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

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Running title: Metronomic Topotecan and Pazopanib in pediatric solid tumors

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Statement of 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 anti-tumor efficacy of administration of Low Dose Metronomic topotecan alone and its combination with a VEGFR tyrosine kinase inhibitor pazopanib in three aggressive pediatric extracranial solid tumors mouse models: Neuroblastoma, Osteosarcoma and Rhabdomyosarcoma. Administration of Low Dose Metronomic topotecan demonstrated a significant antitumor efficacy in most tumor type, 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 trial in these three categories of aggressive childhood solid tumors.
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 VEGFR inhibitor, in three pediatric extracranial solid tumors 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 combination were tested on four subcutaneous xenograft models and on two neuroblastoma metastatic models. Circulating Angiogenic Factors (CAFs) such as Circulating Endothelial Cells (CECs) Circulating Endothelial Progenitor cells (CEPs) as well as 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) demonstrated significant anti-tumor activity and significant enhancement in survival compared to 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 to respective single agents in pediatric tumor mouse models and represent a valid option as a maintenance therapy in aggressive pediatric solid tumors.
Introduction:

Neuroblastoma and Sarcoma are the most common extracranial childhood solid tumors. Though 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 mechanism 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 intensitiy chemotherapy as an induction or consolidation therapy, therefore 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 CEPs from the bone marrow, represents an attractive and effective antiangiogenic strategy [3,4,5]. Drawbacks to using pulse topotecan include resistance of tumor cells and the cross-resistance with topoisomerase-II inhibitors in neuroblastoma cell lines [6,7]. LDM regimens of topotecan, which may target endothelial cells and tumor cells by other mechanism, can possibly overcome some of these limitations.
Despite its advantages, relapses may occur in patients who have initially responded to single agent LDM chemotherapy. Vascular Endothelial Growth Factor (VEGF), a survival factor for endothelial cells, 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 anti-tumor activity. We have previously demonstrated the therapeutic advantage of such combinations by combining LDM vinblastine with VEGFR2-neutralizing antibody, DC101, in a human neuroblastoma xenograft preclinical study [9]. Such combinations have also been found to be effective in other pre-clinical and clinical trials [10,11,12]. Recently, two 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 to 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 three types of pediatric solid tumors and to evaluate CAFs such as viable CEPs and CECs and tumor microvessel densities as surrogate biomarker 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-dihydroxy-1H-yrano [3’,4’:6,7] indolizino[1,2-b] quinoline-3,14 (4H,12H)-dione monohydrochloride and Pazopanib (5-[[4-[(2,3-Dimethyl-2H-indazol-6-yl)methylamino]-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 [16], osteosarcoma cell line KHOS and rhabdomyosarcoma cell lines RH30 and RD, Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from American Type Culture Collection (ATCC) (Manassas, VA); BE(2)-c sub-clone 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 DMEM (# 319-010-CL, Wisent bioproducts), both containing 10% fetal bovine serum and 1% antibiotic mixture in humidified atmosphere at 37°C with 5% CO2,
In-vitro cytotoxicity:

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

In vivo models:

For subcutaneous xenograft studies, we used SK-N-BE(2), SH-SY5Y, KHOS and RH30. 1 x 10^6 cells were implanted subcutaneously into the inguinal fat pad of each of non-obese diabetic/severe combined immune deficient (NOD/SCID) mice. When tumors reached 0.5 cm in diameter, the animals were randomized into four 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). In order to compare pulse topotecan to 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 end point were tumor sizes exceeding 2.0 cms in diameter or animals showing signs of morbidity. The tumor sizes were measured on a daily basis until the end point or sacrifice. The long (D) and short diameters (d) were measured
with calipers. Tumor volume (cm$^3$) was calculated as $V = 0.5 \times D \times d^2$. When the end point was reached or at the end of the treatment, the animals were sacrificed by cervical dislocation.

