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Metronomic Dosing Enhances the Antiangiogenic Effect of Epophilone B

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Abstract

Background—High doses (10nM) of epothilone B, a microtubule stabilizer, will inhibit the development of human tumor-derived angiogenesis following short (14 day) drug exposure times. Metronomic dosing regimes use lower drug doses and prolonged drug exposure times in an attempt to decrease toxicity compared to standard dosing schedules.

Hypothesis—We hypothesized that epothilone B would be an effective antiangiogenic agent when administered at very low doses over an extended period of time.

Methods—Fragments of four fresh human tumors were cultured in a fibrin-thrombin matrix and maintained in nutrient media plus 20% fetal bovine serum for 56 days. Tumor fragments (n=40–60 per group) were exposed to weekly doses of epothilone B at concentrations of 10, 5, 1, 0.5, or 0.1 nM. All of these concentrations are clinically achievable. Tumor angiogenesis was assessed weekly on day 14–56 using a validated visual grading system. This system rates neovessel growth, density, and length on a 0–16 scale [angiogenic index, (AI)]. The average change in AI between day 14 and 56 was calculated for all samples and used to evaluate the metronomic response.

Results—Epothilone B produced a dose-dependent antiangiogenic response in all tumors. Two of the four tumors demonstrated a clear and significant metronomic antiangiogenic effect over time.

Conclusions—Epothilone B, when dosed by a metronomic schedule may have a significant antiangiogenic effect on human solid tumors. This study provides evidence for the potential use of epothilone B on a metronomic dosing schedule.

Keywords

Angiogenesis; Metronomic; Human; Epophilone B; Cancer

Introduction

The commonly accepted paradigm that drives the selection of chemotherapeutic treatment regimens for human solid tumors involves the administration of the Maximum Tolerable Dose (MTD). Using a MTD regimen, the patient receives the highest possible concentration

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of a cytotoxic agent with the objective of achieving maximum tumor cyto-reduction while inducing “tolerable” side effects (1). The unintended and deleterious consequences of this approach are well-characterized, and include toxic side effects such as myelosuppression, neuropathy, alopecia, and gastrointestinal upset. These toxicities often require a significant recovery period to be built into MTD dosing regimens so that the patient’s symptoms can subside before the next dose is administered. While there is often a substantial initial reduction in tumor size, the recovery phase of the patient’s treatment schedule can allow regrowth of tumor cells not eradicated by the treatment. The MTD schedule of anti-tumor therapy allows susceptible tumor cells to be destroyed, but fosters the selection of a population of resistant tumor cells that are refractory to subsequent treatment. The required recovery phase permits further expansion of the resistant cell population. This “selection effect” has a significant negative influence on the likelihood of a long term clinical response.

Administering chemotherapy on a metronomic dosing schedule represents a novel approach to the treatment of solid tumors. Rather than pursuing maximum tumor regression in the short term, the objective of this dosing schedule is to slowly cyto-reduce the tumor by targeting the tumor vasculature over the long term. In a metronomic dosing schedule, traditional cytotoxic agents are administered continuously (or at extremely short treatment intervals) in low doses over an extended course, with no recovery times built into the regimen. The endothelial cells that comprise the tumor vasculature are in a state of rapid growth, and thus are susceptible to treatment. However, because of the continuous nature of the dosing regimen and the presumed genetic stability of the endothelial cells, these cell populations are not as susceptible to the “selection effect.” This regime has the potential to prevent cultivation of resistant endothelial cell populations. The low drug concentrations used in metronomic dosing should also reduce or eliminate the toxic side effects commonly associated with MTD therapy. Continuous low dose therapy eliminates the need for a recovery period and consequently allows for continuous drug exposure for the tumor cells and vasculature.

There are a growing number of studies that support a shift in focus towards clinical application of an antiangiogenic approach, and specifically, the metronomic scheduling of antiangiogenic chemotherapy. Recent *in vitro* studies have demonstrated that cultured human endothelial cells undergo apoptosis when continuously exposed to traditional cytotoxic agents at concentrations well below the accepted MTD. The apoptosis of tumor cells occurs while other normal human cells are not affected (2–5). A number of animal studies support these findings. In these *in vivo* studies, metronomic dosing schedules produced favorable results when compared to standard MTD schedules. Both cyclophosphamide and docetaxel, when administered as single agents on a metronomic schedule to mice with human tumor xenografts, caused a greater reduction of tumor mass and increased survival compared to the identical agents used in a traditional MTD dosing schedule (2,6–8). Even more striking results have been observed when cytotoxic chemotherapeutics such as etoposide, carboplatin, and vinblastine were administered on metronomic schedules simultaneously with specific antiangiogenic agents such as PEX (a fragment of human metalloprotease-2) or monoclonal anti-VEGF antibodies (9–11). These results were generally sustained over extended periods of time, and were accompanied by a significant reduction of negative side effects relative to MTD therapy (6,11,12). Additionally, several clinical trials are currently underway that are investigating the potential clinical benefits of many of these cytotoxic agents when administered on a metronomic schedule. Metronomic treatment regimens involving vinblastine, cyclophosphamide, and methotrexate have also been associated with clinically beneficial results when applied to heavily pre-treated patient populations; results that were, in several instances, sustained over the long term. The patients in these studies also generally

