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ORIGINAL PAPER

Preclinical impact of high dose intermittent antiangiogenic tyrosine kinase inhibitor pazopanib in intrinsically resistant tumor models

Elaine Reguera-Nuñez[1,2] · Shan Man[2] · Ping Xu[2] · Robert S. Kerbel[1,2,3]

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Abstract

Antiangiogenic tyrosine kinase inhibitors (TKIs) target vascular endothelial growth factor receptors and other receptor tyrosine kinases. As a result of toxicity, the clinical failures or the modest benefits associated with antiangiogenic TKI therapy may be related in some cases to suboptimal drug dosing and scheduling, thereby facilitating resistance. Most antiangiogenic TKIs, including pazopanib, are administered on a continuous daily basis. Here, instead, we evaluated the impact of increasing the dose and administering the drug intermittently. The rationale is that using such protocols, antitumor efficacy could be enhanced by direct tumor cell targeting effects in addition to inhibiting tumor angiogenesis. To test this, we employed two human tumor xenograft models, both of which manifest intrinsic resistance to pazopanib when it is administered continuously: the VHL-wildtype SN12-PM6-1 renal cell carcinoma (RCC) and the metastatic MDA-MB-231/LM2-4 variant breast cancer cell line, when treated as distant metastases. We evaluated four different doses and schedules of pazopanib in the context of primary tumors and advanced metastatic disease, in both models. The RCC model was not converted to drug sensitivity using the intermittent protocol. Using these protocols did not enhance the efficacy when treating primary LM2-4 tumors. However, one of the high-dose intermittent pazopanib protocols increased median survival when treating advanced metastatic disease. In conclusion, these results overall suggest that primary tumors showing sensitivity to continuous pazopanib treatment may predict response to this drug when given at high doses intermittently in the context of advanced metastatic disease, that are otherwise resistant to the conventional protocol.

Keywords Antiangiogenic · TKI · High dose · Intermittent schedule · Metastasis

Introduction

Currently, more than ten different antiangiogenic drugs have been approved for over ten different types of cancer [1]. These drugs include vascular endothelial growth factor (VEGF) pathway-targeting antibodies and oral small molecule tyrosine kinase inhibitors (TKIs) targeting VEGF receptors (VEGFRs), among a number of other cytoplasmic and receptor tyrosine kinases (RTKs). The majority of antiangiogenic TKIs are designed to be taken orally on a daily continuous basis with no breaks, exceptions being sunitinib which is administered using a 4-weeks ON/2-weeks OFF schedule [2], and regorafenib, which is given using a 3-weeks ON/1-week OFF schedule [3, 4]. Most monotherapy or combination antiangiogenic TKI treatments have failed to show meaningful, if any, efficacy in most types of common solid tumors, such as breast, colorectal, lung, and prostate cancers, based on results obtained in numerous randomized phase III clinical trials [1]. Major exceptions are first line therapy with sunitinib in highly angiogenic clear cell renal cell carcinoma (ccRCC) and sorafenib in hepatocellular carcinoma (HCC). Also, regorafenib has shown modest efficacy as a second or third line therapy in refractory colorectal carcinoma patients [4] and advanced gastrointestinal stromal tumors [3]. With respect to combination therapy, the antiangiogenic TKI, nintedanib, provided a modest clinical benefit in survival when combined with docetaxel as second line treatment for non-small cell lung

✉ Robert S. Kerbel
robert.kerbel@sri.utoronto.ca

[1] Department of Medical Biophysics, University of Toronto, Toronto, ON M5S 2J7, Canada

[2] Biological Sciences Platform, Sunnybrook Research Institute, Toronto, ON M4N 3M5, Canada

[3] Biological Sciences Platform, Sunnybrook Research Institute, 2075 Bayview Ave, Room S-217, Toronto, ON M4N 3M5, Canada

carcinoma patients who had rapidly progressed after first line chemotherapy [5].

There are a number of possible reasons to explain some of the aforementioned failures or very limited benefits of antiangiogenic TKIs, one being the possibility that the "flat" recommended doses and continuous schedules used may be sub-optimal, at least in some patients. In this regard, Rini and colleagues hypothesized that adaptive titration of the antiangiogenic TKI axitinib, administered daily to RCC patients, based on individual tolerability (assessed in terms of hypertension and other toxicities) might improve antitumor efficacy [6]. However, although improved objective response rates (i.e., tumor shrinkage) were observed in the axitinib titration arm, this did not translate into a superior progression-free survival (PFS) [6]. In another clinical study, Bjarnason and colleagues reported retrospective evidence, in RCC patients, suggesting that adaptively individualizing sunitinib dose, and shortening the usual 2-week breaks, based on toxicity (as a surrogate for either sufficient on insufficient drug exposure), significantly improved efficacy [7]. This adaptive individualized therapy protocol is now being evaluated in a prospective phase II clinical trial, together with dose escalation of sunitinib in patients showing minimal toxicity when treated with the conventional protocol (NCT01499121). Recently, Maráz et al. reported results of a clinical study involving a relatively small group of metastatic RCC patients showing modest progression after an initial response to sunitinib, but still classified as stable disease according to RECIST 1.1 criteria, who subsequently benefited from dose escalation of the drug [8]. These investigators determined dose escalation based on tolerable toxicity to the drug, observing prolonged PFS and overall survival in the study group compared to the control group (i.e., patients who continued receiving the standard protocol despite slight progression) [8].

A potentially important consideration regarding altering TKI dose is that antiangiogenic TKIs, as mentioned above, target multiple other cytoplasmic and cell surface RTKs, not just VEGFRs. Thus, they could conceivably also cause antitumor effects by direct tumor cell killing or inhibition of proliferation, similar to conventional cytotoxic chemotherapeutic drugs. This possibility has likely been under-appreciated because TKIs are not generally administered at higher tolerated doses, using intermittent schedules. In this regard, there is some in vitro evidence [9–13] and also some limited in vivo evidence [14] suggesting dose-dependent anti-proliferative/anti-tumor effects mediated by TKIs (e.g., pazopanib, sunitinib and axitinib) when used to treat different cancer cell lines. Such in vivo anti-proliferative effects of TKIs, at least in one case, are lost when the tumor cells were previously made resistant overtime due to continuous and increasing exposures in vitro to this drug, as previously reported by Gotink and colleagues with the HT29 colon cancer cell line [15]. This group observed that exposure to high dose sunitinib in a pulsatile fashion did not induce in vitro resistance in the ccRCC 786-0 cell line [16]. Thus, taken together, these results suggest that the response of tumor cells, not just the tumor vasculature, may also contribute to acquired resistance to such TKIs and highlight the importance of optimizing the dose and schedule. Moreover, preclinical studies have shown that such antitumor effects of an antiangiogenic TKI could be enhanced when administered in an intermittent fashion, without altering the cumulative drug dose per week [16, 17]. Therefore, a question that arises is whether this broad, dose-dependent antitumor activity using a TKI such as pazopanib is maintained or even improved when given at higher doses intermittently compared to the conventional, continuous antiangiogenic dose.

