resistance can be exploited to develop new promising therapeutic regimens. In this study, we have tested the overall antitumor efficacy of administration of low dose metronomic (LDM) topotecan alone and its combination with a VEGF receptor tyrosine kinase inhibitor pazopanib in 3 aggressive pediatric extracranial solid tumors mouse models: neuroblastoma, osteosarcoma, and rhabdomyosarcoma. Administration of LDM topotecan showed a significant antitumor efficacy in most tumor types, either localized or metastatic, which was amplified in all tumor types by the adjunction of pazopanib. These results support the development of phase-I clinical trials in these 3 categories of aggressive childhood solid tumors.

may be responsible, at least in part, for the loss of antitumor efficacy of LDM chemotherapy (8). As a result, combining metronomic therapy with agents that target the VEGF-signalling pathways is associated with an overall increase in antitumor activity. We have previously shown the therapeutic advantage of such combinations by combining LDM vinblastine with VEGF receptor 2 (VEGFR2)-neutralizing antibody, DC101, in a human neuroblastoma xenograft preclinical study (9). Such combinations have also been found to be effective in other preclinical and clinical trials (10–12). Recently, 2 independent studies have reported the marked superiority of the combination of daily oral LDM topotecan and the small molecule receptor tyrosine kinase inhibitor (RTKi) pazopanib compared with either single agent therapy in models of advanced ovarian cancer (13, 14). This combination and their relevant pharmacokinetic and pharmacodynamic markers have never been explored in multiple preclinical pediatric tumor models.

The main objective of this study was to compare the antitumor efficacies of LDM topotecan and its combination with pazopanib, a multikinase inhibitor (15) in 3 types of pediatric solid tumors and to evaluate circulating angiogenic factors (CAF) such as viable CEPs and circulating endothelial cells (CEC) and tumor microvesSEL densities as surrogate markers of drug activity. Pharmacokinetic (PK) analysis was conducted as well, to detect possible drug-drug interaction.

Materials and Methods

Drugs and reagents

Topotecan (S)-10-[(dimethylamino)methyl]-4-ethyl-4, 9-dihydropyrido[3,4-d,6,7]indolizino[1,2-b:4,5-d'] quinoline3,14(4H,12H)-dione monohydrochloride and pazopanib (5-[4-(2,3-Dimethyl-2H-indazol-6-yl) methyl amino]-2-pyrimidinyl)amino]-2-methylbenzolsulfonamide were provided by GlaxoSmithKline. Topotecan-d6 was purchased from Toronto Research Chemicals (catalogue # T542502).

Cell lines

SK-N-BE(2) (N-Myc amplified) and SH-SY5Y (non-N-Myc amplified neuroblastoma cell lines; ref. 16), osteosarcoma cell line KHOS and rhabdomyosarcoma cell lines RH30 and RD, human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection; BE(2)-c subclone of SK-N-BE(2) (17) was obtained from Dr. Michelle Haber (Children’s Cancer Institute for Medical Research, Lowry Cancer Research Centre, Randwick, Australia); NUB-7, an I-type neuroblastoma cell line (18) was obtained from Dr. Herman Yeger (The Hospital for Sick Children, Toronto, Ontario). Neuroblastoma cell lines were grown in alpha-MEM (# 310-010-CL, Wisent Bioproducts), while sarcoma cell lines were grown in Dulbecco’s Modified Eagle’s Medium (# 319-010-CL, Wisent bioproducts), both containing 10% FBS and 1% antibiotic mixture in humidified atmosphere at 37°C with 5% CO2.

In vitro cytotoxicity

A total of 50,000 cells were seeded in 48 well plates and incubated for 48 hours, after which they were treated with topotecan and/or pazopanib for 72 hours. Cell viability was determined by Alamar blue assay. Alamar blue (10% of total volume) was added to each well 3 hours before fluorometric detection. Fluorometric detection was done using the SPECTRAMax Gemini Spectrophotometer at excitation wavelength of 540 nm and emission wavelength of 590 nm.