**Metastatic mouse model:**

$1 \times 10^6$ 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 four groups and treated in the same way as the inguinal xenograft model. The treatment was continued until death or end point for BE(2)-c model and till fourteen days for NUB-7 model.

Protocol and endpoints were approved by Sickkids animal committee facility.

**Immunohistochemistry and histopathology:**

Formalin fixed tissues were paraffin embedded and sections cut at 7um. These sections were deparaffinized through xylene and ethanol, rehydrated in Phosphate-buffered Saline (PBS) (# 311-010-CL, Wisent Bioproducts) and incubated overnight with primary antibodies for von Willebrand factor (vWF) (# A0082; DakoCytomation, Glostrup, Denmark) at 4°C. After the primary antibody treatment, all the slides were washed three times with PBS and incubated with broad spectrum poly-horse radish peroxidase (HRP) conjugated secondary antibody (# 87-9663, Invitrogen) for 1h at room temperature. After washing three
times with PBS, slides were stained with diaminobenzidine (DAB) and counterstained with hematoxylin. Microscopic images were captured by Olympus UTV1-X microscope mounted with Qimaging Retiga 2000R camera.

Frozen sections from SH-SY5Y tumor model were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100. After blocking with 5% bovine serum albumin in PBS for one hour, the sections were incubated overnight with rabbit polyclonal anti-CD31 antibody (#ab28364, Abcam, dilution 1:50). The sections were washed three times with PBS containing 0.1% tween 20 (PBST) and incubated with Alexa fluor 594 donkey anti-rabbit IgG (#A21207, Invitrogen, dilution 1:300) for one hour. After washing with PBST, 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 six fields of high vascular density were digitally captured and the pixel values for stained areas were quantified using ImageJ software. Tumor angiogenesis was quantified as the number of pixels of regions positive for von Willebrand Factor or CD31.

Analysis of CAFs (CEPs and CECs):
Approximately 160µl of mouse blood was collected in K2-EDTA tubes by saphenous vein puncture in SH-SY5Y, KHOS and RH30 model after twenty, twenty eight and thirty one days respectively. Blood was immediately stored at 4°C until analysis. The CEP/CECs were measured by flow cytometry within 48hrs.
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 CD13+. 7-AAD was employed 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 performed as previously published by our group [21]. After the sacrifice of animals belonging to RH30 model, the femur was isolated. Bone marrow was flushed out of the femur 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 35mm culture dish, in triplicate. Bone marrow cells of non tumor bearing mice (n=3), pooled and cultured (in triplicate), concurrently with those of each group of mice, were used as reference culture plates. After fourteen days, Colony Forming Units- Granulocyte, Macrophages (CFU-GM) were counted under the optical microscope.

**Pharmacokinetics of topotecan and pazopanib:**

Non-tumor bearing animals were randomized into four 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 (30µl) were collected in heparinized microcentrifuge tubes at 30 min, 1h, 2h, 4h, 8h, 12h, 18h and 24 h as per the Laboratory Animal Services protocol. Plasma was immediately isolated after blood collection by centrifugation. For the topotecan assay, 10µl plasma was immediately precipitated with 20µl methanol and centrifuged. The supernatant and rest of the plasma were stored at -80µC until analysis.

Assay of pazopanib: 5µl of plasma was precipitated with 40µl methanol and centrifuged. 30 µl of supernatant was injected into the HPLC system, which consisted of Phenomenox C18 column (Luna; 150 x 4.6 mm; particle size 5 µ), UV detector (267 nm). The mobile phase was 50:50 mixture of 10mM potassium phosphate and methanol, with flow rate 1.0 ml/min. The concentrations of calibration standards were 5.0, 10, 50,100 and 200µg/ml.

Assay of topotecan: Prior to analysis, the previously prepared 20µl methanolic extract was mixed with 50µl of internal standard solution (5ng/ml d6 topotecan dissolved 0.1% formic acid in acetonitrile). The mixture was then centrifuged and the supernatant was transferred to auto- sampler vials.