Another observation relevant to antiangiogenic TKI monotherapy and drug dose/schedule is the fact that a substantial proportion of patients can be intrinsically resistant "up front" to such drugs. Thus, about 20% of RCC patients do not respond initially to antiangiogenic TKIs [18]. Furthermore, patients whose tumors are initially responsive, almost always develop acquired resistance over time [19]. In this regard, there is some limited evidence showing that when tumors become resistant to TKIs administered daily and continuously, such refractory tumors may be re-sensitized to the same drug after a break from therapy and increasing the dose once the treatment is resumed [20–26], or by switching the treatment to an alternative antiangiogenic TKI, i.e., by undertaking "sequential" TKI therapy [27, 28].

In this preclinical study, we tested the hypothesis that increasing the recommended generally used antiangiogenic TKI drug dose and including break periods could be a putative strategy to use such drugs as more effective direct inhibitors of tumor growth. Importantly, to do so, we took advantage of two tumor models we have used that are known to be resistant to pazopanib when given continuously on a daily basis as an angiogenesis treatment strategy. We asked whether these tumor models could be converted to a drug sensitive state by substantially increasing the dose. The first model consists of advanced, late stage, metastatic breast cancer using the metastatic variant LM2-4, derived from the established human "triple negative" breast cancer cell line MDA-MB-231 [29]. Four negative phase III results of sunitinib in metastatic breast cancer patients [30–33] were preclinically recapitulated with the LM2-4 model when mice with metastatic disease were treated with sunitinib alone or with paclitaxel chemotherapy [34]. Similarly, pazopanib lacked anti-tumor efficacy in the metastatic setting, whereas both TKIs caused anti-tumor efficacy when treating established primary tumors using this model [34]. The lack of efficacy was found to be related to a lack of angiogenesis observed in the lung metastases, the main site of distant metastasis in this model [29], where instead vessel co-option

was detected [35]. The second model involves a VHL-wildtype human RCC cell line, also selected for increased metastatic ability, derived from the human cell line SN12-PM6 [13]. Treatment with conventional antiangiogenic doses and continuous schedules of pazopanib causes an insignificant primary tumor inhibition effect, and metastatic disease was found to be completely resistant [13]. We tested four different protocols of increased pazopanib dose, given intermittently in different schedules in these two models. With one exception, no evidence for conversion to drug sensitivity assessed by changes in tumor growth and survival times was observed in these models. However, despite high dose intermittent pazopanib failing to improve the antitumor efficacy when treating established orthotopic primary LM2-4 tumors, one protocol we tested caused increased median survival in the advanced metastasis model. As such, these studies may serve as basis for further evaluation of the concept of altering conventional dose and schedule protocols of antiangiogenic TKIs, either as monotherapy or in combination with other therapeutic modalities.

Materials and methods

Cell lines and mice

MDA-MB-231/LM2-4 is a variant of the "triple negative" human breast cancer cell line MDA-MB-231 (originally obtained from Dr. Jeff Lemontt, Genzyme Corp.) selected in vivo for aggressive spontaneous metastatic spread after the established orthotopic primary tumor has been resected by mastectomy [29]. The LM2-4 cell line is cultured in RPMI 1640 medium with 5% fetal bovine serum (FBS), at 37 °C in 5% CO_2, as previously described [29]. SN12-PM6-1 is a variant of the VHL-wildtype human ccRCC cell line SN12-PM6 (kindly provided by Dr. I.J. Fidler, MD Anderson Cancer Center, Houston) [36], which was serially selected in vivo for a more aggressive metastasizing ability after resection (nephrectomy) of the established orthotopic primary tumor, and is tagged with luciferase to allow for whole body optical bioluminescent imaging [13]. This cell line is cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% FBS, at 37 °C in 5% CO_2. Both cell lines were authenticated to confirm their human origin by STR DNA analysis (Genetica DNA Laboratories). They were also screened for mycoplasma contamination using commercial kits (Lonza) and were certified as being mycoplasma-free.

CB17 severe combined immunodeficient (SCID) mice expressing the yellow fluorescent protein (YFP CB17 SCID mice) were bred in house from breeding pairs originally provided by Dr. Janusz Rak (McGill University, Montreal). Mice were used when they reached 6–8 weeks of age. All surgical procedures were undertaken in accordance with the animal care guidelines of Sunnybrook Health Sciences Centre (Canada) and the Canadian Council of Animal Care.

Surgical procedures

Experiments performed with the MDA-MB-231/LM2-4 metastatic variant were done as previously described [29]. Briefly, 2×10^6 cells of the MDA-MB-231/LM2-4 cell line, were implanted in the mammary fat pad of female YFP CB17 SCID mice. To study the effect of pazopanib on tumor growth, treatment was initiated once the primary tumor was established (150 mm^3). Studies of metastatic disease treatment were undertaken after resection of primary tumor (400 mm^3) when presence of overt spontaneous metastasis was known (i.e., beginning 3 weeks after tumor resection), based on previous studies with the LM2-4 variant [29, 34]. All mice were randomized just before initiating treatment to obtain similar average tumor burden among groups. In the primary breast cancer model, tumor growth was recorded once a week by measurements with Vernier calipers. Tumor volumes were calculated using the formula $a^2b/2$, where a is the width and b is the length. Endpoint was considered when volume of primary tumors reached 1700 mm^3. In post-surgical treatment of advanced metastatic visceral disease, survival based on clinical symptoms (i.e., labored breathing) was considered as endpoint.