In vivo models

For subcutaneous xenograft studies, we used SK-N-BE(2), SH-SY5Y, KHOS, and RH30. 1 × 10⁶ cells were implanted subcutaneously into the inguinal fat pad of each of nonobese diabetic/severe combined immune deficient (NOD/SCID) mice. When tumors reached 0.5 cm in diameter, the animals were randomized into 4 groups and treated daily by oral gavage. The animals were grouped as: Control group, LDM topotecan group or LDM TP (1.0 mg/kg topotecan), pazopanib group or PZ (150 mg/kg pazopanib) and combination group or TP + PZ (1.0 mg/kg topotecan + 150 mg/kg pazopanib). To compare pulse topotecan with LDM TP in KHOS osteosarcoma model, PZ was replaced by weekly oral dose of pulse topotecan or Pulse TP (15 mg/kg topotecan). The criteria for endpoint were tumor sizes exceeding 2.0 cm in diameter or animals showing signs of morbidity. The tumor sizes were measured on a daily basis until the endpoint or sacrifice. The long (D) and short diameters (d) were measured with calipers. Tumor volume (cm³) was calculated as V = 0.5 × D × d². When the endpoint...
was reached or at the end of the treatment, the animals were sacrificed by cervical dislocation.

**Metastatic mouse model**
A total of $1 \times 10^6$ BE(2)-c cells or NUB-7 cells were injected into lateral tail veins of NOD/SCID mice to generate experimental metastases as previously described (19). Fourteen days after injection, the mice were randomized into 4 groups and treated in same way as the inguinal xenograft model. The treatment was continued until death or endpoint for BE(2)-c model and till 14 days for NUB-7 model.

Protocol and endpoints were approved by the Sickkids animal committee facility following the CACC guidelines.

**Immunohistochemistry and histopathology**
Formalin-fixed tissues were paraffin embedded and sections cut at 7 um. Sections were deparaffinized through xylene and ethanol, rehydrated in PBS (# 311-010-CL, Wisent Bioproducts) and incubated overnight with primary antibodies for von Willebrand factor (vWF; # A0082; DakoCytomation) at 4°C. After the primary antibody treatment, all the slides were washed 3 times with PBS and incubated with broad spectrum poly-horse radish peroxidase (HRP) conjugated secondary antibody (# 87-9663, Invitrogen) for 1 hour at room temperature. After washing 3 times with PBS, slides were stained with diaminobenzidine and counterstained with hematoxylin. Microscopic images were captured by Olympus UTV1-X microscope (##A21207, Invitrogen) for 1 hour at room temperature. After washing before and with Alexa fluor 594 donkey anti-rabbit IgG (#A21207, Invitrogen, dilution 1:300) for 1 hour. After washing with PBS, the slides were mounted with Vectashield mounting medium (#H-1200, Vector, with DAPI). The microscopic images of the stained sections were captured by Nikon ECLIPSE Ti series fluorescence microscope, using NIS Elements (BR 3.10) software.

Microscopic images of 6 fields of high vascular density areas were quantified by using ImageJ software. Tumor angiogenesis was quantified as the number of pixels of calibration standards were 5.0, 10, 50, 100, and 200 ng/mL. The concentrations of particle size 5

**Analysis of CAFs (CEPs and CECs)**
Approximately 160 μL of mouse blood was collected in K2-EDTA tubes by saphenous vein puncture in SH-SY5Y, KHOS, and RH30 model after 20, 28, and 31 days, respectively. Blood was immediately stored at 4°C until analysis. The CEP/CECs were measured by flow cytometry within 48 hours of blood collection as previously described (20). CEPs were defined as CD45+, VEGFR-2+, CD117+, and CD13+, while CECs were defined as CD45−, VEGFR-2+, CD117+, and 7-AAD was used to exclude the apoptotic cells. The absolute number of CEPs was calculated as the percentage of events collected in CEP enumeration gates multiplied by the total white blood cell (WBC) count.

**Bone marrow progenitor assay**
The bone marrow progenitor culture was done as previously published by our group (21). After the sacrifice of animals belonging to the RH30 model, the femurs were isolated and the bone marrow was flushed out with AMEM containing 2% FBS. Bone marrow cells of mice belonging to each group was pooled and 200,000 cells were cultured in methyl cellulose media (Methocult #3434, Stem Cell Technologies) in 35 mm culture suspension dish, in triplicate. Bone marrow cells of nontumor-bearing mice (n = 3) were pooled and cultured (in triplicate) concurrently with those of each group of tumor-bearing mice and were used as reference culture plates. After 14 days, colony forming units-granulocyte, macrophages (CFU-GM) were counted under the optical microscope.