experienced significantly reduced side effects relative to those typically encountered with MTD therapy (13–15).

Microtubule stabilizers, specifically the taxanes, are currently among the most commonly used chemotherapeutic agents in clinical treatment of solid tumors. Their effects on microtubules act to slow mitotic activity in rapidly dividing cells, and ultimately result in apoptosis. Epothilone B (Epo B, EPO-906, patupilone; Novartis Pharmaceuticals, East Hanover, NJ) is a member of a new class of naturally occurring microtubule stabilizers, originally isolated from the myxobacterium *Sorangium cellulosum*, and is currently undergoing extensive investigation. Epothilone B has an *in vitro* mechanism of action that is very similar to the taxanes, and binds to tubulin in the same molecular location, though with greater affinity (16,17). *In vitro* studies have shown patupilone to be effective at inducing apoptosis in cultured human tumor cell lines, often with greater efficacy than the taxanes (3,16,18,19). This evidence has been further supported with animal studies involving human tumor xenografts (20). There are also indications from clinical trials that epothilone B therapy is associated with a reduced incidence of clinically significant myelosuppression, neuropathy, alopecia, and hypersensitivity relative to other traditional cytotoxic agents (21).

Though few studies have investigated the therapeutic efficacy of sub-toxic concentrations of epothilone B, there is some evidence that supports its potential use in a metronomic dosing schedule as an antiangiogenic chemotherapeutic agent. *In vitro* studies have shown that low-dose epothilone B, when administered on a metronomic schedule, inhibits the formation of angiogenic tubules, and induces apoptosis in cultured human endothelial cells (3,22). Additionally, at *in vitro* concentrations approximating the clinical MTD, epothilone B has a striking antiangiogenic effect when applied to cultured human solid tumors (23).

Based on these concepts we hypothesized that low-dose patupilone, when administered on a continuous metronomic schedule will produce antiangiogenic effects that approximate those associated with a high-dose/short drug exposure time patupilone treatment (MTD) schedule.

Methods

Tissue Preparation

Four primary human tumor specimens were obtained anonymously with the approval of the LSUHSC Institutional Review Board. The four specimens collected included a primary Gastrointestinal Stromal Tumor (GIST), a primary small bowel carcinoid tumor, a carcinoid tumor metastatic to the liver and a carcinoid tumor metastatic to the seminal vesicles (carcinoid seminal vesicle implant). Surgical samples were obtained using sterile techniques, and placed in chilled, serum-containing culture media.

For assay, each tissue specimen was sliced into 1 mm³ fragments and distributed randomly into wells of standard 96-well plates preloaded with thrombin (0.05 IU in 1.0 µL/well) (Sigma Chemical Co., St. Louis, MO) (Figure 1). The tumor fragments were then covered with 100 µL of a clot-forming medium containing human fibrinogen (3 mg/mL), and 0.5 % ε-aminocaproic acid (Sigma) in HPVAM medium. The HPVAM medium consisted of Medium 199 with an antibiotic/antimycotic solution of 100 U penicillin, 100 U streptomycin sulfate and 0.25 µg amphotericin/mL (Gibco BRL, Gaithersburg, MD). The mixture was placed in a humidified incubator and allowed to clot at 37°C in 6.0% CO₂, 94.0% air environment for 30–60 minutes. When fully polymerized, the tissue-containing clots were overlaid with a liquid nutrient medium containing the HPVAM supplemented with 20% fetal bovine serum (Gibco BRL) and 1.0% thrombin, to give a total well volume of 200 µL. Drug-treated wells contained the HPVAM nutrient medium supplemented with epothilone B at appropriate concentrations.