Experiments performed with the VHL-wildtype RCC cell line metastatic variant SN12-PM6-1 were undertaken as previously described [13]. Briefly, 10^6 cells from the SN12-PM6-1 cell line were implanted orthotopically in the renal capsule of male YFP CB17 SCID mice. To study the effect of the TKI in the primary tumor RCC model, treatment started when the luciferase-tagged tumor becomes established (i.e., equivalent to 5×10^6 photons/s). All mice were randomized just before initiating treatment to obtain similar average tumor burden among groups. To study the effect of TKI therapy on advanced metastatic disease, the primary tumor was resected by nephrectomy and treatment initiated when there was evidence of overt distant metastases, detected by whole body bioluminescence imaging [13]. Primary tumor growth and progression of visceral metastases were evaluated once a week using whole body imaging bioluminescence. Clinical symptoms determined endpoint (i.e., labored breathing, lethargy, distress).

Drug and treatments

Pazopanib was purchased from LC Laboratories and reconstituted following the manufacturer's instructions. A group of mice received pazopanib vehicle daily as control. The doses and schedules of pazopanib tested in this study were the following: (1) 150 mg/kg by gavage (po), daily (qd) (i.e.,

the "conventional" antiangiogenic protocol) as previously described [37]; (2) 350 mg/kg po once a day in a 3-days ON, 4-days OFF schedule (equivalent cumulative amount of drug over a week as the conventional protocol); (3) 225 mg/kg po twice a day (bid), in a 3-days ON, 4-days OFF schedule; (4) 300 mg/kg po bid, twice a week (biw); and (5) 400 mg/kg po bid biw. Intermittent schedules were chosen to test whether this could be an alternative to manage very high doses of pazopanib and reduce risk of metastasis.

Histology and immunohistochemistry (IHC)

Tumors and lungs were fixed with 10% buffered formalin and embedded in paraffin. Tumor sections (5-µm-thick) were deparaffinized and stained with hematoxylin and eosin (Leica) to determine local invasion of body wall and analyze necrosis. For IHC, sections were quenched in 1% H_2O_2, unmasked in boiling sodium citrate buffer (10 mmol/L, pH 6, 5 min), and stained using the following specific antibodies: CD31 (1:50, Dianova), Ki67 (1:1000, Vector) and vimentin (1:100, Invitrogen) (to check lung metastasis). Biotin-conjugated secondary antibodies (Jackson ImmunoResearch) were used and detected with Vector Elite HRP kit and DAB chromogen (Dako). Sections were counterstained with hematoxylin (Leica). Sections were visualized with a Leica DM LB2 microscope and digital camera DFC300FX and images acquired using AxioVision 3.0 software. Images were analyzed using ImageJ 1.38d software.

Statistical analyses

Statistical analyses were performed using the GraphPad Prism software package version 4.0 (GraphPad Software, Inc, San Diego, CA). Results are reported as mean ± SD, except bioluminescence data which are reported as mean ± SE, based on its high variability. Based on the small sample size and lack of homogeneity of variance in some sets of results, data were subjected to non-parametric analysis using Mann–Whitney test ($\alpha = 0.05$). Whenever possible, measurements were done without knowledge of treatment group, especially for immunohistochemistry analysis. Survival curves were analyzed using the Gehan-Breslow-Wilcoxon Test. Differences were considered statistically significant when p values were < 0.05.

Results

The metastatic RCC mouse model using the human VHL-wildtype cell line SN12-PM6-1 is an ideal model to determine whether tumors that express an intrinsically resistant phenotype to pazopanib therapy using a conventional daily schedule at recommended dose to inhibit tumor angiogenesis [13], can nevertheless be rendered sensitive simply by increasing the dose and altering the schedule of the drug from continuous to intermittent, i.e., by including breaks. This would seem a reasonable possibility considering the wide spectrum of tyrosine kinase targets of this TKI, some of which are involved in proliferation (such as Src) and highly expressed by this cell line [38]; which likely explains its dose-dependent in vitro sensitivity to pazopanib [13].

A second model, which can act as a control for primary tumor experiments with the VHL-wildtype RCC model, is the LM2-4 variant of the human breast cancer cell line MDA-MB-231 [29]. As reported previously, orthotopic primary tumors are sensitive to standard antiangiogenic protocols of pazopanib by being growth delayed [34]. In contrast, advanced metastatic disease is resistant to such drug given daily and at "flat" doses [34].

In this study, we analyzed the effect on primary tumor growth, as well as advanced metastasis, in both the SN12-PM6-1 and LM2-4 models, using increasing doses of pazopanib per week administered using different (i.e., continuous versus intermittent) schedules. Such intermittent schedules would also allow a determination of whether it is possible to reduce or avoid extreme toxicity related to higher dose pazopanib and the detrimental effects that can sometimes occur, at least preclinically, when very high doses of TKIs are administered daily for a week (such as increased incidence of metastasis and decreased survival times) [39, 40].

Pazopanib tolerability was analyzed in terms of body weight loss as a surrogate for toxicity, since other TKI-related side effects usually observed in clinic are less common or more difficult to detect in mice compared to humans (e.g., nausea, diarrhea and changes in skin color, since SCID mice are covered with fur). Doses and schedules of pazopanib used in this study were in general well tolerated in both the RCC and breast cancer mouse models (Fig. 1); although mice in the RCC studies showed a modest loss of body weight. This could be associated with the RCC model itself which affects renal function that could have an impact in the general health status of mice since some mice in the control group showed signs of body weight loss.

Primary SN12-PM6-1 tumors were not rendered sensitive when treated with high doses of pazopanib given intermittently (Fig. 2a). Moreover, unexpectedly, after 4 weeks of treatment, primary tumors grew faster in those mice treated with pazopanib 350 mg/kg 3-days ON/4-days OFF, than those in the control and the "conventional" dosing protocol groups, despite the cumulative dose of drug using this intermittent schedule being similar to the conventional treatment (Fig. 2a). However, such increase in tumor growth did not differ statistically from the control group or mice treated with the conventional protocol, presumably because of the high variability in the bioluminescence signal in this treatment group.