**PKs of topotecan and pazopanib**
Nontumor-bearing animals were randomized into 4 groups (n = 3): Control, PZ, LDM TP, and TP+PZ. The doses of the drugs were the same as for the inguinal xenograft and metastatic models described above.

After single drug administration, the saphenous vein blood samples (30 μL) were collected in heparinized microcentrifuge tubes at 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 18 hours, and 24 hours as per the Laboratory Animal Services protocol. Plasma was immediately isolated after blood collection by centrifugation. For the topotecan assay, 10 μL plasma was immediately precipitated with 20 μL methanol and centrifuged. The supernatant and rest of the plasma were stored at −80 μC until analysis.

**Assay of pazopanib**. A total of 5 μL of plasma was precipitated with 40 μL methanol and centrifuged. A total of 30 μL of supernatant was injected into the high-performance liquid chromatography (HPLC) system, which consisted of Phenomenex C18 column (Luna; 150 × 4.6 mm; particle size 5 μ), UV detector (267 nm). The mobile phase was 50:50 mixture of 10 mmol/L potassium phosphate and methanol, with flow rate 1.0 mL/min. The concentrations of calibration standards were 5.0, 10, 50, 100, and 200 μg/mL.

**Assay of topotecan**. Before analysis, the previously prepared 20 μL methanolic extract was mixed with 50 μL of internal standard solution (5 ng/mL d6 topotecan dissolved 0.1% formic acid in acetonitrile). The mixture was then centrifuged and the supernatant was transferred to autosampler vials.

The LC/MS system consisted of an HPLC (Agilent Infinity 1290), Column (Kinetex HILIC, 2.6 μ, 100 A, 50 × 4.6 mm) and a mass spectrometer (Sciex 5500-Q Trap). The analytes were eluted by gradient flow. Mobile phase A was water:acetonitrile (10:90) and mobile phase B was water:acetonitrile (50:50) and a mass spectrometer (Sciex 5500-Q Trap). The analytes were eluted by gradient flow. Mobile phase A was water:acetonitrile (10:90) and mobile phase B was water:acetonitrile (50:50).
10 mmol/L ammonium acetate (pH 3.2). The mobile phase ratio was 5% A for 0 to 2 minutes, 20% A for 4 to 6 minutes and 5% A for 8 to 10 minutes at a flow rate 0.5 mL/min. The samples were analyzed by positive ion electrospray ionization technique in multiple reaction monitoring modes. The following mass transitions were monitored: 422.2 to 377.0 m/z, \((topotecan M^+ + H^-)\) and 428.2 to 377.0 m/z \((topotecan d_6 M^+ + H^-)\). The concentrations of calibration standards were 0.5, 1.0, 5.0, 10, and 100 ng/mL.

Statistical analysis

In vitro dose response, in vivo tumor growth curves and the number of pixels for immunohistochemistry are presented as mean ± SD. Statistical significance was assessed by student’s t test. Prism 5 (Version 5.04) for Windows, GraphPad Software was used for the calculation of IC_{50} and \(P\) values.

Results

Drug-induced in vitro cytotoxicities

Pazopanib did not affect the viabilities of the any of the treated cell lines at any of the concentrations tested (Fig. 1A). Both topotecan and pazopanib caused a dose-dependent reduction in viability of HUVEC with IC_{50} of 4.87 ng/mL and 398.0 ng/mL, respectively (Fig. 1B). Topotecan showed a dose-dependent reduction in the viability of all the tumor cell lines (Fig. 1C and D). Among neuroblastoma cell lines, SH-SY5Y (IC_{50} = 5.3 ng/mL) was more sensitive to topotecan than BE(2)-c (IC_{50} = 45.6 ng/mL) and SK-N-BE(2) (IC_{50} = 65.0 ng/mL; Fig. 1C).