Treatment Schemes

Fragments of the four tumors were treated with nutrient medium or drug-containing medium starting on the first day of culture. Epothilone B was a kind gift from Novartis Pharmaceutical Corporation (East Hanover, NJ), and was applied to fragments of each of the four tumor samples at five different concentrations within the nutrient medium. The drug concentrations that were used included 10nM as an *in vitro* approximation of the MTD, 5nM, 1nM, 0.5nM, and 0.1nM (23). The nutrient medium alone was used as an untreated control for each tumor sample. Forty to sixty (40–60) tissue fragments/tumor were plated for the control and for each of the five drug-treatment groups. The fragments were maintained in culture over 56 days, with fresh aliquots of nutrient or drug-containing medium applied to each individual well every seven days.

Angiogenic Assay

Angiogenesis was evaluated by visual inspection (20×) using an inverted microscope prior to the addition of fresh nutrient or drug-containing medium. Two different parameters of angiogenic growth were assessed: initiation of angiogenesis (%I), and the extent of neovessel growth [angiogenic index, (AI)]. Evaluation was performed every 7 days starting on day 14, through day 56 of culture.

Initiation of an angiogenic response was defined as the development of three or more vessel sprouts around the periphery of the tissue fragment. The percent initiation (% I) was expressed as the percent of the total wells plated for each treatment group that developed an angiogenic response.

The degree of angiogenic growth was expressed as the angiogenic index (AI), and was defined using a semi-quantitative visual rating system developed and validated in our laboratory (Figure 1) (23). During evaluation, each tissue fragment was visually divided into four quadrants. Each of the quadrants was assigned a numeric score from 0 to 4 based on the number of angiogenic vessels (density), the length of vessels, and the percentage of the quadrant's circumference involved with the angiogenic response. Scores from the four quadrants were summed to become the AI, a numerical rating that ranges from 0 to 16. A score of 0 indicated no vessel growth in any of the four quadrants, while a score of 16 indicated long, dense angiogenic vessel growth in all four quadrants. For these studies, the AI included all wells plated, those that exhibited an angiogenic response, as well as those with no evidence of angiogenesis. The AI was expressed as the mean AI value $\pm$ standard error of the mean (SEM) for each treatment group ($n = 40\text{--}60$ wells). The mean AI value can be thought of as a parameter that evaluates both initiation and growth, and represents the overall angiogenic response. These scores correlate well with more objective measures of vessel growth as determined by digital image analysis (24).

For statistical analysis, the change in mean AI over the course of the experiment was calculated (mean AI day 56 – mean AI day 14) for all treatment groups, and expressed as $\Delta\text{AI} \pm \text{SEM}$. The overall percent change in angiogenic growth was also calculated ((mean AI day 56 – mean AI day 14)/mean AI day 14) and expressed as % ΔAI . These values (ΔAI and % ΔAI) can be thought of as describing the overall antiangiogenic effect of the epothilone B treatments. Tissue fragments that were lost at any point during the study due to contamination, human error, or were deemed un-readable due to excess debris were excluded from the analysis.

Statistics

Statistical analysis of ΔAI was performed using the Wilcoxon signed rank test. Statistical analysis of the overall dose-dependent effect was calculated using the non-parametric

Kruskal-Wallis one-way analysis of variance (ANOVA). Statistical significance was set at a value of $P < 0.05$.

Results

Initiation of Angiogenic Response

At the time of the initial reading at day 14, treatment groups from the four tumor samples had, with a few exceptions, approached their maximal angiogenic response. At that time the control groups had initiated an angiogenic response in 62% (primary small bowel carcinoid), 48% (GIST), 90% (carcinoid liver metastasis), and 100% (carcinoid seminal vesicle implant), respectively (Table 1, Figure 2). The two metastatic specimens (carcinoid liver metastasis and the carcinoid seminal vesicle implant) demonstrated a greater rate of angiogenesis initiation compared to the two primary tumor samples (carcinoid and GIST).

In addition to the differences seen in the percent of wells that began to grow in the four different tumor specimens on day 14, there was also a dose-dependent response observed in the percent of plated wells that initiated an angiogenic response following epothilone B treatment. The 10 nM epothilone B treatment, an *in vitro* approximation of the plasma level achieved in a clinical MTD scenario consistently produced the maximum inhibition of initiation of an angiogenic response in all four tissue samples. At the initial evaluation (day 14), the 10 nM treatment groups showed that 0% of the small bowel carcinoid wells had initiated an angiogenic response, while 9.26% of the GIST, 2.44% of the carcinoid liver metastasis, and 20% of the carcinoid seminal vesicle implant specimens had initiated an angiogenic response, respectively (Table 1, Figure 2). The 5 nM epothilone B treatment generally approximated the inhibitory effects seen in the 10 nM epothilone B group. In all groups other than the carcinoid liver metastasis, the 0.1 nM epothilone B treatment had a negligible effect on angiogenic initiation, and their angiogenic responses generally approximated those of the control group. For the seminal vesicle implant carcinoid specimen, neither the 0.5 nM (100%I) nor the 1 nM (97%I) epothilone B treatments significantly inhibited initiation of angiogenesis.