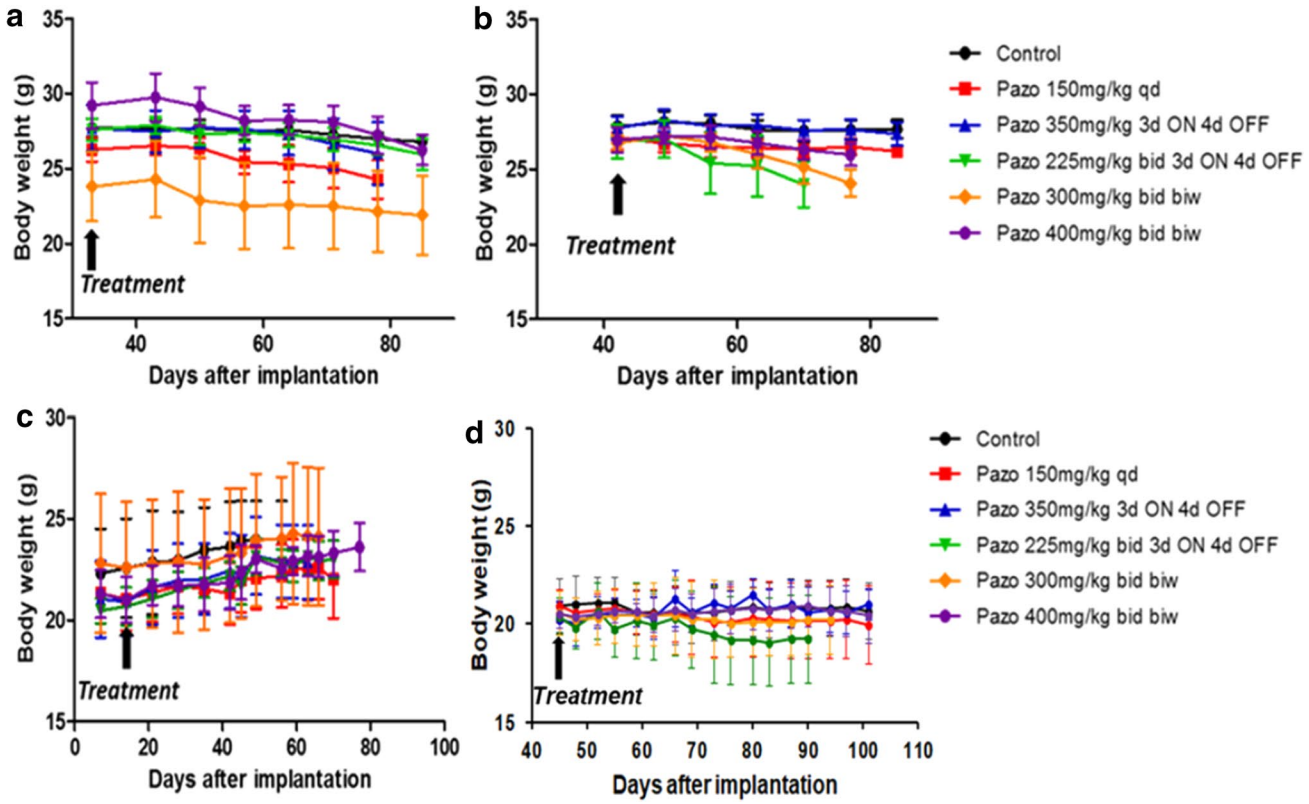
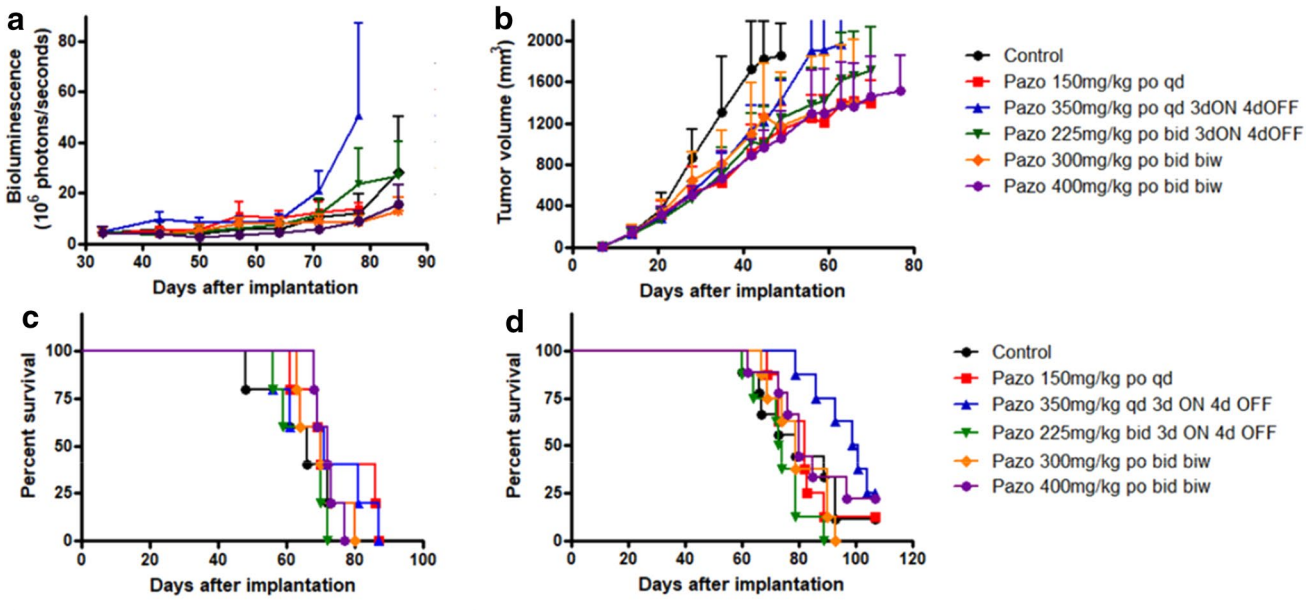


Fig. 1 Different doses and schedules of pazopanib were in general well tolerated. Body weight was used as a surrogate for toxicity in mice. **a** Body weight of mice in the primary RCC SN12-PM6-1 model. **b** Body weight of mice in the advanced metastases RCC SN12-PM6-1 model. **c** Body weight of mice in the primary breast cancer model LM2-4. **d** Body weight of mice in the advanced metastases breast cancer LM2-4 model. All groups $n \geq 5$. Data are presented as means ± SD

Primary LM2-4 tumors responded to treatment with pazopanib when administered both conventionally and at high doses intermittently (Fig. 2b). All these treatments induced a statistically significant delay in tumor growth compared to the control group after 4 weeks of therapy (a time at which all mice in the control group reached endpoint). However, compared to the conventional daily therapy, the antitumor effect of pazopanib in this model did not improve when given at high doses intermittently (Fig. 2b). On the other hand, LM2-4 tumors treated with pazopanib 350 mg/kd 3-days ON/4-days OFF seemed to grow faster after 4 weeks of treatment (Fig. 2b), similar to the results obtained with the primary SN12-PM6-1 model (Fig. 2a), described above.