Among sarcoma cell lines, the IC_{50} of topotecan on RH30, RD, and KHOS cell lines were 7.4 ng/mL, 7.5 ng/mL, and 4.9 ng/mL, respectively (Fig. 1D). Among all tumor cell lines tested, addition of 5,000 ng/mL pazopanib only caused a significant reduction of IC_{50} in SK-N-BE(2) cells (IC_{50} = 35.1 ng/mL, \(P = 0.046\); Fig. 1C).

LDM topotecan and pazopanib in neuroblastoma mouse models

The criteria for effectiveness of the treatments in SK-N-BE (2) xenograft model were tumors regression and enhancement of survival times. Drugs were administered daily over a period of 56 days. In addition, the animals belonging to the TP + PZ group, which remained alive, were also...
retracted from the 103rd day to evaluate the impact of resuming treatment on reversing drug resistance (Fig. 2A). Retreatment was continued until the 125th day, after which the mice were sacrificed. In this model, no significant difference was observed between the tumor growth rates of the LDM TP and PZ-treated groups. Compared with control, both the single agents significantly enhanced survival of animals (\( P < 0.05 \)). The survival in TP + PZ group was significantly higher compared with both control (\( P < 0.005 \)) and the single agents’ groups (\( P < 0.005 \)). Retreatment was associated by transient tumor growth delay up to 120 days. All the animals were sacrificed by 125th day.

In SH-SY5Y xenograft model, animals belonging to all the 4 groups were sacrificed after 20 days treatment, when the tumor endpoint was reached. Tumor growth delay and the difference in tumor weights at the end of the treatment were the criteria for assessment of treatment effectiveness. The treatments caused tumor growth delay in the order PZ < LDM TP < TP + PZ (Fig. 2B). All the 3 treatment groups showed significant difference in tumor weights compared with the control (Fig. 3A). LDM TP caused significant tumor weight reduction compared with PZ, while TP + PZ caused significant tumor weight reduction compared with both LDM TP and PZ.

BE(2)-c and NUB-7 are N-Myc amplified, I-type malignant neuroblastoma cells which have high potential to migrate and metastasize (17, 18). Survival time was used as the parameter to assess the efficacy of treatments in our BE(2)-c metastatic model. All the treatment groups showed
a statistically significant enhanced survival (Fig. 2C). Survival of LDM TP–treated animals was higher than PZ–treated animals \( (P < 0.05) \). The mean survival span of animals in TP + PZ group was approximately 2-fold (100.8 days) compared with the LDM TP group (52.4 days), \( P < 0.005 \). At the time of death or endpoint, the animals belonging to control, PZ and LDM TP groups had macroscopically detectable tumors in liver. Animals belonging to TP + PZ group did not reveal any evidence of liver metastasis (Fig. 3D).

**Effect of LDM topotecan and pazopanib on the tumor growth in sarcoma models**

Because PZ had shown limited efficacy in neuroblastoma models, we decided to evaluate the antitumor activity of Pulse TP and compare it with LDM TP in KHOS osteosarcoma model, in which the mice were sacrificed after 28 days treatment. Here, both Pulse TP and LDM TP delayed the tumor growth, with significantly lower tumor weight at the end of the treatment (Fig. 2D, Fig. 3B). The tumor growth rate curve (Fig. 2D) reveals that the single agents caused tumor growth delay, but not tumor size reduction, while TP + PZ, induced tumor growth delay until 22 days, after which tumor size reduction was observed. The TP + PZ group had significantly lower tumor weights compared with the control, Pulse TP and LDM TP (Fig. 3B).
In RH30 rhabdomyosarcoma RH30 xenograft model, the animals were treated for 56 days. The animals belonging to control and LDM TP reached the endpoint before this period, while those in PZ and TP + PZ–treated groups remained alive after the discontinuation of treatment (Fig. 2E). LDM TP was ineffective in controlling the tumor growth. In view of activity of PZ in soft tissue sarcoma, we decided to test PZ. PZ as a single agent and the combination TP + PZ delayed the tumor growth and enhanced the survival by 2-fold, compared with both control and LDM TP. TP + PZ group had significantly lower tumor size ($P = 0.03$), compared with those of PZ group.