The LC/MS system consisted of an HPLC (Agilent Infinity 1290), Column (Kinetex HILIC, 2.6u, 100A, 50x4.6mm) and a mass spectrometer (Sciex 5500-QTrap). The analytes were eluted by gradient flow. Mobile phase A was water:acetonitrile (10:90) and mobile phase B was 10mM ammonium acetate (pH 3.2). The mobile phase ratio was 5% A for 0-2 min, 20% A for 4-6min and 5%A for 8-10min 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 d6 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, San Diego California USA, was used for the calculation of IC50 and P values.
Results:

Drug-induced in vitro cytotoxities:

Pazopanib did not affect the viabilities of the any of the treated cell lines at any of the concentrations tested (Figure 1A). Both topotecan and pazopanib caused a dose-dependent reduction in viability of HUVEC with IC50 of 4.87 ng/ml and 398.0 ng/ml respectively [Figure 1B]. Topotecan demonstrated a dose-dependent reduction in the viability of all the tumor cell lines [Figure 1C, D]. Among neuroblastoma cell lines, SH-SY5Y cells (IC50 = 5.3 ng/ml) was more sensitive to topotecan than BE(2)-c (IC50= 45.6 ng/ml) and SK-N-BE(2) cells (IC50= 65.0 ng/ml) [Figure 1C].

Among sarcoma cell lines, the IC50 of topotecan on RH30, RD and KHOS cell lines were 7.4 ng/ml, 7.5 ng/ml and 4.9 ng/ml respectively [Figure 1D]. Among all tumor cell lines tested, addition of 5000 ng/ml pazopanib only caused a significant reduction of IC50 in SK-N-BE(2) cells (IC50=35.1ng/ml, P=0.046) [Figure C].

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 fifty six days. In addition, the animals belonging to the TP+PZ group, which remained alive, were also retreated from the 103rd day to evaluate the impact of resuming treatment on reversing drug
resistance [Figure 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 to control, both the single agents significantly enhanced survival of animals (P<0.05). The survival in TP+PZ group was significantly higher compared to 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 four groups were sacrificed after twenty days treatment, when the tumor end point 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 [Figure 2B]. All the three treatment groups showed significant difference in tumor weights compared to the control [Figure 3A]. LDM TP caused significant tumor weight reduction compared to PZ, while TP+PZ caused significant tumor weight reduction effective compared to both the 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 demonstrated a statistically significant
enhanced survival [Figure 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 two fold (100.8 days) compared to the LDM TP group (52.4 days), P<0.005. At the time of death or end point, 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 [Figure 3D]. Animals in all the four groups of BE(2)-c model had evidence of tumors present in kidney, adrenal gland and bone marrow.

NUB-7 metastatic model, the animals belonging to all the four groups were sacrificed after fourteen days treatment. Compared to the control, LDM TP and TP+PZ liver weights were significantly lower in TP+PZ treated animals, compared to PZ [Figure 3C]. Microscopic tumors were visible in the livers of mice belonging to all the groups except TP+PZ confirming the ability of TP+PZ to control liver metastasis [Figure 3D].

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

Since PZ had demonstrated limited efficacy in neuroblastoma models, we decided to evaluate the anti-tumor activity of Pulse TP and compare it with LDM TP in KHOS osteosarcoma model, in which the mice were sacrificed after twenty eight days treatment. Here, both Pulse TP and LDM TP delayed the tumor growth, with significantly lower tumor weight at the end of the treatment [Figure 2D, 3B]. The tumor growth rate curve [Figure 2D] reveals that the single agents caused tumor growth delay, but not tumor size reduction, while TP+PZ, induced
tumor growth delay until twenty two days, after which tumor size reduction was observed. The TP+PZ group had significantly lower tumor weights compared to the control, Pulse TP and LDM TP [Figure 3B].