Different doses and schedules of pazopanib appear to have contrasting effects on angiogenesis, based on immunohistochemistry analysis for CD31 (Fig. 3a), suggesting that when giving the drug intermittently, it may be necessary to use very high doses to induce an antiangiogenic effect. We did not observe any difference among treatments in terms of proliferation at the viable tumor rim (Fig. 3b). In general, tumors were highly necrotic (based on H&E staining), but this increased significantly when using pazopanib 350 mg/kg 3-days ON 4-days OFF treatment (Fig. 3c). This suggests that pazopanib when used at 350 mg/kg 3-days ON 4-days OFF may act through a mechanism other than inhibiting angiogenesis, while promoting necrosis of the tissue, presumably by inducing cell death, but this requires further evaluation.

In the primary LM2-4 tumor study, we observed that despite the antitumor effect induced by the continuous conventional pazopanib protocol, it also increased the local tumor invasiveness into the body wall (based on H&E staining) compared to the control group, which resulted in ascites formation (Table 1). This effect on invasiveness seems to be affected by the dose and schedule of the drug. The frequency of body wall invasion and ascites decreased when mice were treated with the same cumulative dose per week as the conventional protocol, but in an intermittent fashion (i.e., 350 mg/kg 3-days ON/4-days OFF) (Table 1). A similar effect was observed for the other intermittent schedules, but to a lesser extent (Table 1). In addition, administering pazopanib intermittently seemed to decrease metastasis to the lymph nodes induced by the conventional protocol (Table 1). Moreover, analysis of the incidence of metastasis in lungs from primary LM2-4 tumor-bearing mice showed that some pazopanib protocols promoted lung metastasis,



Treatment	Median survival (days)
Control	66
Pazo 150mg/kg po qd	70
Pazo 350 mg/kg qd 3d ON 4d OFF	71
Pazo 225mg/kg bid 3d ON 4d OFF	70
Pazo 300mg/kg po bid biw	70
Pazo 400mg/kg po bid biw	72

Treatment	Median survival (days)	
Control	79	p=0.0354
Pazo 150mg/kg po qd	82	p=0.0292
Pazo 350 mg/kg qd 3d ON 4d OFF	100	
Pazo 225mg/kg bid 3d ON 4d OFF	73.5	
Pazo 300mg/kg po bid biw	79	
Pazo 400mg/kg po bid biw	80	

Fig. 2 Effect of different doses and schedules of pazopanib on tumor growth and median survival. **a** Tumor growth in the primary RCC SN12-PM6-1 model measured by bioluminescence. Pazopanib did not cause a delay of tumor growth. $n=5$; means ± SE. Mann–Whitney test was used for statistical analysis. The dose 350 mg/kg 3-days ON, 4-days OFF, enhanced tumor growth in the primary RCC SN12-PM6-1 model but did not reach statistical significance. **b** Tumor growth in the primary breast cancer model LM2-4 measured with Vernier calipers. Conventional daily and high dose intermittent protocols showed statistically significant tumor growth delay compared to control group 49 days after implantation (Mann–Whitney test, $p<0.05$. $n\geq5$; means ± SD). **c** Kaplan–Meier survival curves and median survival values for the advanced metastases RCC SN12-PM6-1 model. In this model, tested doses and schedules did not have any benefit in improving median survival (according to Gehan-Breslow-Wilcoxon Test, $n=5$). **d** Kaplan–Meier survival curves and median survival values for the advanced metastases breast cancer LM2-4 model. In this model, only treatment with pazopanib 350 mg/kg 3-days ON/4-days OFF increased median survival significantly compared to the control group ($p=0.0354$) and to the conventional treatment group ($p=0.0292$) (according to Gehan-Breslow-Wilcoxon Test, $n=8$–9)

particularly, the daily conventional dose protocol (Fig. 3d). However, such a detrimental effect was highly reduced when pazopanib was administered at 350 mg/kg 3-days ON/4-days OFF, lung metastasis being similar to the control group (Fig. 3d). In the case of primary RCC SN12-PM6-1 tumors study, the only treatment that significantly induced lung metastasis was the conventional protocol (based on immunohistochemistry analysis for vimentin staining), while mice treated with the other protocols had levels of lung metastasis similar to control group (data not shown).

In the advanced metastatic SN12-PM6-1 disease model, high-dose intermittent pazopanib promoted metastasis in a dose-dependent manner, both to the lungs and elsewhere (Fig. 4), reaching statistical significance when the drug was given at 400 mg/kg bid biw compared to control group and the conventional protocol. Thus, such intermittent schedules used in this study did not avoid the detrimental (pro-malignant) effects of short course, high dose continuous of TKIs (particularly sunitinib) that we have previously reported [39]. However, despite increases in bioluminescence regarding metastasis, no detrimental effect on median survival was observed in the metastatic RCC model with the different doses/schedules of pazopanib (Fig. 2c). However, neither was a benefit observed.