**Effect of treatment on tumor microvessel densities**

Comparison of the pixel counts of 6 fields of highly vascularized regions of tumor sections stained for CD31 and vWF revealed that TP + PZ significantly reduced the microvessel density of the tumors, compared with the control in SH-SY5Y, RH30, and KHOS models (Fig. 4A–C). In SH-SY5Y model, PZ but not LDM TP caused significant reduction in microvessel densities compared with the control. In RH30 model, none of the single agents caused reduction in microvessel densities, compared with the control, while in KHOS model, both Pulse TP and LDM TP caused reduction in microvessel densities.

**Effect of the treatments on CAFs (CECs/CEPs)**

CECs originate from the bone marrow and also the adipose tissue (22). VEGF stimulates the recruitment of CECs into the tumor neovasculature and thus contributes to the endothelial lining (4). Therefore blockade of the VEGF-signalling pathway is expected to reduce the CEP level in blood and thus inhibit angiogenesis. In our SH-SY5Y neuroblastoma model, after 20 days treatment, TP + PZ significantly reduced both viable CEC and CEP levels compared with the control and single agents groups (Fig. 5A). Though the single agents caused reduction in both CEC and/or CEP levels, compared with the control, the differences were not statistically significant.

In RH30 rhabdomyosarcoma model, after 31 days treatment, TP + PZ caused significant reductions in both viable CECs and CEPs levels compared with the control and LDM TP (Fig. 5B). Comparison with PZ, TP + PZ caused significant viable CEP reduction. PZ–treated group had significantly lower viable CEP levels compared with the control.

In KHOS osteosarcoma model, CEP and CEC levels were measured after 28 days treatment (Fig. 5C). TP + PZ caused significant reduction in viable CEC and CEP levels compared with the control and Pulse TP. Also, LDM TP caused a significant reduction in viable CEP levels compared with Pulse TP.

WBC count was used as a parameter to assess bone marrow toxicity. In SH-SY5Y and KHOS model model, all the 3 treatment regimens significantly reduced WBC. TP + PZ–treated group had significantly lower WBC levels compared with both the single agents (Fig. 5A and C). Surprisingly, LDM TP had significantly lower WBC count compared with pulse TP. In RH30 model, PZ and TP + PZ reduced WBC level significantly, whereas LDM TP did not (Fig. 5B). Despite the significant lowering of WBC induced by the combination, compared with the control and the single agents, the animals belonging to this group in all the models were active and showed no signs of illness during or after this period until tumor sizes reached the endpoint.

**Bone marrow CFU-GM assay**

To better understand the impact of LDM TP and combination on bone marrow function, CFU-GM were counted in RH30 model, where the mice were sacrificed at different times, that is, day 32, day 35, day 71, and day 73 for control, LDM TP, PZ, and TP + PZ, respectively. Percentage CFU-GM count for each plate was calculated as the percentage of CFU-GM number in that plate to the average CFU-GM number in reference plates (Fig. 5D). LDM TP–treated group had significantly lower CFU-GM counts compared with the control. TP + PZ–treated group had significantly lower CFU-GM number compared with the control but not compared with the single agent groups.

**PK did not reveal drug interaction between topotecan and pazopanib in TP + PZ group**

The PK of topotecan and pazopanib was conducted to detect any PK interaction between topotecan and pazopanib in TP + PZ group. The peak plasma concentration of pazopanib was reached in 2 hours in both PZ and TP + PZ groups (Fig. 6A and B). The $C_{\text{max}}$ of pazopanib was 133.5 ng/mL and 122.4 ng/mL in PZ and TP + PZ groups, respectively, while the trough concentration was 9.46 ng/mL and 14.56 ng/mL, respectively. Peak plasma concentrations of topotecan in LDM TP and TP + PZ groups were 19.75 ng/mL and 33.05 ng/mL, respectively, while the trough concentration was 0.77 ng/mL and 2.79 ng/mL (Fig. 6A and B). For both drugs, no significant difference was observed between plasma concentrations of single agent and combination treated animals at any time point. A significant interanimal drug concentration variability was detected and larger group studies may be necessary to detect drug–drug interactions and changes in trough concentration. The previously reported optimal plasma concentration of pazopanib effectiveness ($40 \mu$mol/L or $\approx 18 \mu$g/mL; ref. 15) was maintained until at least 18 hours in both PZ and TP + PZ groups.