In RH30 rhabdomyosarcoma RH30 xenograft model, the animals were treated for fifty six days. The animals belonging to control and LDM TP reached the end point before this period, while those in PZ and TP+PZ treated groups remained alive after the discontinuation of treatment [Figure 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 as well as the combination TP+PZ delayed the tumor growth and enhanced the survival by two fold, compared to both control and LDM TP. TP+PZ group had significantly lower tumor size (P=0.03), compared to those of PZ group.

Effect of treatment on tumor microvessel densities:

Comparison of the pixel counts of six 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 to the control in SH-SY5Y, RH30 and KHOS models. [Figure 4 A, B and C]. In SH-SY5Y model, PZ but not LDM TP caused significant reduction in microvessel densities compared to the control. In RH30 model, none of the single agents caused reduction in microvessel densities, compared to 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)

CEPs originate from the bone marrow and also the adipose tissue [22]. VEGF stimulates the recruitment of CEPs 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 twenty days treatment, TP+PZ significantly reduced both viable CEC and CEP levels compared to the control and single agents groups [Figure 5 A]. Though the single agents caused reduction in both CEC and / or CEP levels, compared to the control, the differences were not statistically significant.

In RH30 rhabdomyosarcoma model, after thirty one days treatment, TP+PZ caused significant reductions in both viable CECs and CEPs levels compared to the control and LDM TP [Figure 5B]. Compared to PZ, TP+PZ caused significant viable CEP reduction. PZ treated group had significantly lower viable CEP levels compared to the control.

In KHOS osetosarcoma model, CEP and CEC levels were measured after twenty eight days treatment [Figure 5C]. TP+PZ caused significant reduction in viable CEC and CEP levels compared to the control and Pulse TP. Also, LDM TP caused a significant reduction in viable CEP levels compared to Pulse TP.

White Blood Cell (WBC) count was used as a parameter to assess bone marrow toxicity. In SH-SY5Y and KHOS model model, all the three treatment regimens significantly reduced WBC. TP+PZ treated group had significantly lower WBC levels compared to both the single agents [Figure 5A, 5C]. Surprisingly LDM TP
had significantly lower WBC count compared to pulse TP. In RH30 model, PZ and TP+PZ reduced WBC level significantly, while LDM TP did not [Figure 5B]. Despite the significant lowering of WBC induced by the combination, compared to 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 reaching the end point.

**Bone Marrow CFU-GM assay:**

In order 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 i.e. 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 [Figure 5D]. LDM TP treated group had significantly lower CFU-GM counts compared to the control. TP+PZ treated group had significantly lower CFU-GM number compared to the control, but not compared to 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 pharmacokinetic interaction between topotecan and pazopanib in TP+PZ group.
The peak plasma concentration of pazopanib was reached in 2h in both PZ and TP+PZ groups [Figure 6 A, B]. The Cmax 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 19.75ng/ml and 33.05ng/ml, respectively, while the trough concentration was 0.77ng/ml and 2.79 ng/ml [Figure 6 A,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 inter-animal 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 µM or ≈ 18 µg/ml) [15] was maintained until at least 18 h 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 [23,24,25].

LDM chemotherapy alone has demonstrated clinical benefit in several pediatric cancers and its maximum tolerated dose has been established in phase-I trials [26,27,28]. 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 anti-angiogenic therapy [9]. However the mechanism of increased efficacy and safety of metronomically administered of drug combinations and their PKs have never been studied widely in pediatric cancers. Despite reports regarding anti-tumor 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 metronomic 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 RTKi, pazopanib, in the murine models of three 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 et al 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 one 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 15mg/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 anti-tumor 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-vitro, 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-BE(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 anti-tumor efficacy compared to 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 demonstrated the potential 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-BE(2) and SH-SY5Y models, and reduced micrometastasis 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 three markers: Viable CECs, viable CEPs and tumor microvessel density, by the TP+PZ, compared to the both LDM TP and PZ in SH-SY5Y neuroblastoma models. However, among the single agents, only PZ demonstrates 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 twenty eight 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 to both control and LDM TP. In addition, significant reduction in viable CEP level was demonstrated with PZ alone after thirty one 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 two 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.