When different doses and schedules of pazopanib were evaluated in the advanced metastatic setting in the LM2-4

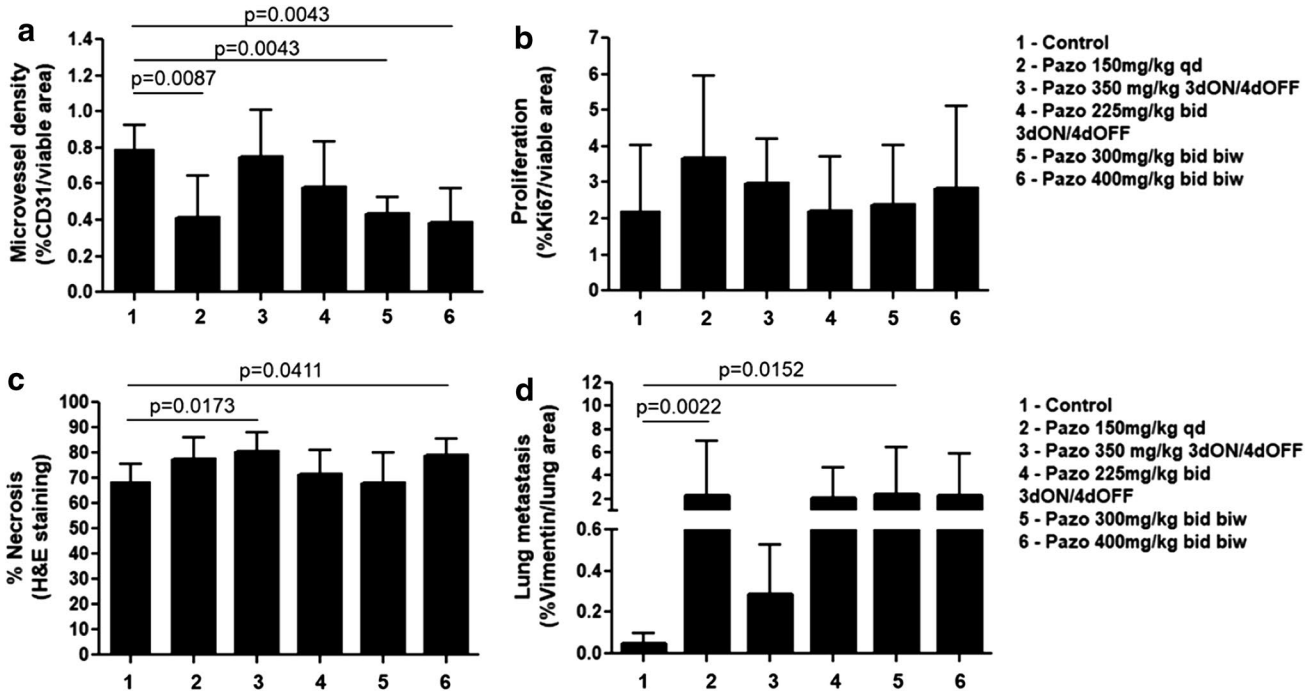


Fig. 3 Effect of different doses and schedules of pazopanib on **a** angiogenesis; **b** proliferation; **c** level of necrosis; and **d** lung metastasis. Histology and immunohistochemistry analyses were performed on samples obtained from the primary tumor study with the LM2-4 cell line. **a** Groups 2 and 3 received same cumulative dose per week, but daily administration is required to induce an antiangiogenic effect. **b** There were no differences in proliferation among groups. **c** Level of necrosis also varied depending on dose and schedule, being higher for groups 3 and 6, compared to the control group. **d** The most marked difference among treatments was in the incidence of lung metastasis. All treatments, except 350 mg/kg 3-days ON/4-days OFF seemed to increase incidence of lung metastasis. All groups $n \geq 5$. The Mann–Whitney test was used for statistical analyses. Data are presented as means ± SD

Table 1 Effect of different doses and schedules of pazopanib on invasiveness and distant metastases in the primary LM2-4 tumor study, reported as frequency (%) of occurrence per group analyzed at necropsy

Treatment	Lymph node metastases (%)	Ascites (%)	Invasion into body wall[a] (%)
Control	0	0	33.3
Pazo 150 mg/kg qd	83.3	66.7	83.3
Pazo 350 mg/kg 3-days ON/4-days OFF	0	0	40
Pazo 225 mg/kg bid 3-days ON/4-days OFF	50	33.3	83.3
Pazo 300 mg/kg bid biw	16.7	16.7	66.7
Pazo 400 mg/kg bid biw	50	50	50

[a]Invasion of tumor into the body wall was confirmed with H&E staining

breast cancer model (Fig. 2d), we observed that only treatment with pazopanib 350 mg/kg 3-days ON/4-days OFF increased median survival compared to the control group and the conventional protocol. This benefit in survival seems to be related to a later onset in lung metastasis and lack of lymph node metastasis in mice treated using this protocol, compared to the control group and the conventional treatment (based on necropsy observations) (Table 2). In this intermittent protocol, mice receive the same cumulative dose per week as those mice treated with the conventional protocol. Thus, it seems that the benefit observed in the median survival when pazopanib was given at 350 mg/kg 3-days ON/4-days OFF is not directly related to the drug dose per week but to the treatment schedule, since none of the other protocols involving higher doses of pazopanib had a survival benefit in the advanced metastatic LM2-4 model. Our results suggest that in this model, as the pazopanib dose is increased, it is necessary to increase the time off therapy since mice treated with 225 mg/kg bid 3-days ON/4-days OFF did worse than the control group, mainly because of some mice showing signs of toxicity, as manifested by sudden loss of body weight, which affected the median survival.