**Discussion**

Angiogenesis plays important roles in cancer growth, metastasis, and response to therapy. In pediatric tumors such as neuroblastoma, osteosarcoma, and rhabdomyosarcoma, *in situ* tumor angiogenesis and the levels of circulating angiogenic factors correlates with metastatic disease and poor prognosis (23–25). LDM chemotherapy alone has shown clinical benefit in several pediatric cancers and its maximum-tolerated dose has been established in phase-I trials (26–28). The combination of LDM chemotherapy with RTKis have been tested in several preclinical studies, including metronomic...
topotecan and pazopanib in ovarian cancer (13, 14, 29, 30) and in clinical trials (31, 32).

Neuroblastoma was the first preclinical tumor model to validate the concept of combining metronomic chemotherapy with antiangiogenic therapy (9). However, the mechanism of increased efficacy and safety of metronomically administered drug combinations and their PKs have never been studied widely in pediatric cancers.

Despite reports regarding antitumor activity of such combinations, their effectiveness in a particular pediatric cancer model cannot be predicted on the basis of its effects on other cancer models. In our experience combination of metronomic cyclophosphamide and sunitinib did not have any advantage over sunitinib monotherapy when tested in a neuroblastoma preclinical xenograft model (19). Also, in a previous study, the combination of axitinib with
metronomic cyclophosphamide was less effective than metronomic cyclophosphamide alone in gliosarcoma model (33). Therefore, the benefit of combining metro-

Figure 6. Plasma concentration-time profiles of topotecan and pazopanib in single agents group (A) and in combination group (B).

Figure 5. The effect of the treatment regimens on CEP/CEC levels in blood. A, comparison of CAF and WBC levels in SH-SYSY xenograft model after 20 days treatment; B, the comparison of CAF and WBC levels in RH30 model after 31 days treatment; C, the comparison of CAF and WBC levels in KHOS xenograft model after 28 days treatment; and D, the comparison of percentage CFU-GM among the 4 groups in RH30 xenograft model.

nomic chemotherapy with a particular RTKi should be confirmed preclinically and the proper dose and preclinical PK need to be established before moving to phase-I clinical trials. Here, we evaluated the effectiveness of LDM regimen of oral topotecan and its combination with one of the clinically approved RTKis, pazopanib, in the murine models of 3 pediatric solid tumors, with particular emphasis on the antiangiogenic mechanism and their potential bone marrow toxicity.

The doses of drugs were selected on the basis of previous studies. The daily oral doses of 1.0 mg/kg topotecan and 150 mg/kg pazopanib have been previously found to be effective in ovarian cancer mouse models (14). Shaked and colleagues has previously defined the optimal biologic dose (OBD) of LDM chemotherapy as the dose causing maximum reduction in CEPs with minimal or no toxicity after daily treatment for 1 week; this dose is associated with maximum antiangiogenic efficacy (34). In a previous dose-response study, the daily dose of oral metronomic topotecan (0.5, 1.0, and 1.5 mg/kg) caused greater reduction in microvascular density compared with weekly maximum-tolerated dose regimen (7.5 and 15 mg/kg) in an ovarian cancer model, but the mice treated with 1.5 mg/kg daily,
oral topotecan showed decreased food intake, and a lesser antitumor effect (35). By applying the aforementioned definition of OBD, we postulated that 1.0 mg/kg oral topotecan administered daily, would be the OBD, or within the range of the OBD. The antiangiogenic efficacy of weekly pulse topotecan and daily LDM topotecan has also been compared in our osteosarcoma model.

In vivo, pazopanib neither had any effect on the viability of any of the cell lines, nor did it enhance the cytotoxicity of topotecan on any of the cell lines except SK-N-EB(2) but was active on HUVEC cell lines. In agreement with our hypothesis, in vivo, LDM topotecan and its combination with pazopanib delayed the tumor growth and significantly enhanced the animal survival in all the models, TP + PZ showing higher antitumor efficacy compared with LDM TP and PZ or Pulse TP. LDM TP was more effective than PZ in neuroblastoma models, while in RH30 model, PZ was more effective in delaying tumor growth than LDM TP. The delay of tumor growth at metastatic sites by TP + PZ in NUB-7 and BE(2)-c metastatic models indicates that the combination of LDM topotecan and pazopanib can potentially control minimal residual disease and enhance the survival in high risk neuroblastoma.