Pharmacokinetic interaction between two co-administered 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 inter-animal 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, 40µM (≈ 18µg/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 18h, it can be concluded 150 mg/Kg pazopanib can inhibit VEGFR2 phosphorylation for at least 18h after oral drug administration.

In summary, combination of LDM topotecan and pazopanib has higher antitumor efficacy compared to single agents in neuroblastoma, rhabdomyosarcoma and osteosarcoma. This combination caused significant lowering of CAFs, compared to 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 three 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 post-stem cell transplantation regimen. This new maintenance strategy would potentially target highly resistant minimal residual disease.
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Legends for Illustrations:

Figure 1: In vitro efficacy of topotecan and pazopanib. (A) Effect of pazopanib on neuroblastoma cell lines. (B) Dose-response plots of topotecan and pazopanib on HUVEC. (C) and (D) Dose-response plots of topotecan alone and in combination with 5000 ng/ml pazopanib on neuroblastoma cell lines and sarcoma cell lines respectively.

Figure 2: In vivo effects of topotecan and pazopanib treatment. (A) tumor growth rate (left) and Kaplan-Meier survival curve (right) for SK-N-BE(2) xenograft model. Dotted line indicates that the treatment was stopped after fifty six days, while solid line indicates that the animals belonging to combination group were retreated starting from 103 days. † One animal in combination group died due to gavaging error on 9th day of treatment. (B) Tumor growth rate in SH-SY5Y xenograft model. (C) Kaplan Meier survival curve for BE(2)-c metastatic model. In BE(2)-c metastatic model, all the three treatment groups had significantly higher life span than control group (P<0.005). (D) and (E) Tumor growth rate for model, KHOS osteosarcoma model and RH30 rhabdomyosarcoma model respectively.

Figure 3: (A) and (B) Photograph and histogram depicting the tumor weight comparison in SH-SY5Y and KHOS models respectively. (C) Photograph and histogram depicting the liver weight comparison in NUB-7 metastatic model. (D) Microscopic images of hematoxylin and eosin stained sections of livers in BE(2)-
c (40x magnification) and NUB-7 metastatic models (10X magnification). The tumor bearing areas in the microscopic sections can be distinguished by dark blue color, compared to pink colored non-tumor bearing areas.

Figure 4: (A) Microscopic images of highly vascularized areas, stained for CD31 in tumor sections from SH-SY5Y xenograft (original magnification x 10). The red color represent regions stained for CD31. (B) and (C) are the sections from RH30 and KHOS xenografts respectively, stained for vWF (original magnification x10). Arrows point toward regions stained for vWF.

Figure 5: The effect of the treatment regimes on CEP/CEC levels in blood. (A) Comparison of CAF and WBC levels in SH-SY5Y xenograft model after twenty days treatment; (B) The comparison of CAF and WBC levels in RH30 model after thirty one days treatment; (C) The comparison of CAF and WBC levels in KHOS xenograft model after twenty eight days treatment and (D) The comparison of percentage CFU-GM among the four groups in RH30 xenograft model.

Figure 6: Plasma concentration-time profiles of topotecan and pazopanib in (A) single agents group and (B) in combination group.
Figure 2
Figure 4
Figure 5
**Figure 6**

(A) **Single agent groups**

- AUC_{0-24} = 93.55 ng/ml.hr
- AUC_{0-24} = 1802 µg/ml.hr

(B) **TP+PZ group**

- AUC_{0-24} = 122.0 ng/ml.hr
- AUC_{0-24} = 1733 µg/ml.hr
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