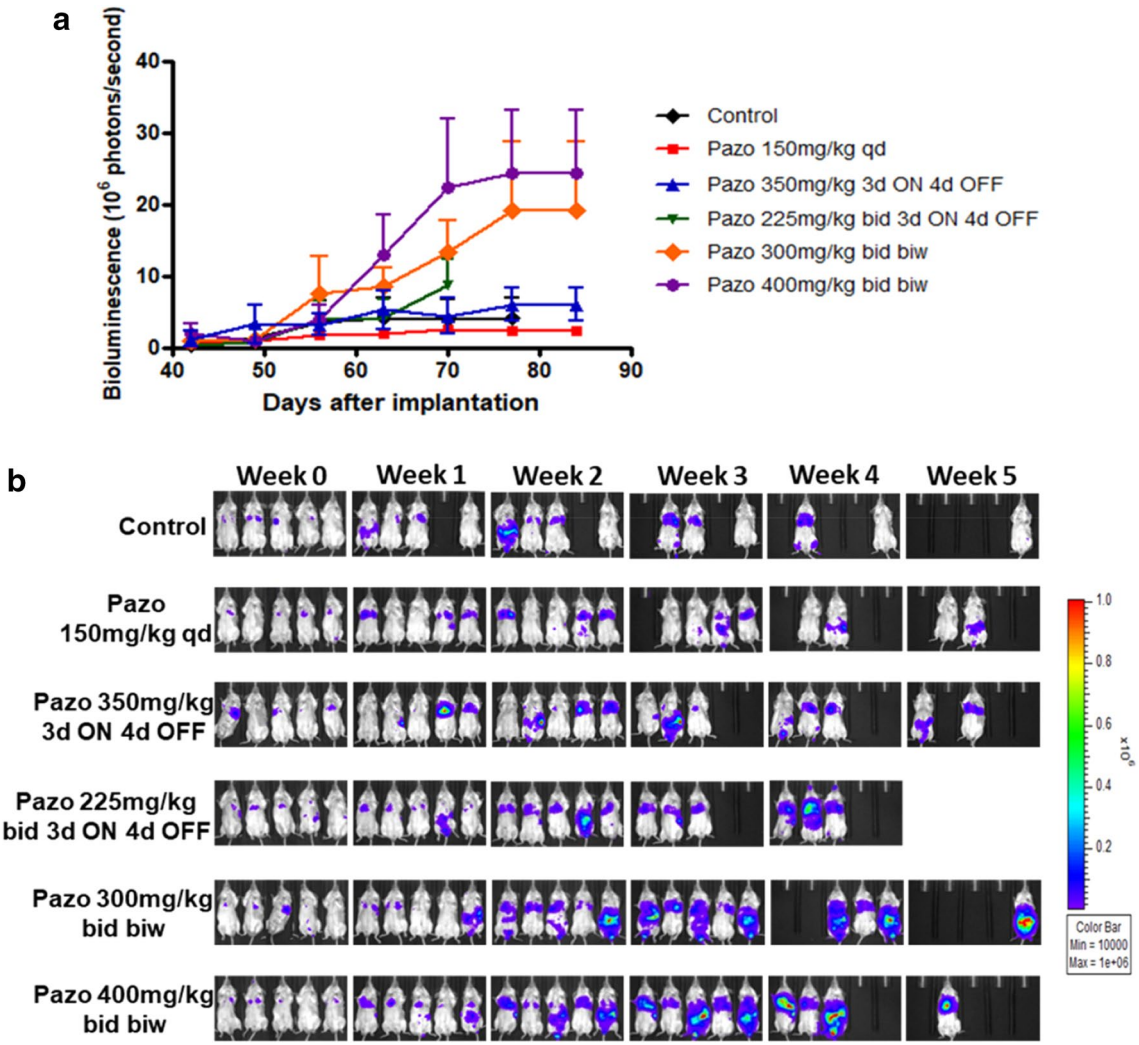


Fig. 4 Pazopanib induced metastasis in a cumulative dose-dependent manner. **a** Bioluminescence signal from a ventral view in mice from the metastatic RCC SN12-PM6-1 model. Mice treated with 300 or 400 mg/kg po bid biw showed higher incidence of metastasis, including lungs and intestines, as well as regrowth of primary tumor, but only 400 mg/kg bid biw reached statistical significance ($p=0.0317$. Mann–Whitney test. All groups $n=5$. Data are presented as means ± SE). **b** Bioluminescence images according to week of treatment corresponding to data represented in **a**

Table 2 Effect of different doses and schedules of pazopanib on distant metastases in the advanced metastasis LM2-4 study, reported as frequency (%) of occurrence per group analyzed at necropsy

Treatment	Lung nodules (%)	Lymph node metastases (%)
Control	66.7	44.4
Pazo 150 mg/kg qd	87.5	75
Pazo 350 mg/kg 3-days ON/4-days OFF	62.5	0
Pazo 225 mg/kg bid 3-days ON/4-days OFF	75	12.5
Pazo 300 mg/kg bid biw	87.5	62.5
Pazo 400 mg/kg bid biw	66.7	33.3

However, such an effect was not observed in mice treated with higher cumulative doses but less frequently (i.e., 300 and 400 mg/kg, both twice a day, twice a week).

Discussion

As outlined in the Introduction, the rationale for investigating the impact of intermittent schedules and higher doses of an antiangiogenic TKI—in this case pazopanib—is that such treatment regimens may induce or increase direct antitumor cell effects, and hence, the possibility of rendering tumors sensitive, that are normally intrinsically resistant to the antiangiogenic effects of the drug when using conventional continuous dosing schedules. To this end, we

evaluated four independent intermittent schedules using different doses of pazopanib in two distinct tumor models; moreover, we evaluated the therapies on both established orthotopic primary tumors and in mice with overt distant metastatic disease at the time therapy was initiated. When the resultant data set is considered as a whole, the results did not support the hypothesis that the intermittent higher dose protocol would convert tumors intrinsically resistant to conventional-administered pazopanib to a drug sensitive phenotype. The only exception was a particular protocol using pazopanib at 350 mg/kg on a 3-days ON/4-days OFF schedule to treat metastatic MDA-MB-231/LM2-4 breast cancer bearing mice, where survival was prolonged compared to all other treatment groups.

There is an increasing interest in giving TKIs intermittently in an individualized fashion, so patients can benefit from treatment without excessive toxicity and development of resistance [7, 8, 41–44]. Some studies suggest adjusting drug dose (i.e., lower or higher than conventional) while giving it in an intermittent/pulsatile fashion to allow optimal drug exposure [7, 8], avoid clonal selection and resistance [42], and potentially induce different mechanisms of action (e.g., cytotoxic and/or antiproliferative effect of antiangiogenic TKIs) [44].

The intermittent administration of high doses of antiangiogenic TKIs has been evaluated previously in preclinical models models that are known to be intrinsically responsive to antiangiogenic protocols involving continuous drug administration [16, 17], with the goal of further increasing the efficacy of the therapy. However, in contrast, we evaluated the prospect that using such protocols might enhance potential direct tumor cell targeting effects of pazopanib (in addition to inhibiting tumor angiogenesis) and thus render tumors that are intrinsically resistant to conventional antiangiogenic pazopanib become sensitive to the drug, as discussed above.

We have previously reported that pazopanib inhibited in vitro cell proliferation of SN12-PM6-1 [13] and LM2-4 cell lines [12] in a dose-dependent manner. Such antiproliferative effects of pazopanib may be mediated not only by targeting cKit, which is expressed in low levels in ccRCC [45] and MDA-MB-231 breast cancer cells [46], but also Src [47], which is highly expressed in both cell lines [38, 48]. When we tested high-dose intermittent pazopanib protocols in SN12-PM6-1 bearing mice grown as primary tumors or as advanced metastases, we did not detect an antitumor effect, and thus the tumors remained refractory to this TKI. However, high dose intermittent pazopanib (350 mg/kg 3-days ON/4-days OFF) increased median survival in the LM2-4 advanced metastasis mouse model. Interestingly, this protocol showing improvement in median survival involves the same total dose of pazopanib per week as the conventional daily dosing protocol. Thus, it seems that scheduling may play a crucial role in pazopanib efficacy, at least in this advanced breast cancer model.