Numerous preclinical and clinical studies have shown the value of CECs and CEPs as potential biomarkers of antiangiogenic activity (36). Dose-dependent decreases of CEPs have been observed with LDM administration of several cytotoxic agents (34). Recently, a similar combination of metronomic topotecan and pazopanib, caused a significant reduction in CEP levels in an ovarian cancer preclinical model (14). Our data confirm these findings in all pediatric tumor models with various degree of responses.

In neuroblastoma, TP + PZ delayed tumor growth in SK-N-EB(2) and SH-SY5Y models, and reduced micro-metastasis in BE(2)-c and NUB-7 models. The superiority of the combination over the single agents could be partially explained by its antiangiogenic activity, as observed by the significant reduction of all the 3 markers: viable CECs, viable CEPs and tumor microvessel density, by the TP + PZ, compared with the both LDM TP and PZ in SH-SY5Y neuroblastoma models. However, among the single agents, only PZ shows antiangiogenic activity, as observed by the significant reduction in the microvessel density.

In KHOS osteosarcoma model, all the regimens tested caused significant reduction in the levels of viable CECs and CEPs and microvessel densities after 28 days treatment. Though there was no significant difference between the tumor weights of Pulse TP and LDM TP upon sacrifice, the viable CEP levels in LDM TP treated group were significantly lower than those in Pulse TP treated group, indicating that metronomic topotecan is more antiangiogenic than the pulse dosing of topotecan.

In RH30 rhabdomyosarcoma model, TP + PZ caused significant reduction in viable CEC and CEP levels and microvessel density compared with both control and LDM TP. In addition, significant reduction in viable CEP level was showed with PZ alone after 31 days treatment, thus correlating with its tumor response. After exposure to single agent PZ, the microvessel densities of tumor xenografts, isolated at the time of tumor progression 2 weeks after discontinuation of treatment, were not different from those of control group. TP + PZ had significantly low viable CEPs than PZ. By analyzing the observations from tumor growth rate, CAF levels, and microvessel density experiment, we are postulating that in rhabdomyosarcoma model, PZ and TP + PZ are more effective than LDM TP and that the antiangiogenic effectiveness of TP+PZ is more sustained than PZ after the discontinuation of the treatment.

PK interaction between 2 coadministered drugs is an important consideration. No such studies have been conducted so far in the context of metronomic chemotherapy and combination with VEGF RTKi agents. Pazopanib is a substrate of CYP3A4 (15), while topotecan is a CYP3A4 inhibitor, which is reported to reduce the clearance of another CYP3A4 substrate (37). Therefore, we compared the plasma concentration–time profiles of each drug when administered alone and in combination. Our PK did not reveal any significant differences in the plasma concentrations of LDM TP or PZ between single agent and the combination groups, at any of the time points examined. However, a significant interanimal variability was detected at the trough level of TP in the TP + PZ group, though it did not reach statistical significance; it was higher in the TP + PZ group than in TP group. For pazopanib, 40 μmol/L (≈ 18 μg/mL) has been reported to be the optimum plasma concentration for the inhibition of VEGFR2 phosphorylation in mice (15). Since the plasma concentration of pazopanib was above this limit until 18 hours, it can be concluded 150 mg/kg pazopanib can inhibit VEGFR2 phosphorylation for at least 18 hours after oral drug administration.

In summary, combination of LDM topotecan and pazopanib has higher antitumor efficacy compared with single agents in neuroblastoma, rhabdomyosarcoma, and osteosarcoma. This combination caused significant lowering of CAFs, compared with control and single agents. Since no other synergism or additive effect have been observed between topotecan and pazopanib in in vitro and PK experiments, the mechanism behind the efficacy of the combination can be attributed to antiangiogenic activity in all 3 solid tumor models. Direct interaction with VEGF signaling pathway within tumor cells cannot be excluded (38). Potential bone marrow toxicity may be expected with the combination of LDM topotecan and pazopanib and caution should be taken before claiming that such a combination is not myelotoxic.

These results support development of this phase-I combination in pediatric solid tumors with a potential, if proven to be safe to be integrated into poststem cell transplantation regimen. This new maintenance strategy would potentially target highly resistant minimal residual disease.
Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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