Previously, Wang et al. observed that this same intermittent schedule (3-days ON/4-days OFF) improved the efficacy of sorafenib in the human VHL-mutant ccRCC cell line 786-0 growing subcutaneously in nude beige mice, when given at high doses but with the same cumulative dose per week as the conventional protocol [17]. Also, Rovithi and colleagues evaluated high-dose pulsatile sunitinib in HT29 colon carcinoma cells growing in the chorioallantoic membrane of the chicken embryo. They observed a more potent antitumor effect than the conventional protocol, despite the intermittent protocol delivering less total drug per week [16]. Differences between our ccRCC model (SN12-PM6-1) and results when treating 786-0 cells with high dose intermittent TKIs [17] may be related to the different in vivo models used, the VHL-status of both cell lines, as well as differences in their aggressiveness and metastatic potential. We have previously shown that SN12-PM6-1 cell line is highly metastatic with 100% penetrance; whereas it was not possible to establish a metastasis mouse model with a variant of 786-0 cell line isolated from lung metastases (786-L16) due to lack of consistency in occurrence of metastases [13].

Our results and those of others [16, 17] suggest that tumors showing sensitivity to antiangiogenic TKIs when treated as primary tumors can predict enhanced efficacy when the drug is administered at higher doses than the conventional dose, and in an intermittent fashion. In our studies reported here, such enhancement was not observed in the primary tumor treatment setting using the LM2-4 breast cancer cell line, but only in the advanced metastasis treatment setting. Wang and colleagues reported that increased tumor growth delay when sorafenib was administered at high doses intermittently was due to a more potent antiangiogenic activity compared to the conventional protocol [17]. On the other hand, Rovithi et al. proposed that high-dose pulsatile sunitinib resulted in higher intratumoral concentration of the TKI (compared to daily administration of the conventional dose), which may mediate prolonged stability of tumor growth, despite an observed lack of effect on microvessel density and cell proliferation [16]. In our study, we observed that the same cumulative dose of pazopanib given intermittently (350 mg/kg 3-days ON/4-days OFF) improved median survival, as mentioned above, in the advanced metastasis model, using the LM2-4 breast cancer cell line (Fig. 2d). Interestingly, this effect may be mediated by a decrease in the invasiveness of the tumor cells, since we observed a lower incidence of lung metastasis, lymph node macrometastases, invasion of the primary tumor into the body wall, and ascites formation at the time of sacrificing the mice (i.e., when primary tumors reached volumes considered as endpoint) (Table 1). A similar effect was observed in the advanced metastasis model, with reduced

incidence of lymph node metastasis detected at necropsy analysis (Table 2). In this regard, we and others have previously reported that in some cases conventional antiangiogenic therapy can increase local cancer cell invasiveness and distant metastasis in different tumor models [49, 50]. Thus, based on results from this study, giving the same cumulative dose of antiangiogenic TKIs as the conventional protocol, but in a dose dense fashion with short breaks seems to be effective in decreasing, not increasing, invasiveness. Also, the effect of high dose intermittent pazopanib on LM2-4 tumors may depend on the tumor size. We did not observe an improved tumor growth delay in the LM2-4 primary tumor model with pazopanib 350 mg/kg 3-days ON/4-days OFF (with the treatment started once tumors reached 150 mm^3); but we observed a later onset in lung metastasis which translated in improved median survival in the advanced metastasis model. Presumably, such an effect in the metastasis model may be mediated by an antitumor effect on micrometastases growing in the lungs, but this requires further evaluation.

There is recent clinical evidence [8] supporting the idea that antitumor effect of antiangiogenic TKIs can be improved when the drug is administered at higher doses than conventional, but this was observed in tumors showing prior (upfront) intrinsic sensitivity to the drug. Thus, metastatic RCC patients who initially respond to conventional sunitinib therapy, but later show signs of tumor progression, may benefit from dose escalation of the drug, achieving improved PFS and overall survival compared to those treated conventionally [8].

An important aspect of our studies, which reinforces earlier reports, is that pazopanib administered intermittently at higher doses had very different effects when the therapy was evaluated in primary tumors compared to advanced metastasis, particularly with the LM2-4 breast cancer cell line where, ironically, the best therapeutic result obtained was in the advanced metastatic setting. We have previously shown the potential translational value of the LM2-4 advanced metastasis model [34]. Thus, this study could be used as a first step for further approaches in evaluating high-dose intermittent protocols of "antiangiogenic" TKIs, as a strategy to increase the exposure to the drug and improve its efficacy in cancer models showing resistance to the conventional protocol (e.g., in the advanced metastatic triple negative breast cancer model). Importantly, future studies should take into consideration that improvement of drug efficacy when used at high doses intermittently may depend on the intrinsic sensitivity of the tumor when treated as primary tumors using conventional antiangiogenic protocols.

We acknowledge one particular weakness in the experimental design which should be considered with respect to the overall conclusions, and that is the empirical nature of the intermittent higher dose regimens we tested. It is possible that a different regimen might have caused more robust anti-tumor effects. Nevertheless, this underscores the problem that would be encountered when similarly trying to determine whether the intermittent dosing concept would improve antitumor efficacy in the clinic when using antiangiogenic TKIs such as pazopanib, especially to try and treat patients whose tumors are intrinsically resistant to conventional continuously administered drug, or which acquire resistance after showing an initial response.

Conclusions

Most oral TKIs have been approved using protocols of flat doses taken continuously. This is often associated with considerable toxicity in some patients. Thus, doses and schedules of TKIs need further evaluation to reduce toxicity while preserving, or even enhancing, their antitumor efficacy. Administering antiangiogenic TKIs at higher doses than used conventionally and in an intermittent fashion seems to be a potential strategy to improve efficacy, particularly in those type of cancers showing resistance in certain circumstances, such as overt/advanced metastatic disease. Importantly, any benefit of altering dose and schedule of TKI therapy may depend on intrinsic sensitivity of the tumor to the drug.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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