In evaluating the antiangiogenic effect of the metronomic dosing schedule, it is essential to consider the angiogenic response observed over time, specifically at the final evaluation point (day 56). For the carcinoid specimen, a slight increase in the %I was seen in the control and 0.1 nM treatment groups from day 14 until day 56. In contrast, no change was noted in the 10 nM and 5 nM epothilone B groups over time. All time points in these groups demonstrated total inhibition of angiogenesis. However, moderate doses of epothilone B resulted in a decrease in the number of wells demonstrating an angiogenic response over time. The 0.5 nM and 1 nM treatments effected decreases from 30% (%I, day 14) to 17% (%I, day 56) and 12% (%I, day 14) to 3% (%I, day 56), respectively (Table 1, Figure 2). For the GIST specimen minor changes in the %I were observed with epothilone B treatment over time. The 10 nM epothilone B completely inhibited any angiogenic response by day 56, but there was also a decline of %I seen in the 5 nM, 1 nM, and 0.1 nM epothilone B groups, while the control and 0.5 nM groups demonstrated small increases in %I. (Table 1, Figure 2) The remaining two tumors used in this study demonstrated pronounced antiangiogenic effects with metronomic epothilone B treatment. For the carcinoid liver metastasis a decrease in the number of wells exhibiting an angiogenic response for the 0.1 nM (89%I to 57%I), 0.5 nM (56%I to 4%I), and 1 nM (29%I to 2%I) epothilone B-treated groups was observed. There was total inhibition of the angiogenic response in the 10 nM treatment group %I by day 56, and an overall decrease to 2% (%I) in the 5 nM. The changes over time in those two groups were minimal due to the low initial value for the %I. A modest increase in %I for the control group was observed with this specimen. (Table 1, Figure 2). Similar results were obtained for the highly angiogenic carcinoid seminal vesicle implant. With this

tissue, the control, 0.1 nM, 0.5 nM, and 1 nM epothilone B-treated samples all demonstrated approximately 100% (%I) at the initial day 14 time point (Table 1, Figure 2). Though the control and 0.1 nM groups maintained the high angiogenic response through the entire 56 day course, the 0.5 nM (100%I to 50%I) and 1 nM (98%I to 60%I) treatment groups each experienced a large decrease in an observable angiogenic response. Due to the high initial inhibition of an angiogenic response, there was only a minimal decline in %I observed for the 10 nM and 5 nM treatment groups over time.

Overall Antiangiogenic Effect (AI) of Epothilone B

For the carcinoid specimen, epothilone B treatment resulted in mean AI values on day 14 of 3.96 (control), 3.30 (0.1 nM), 0.58 (0.5 nM), 0.12 (1 nM), 0.00 (5 nM), and 0.00 (10 nM), demonstrating a significant dose-dependent antiangiogenic effect with epothilone B therapy (Table 1, Figure 3). In order to assess the overall antiangiogenic effect of continuous exposure to epothilone B, the average change in AI over the 56 day course (Δ AI) was calculated for each treatment group (Table 1, Figure 4). The control and 0.1 nM groups demonstrated significant positive overall angiogenic growth of +44% Δ AI and +59% Δ AI respectively. While the 0.5 nM and 1nM epothilone B-treated groups both experienced an overall antiangiogenic effect, the results were not statistically significant (Table 1, Figure 4). The 5 nM and 10 nM epothilone B treatments totally inhibited angiogenic growth from the outset, and thus there were no observable time-dependent antiangiogenic effects.

For the GIST tissue sample, the mean AI values (Table 1, Figure 3) for each treatment group at the time of the first reading (day 14) were 3.26 (control), 2.71 (0.1 nM), 0.88 (0.5 nM), 0.91 (1 nM), 0.17 (5 nM), and 0.09 (10 nM). These results also demonstrate a significant, dose-dependent, antiangiogenic effect. While the control, 0.1 nM, and 0.5 nM treatment groups all demonstrated a positive overall angiogenic response and the 1 nM, 5 nM, and 10 nM treatment groups experienced an overall antiangiogenic effect, only the 0.1nM and 1 nM treatments had statistically significant overall effects of +26% Δ AI and -35% Δ AI respectively (Table 1, Figure 4).

The carcinoid liver metastasis treatment groups also experienced a significant dose-dependent antiangiogenic response, and had initial day 14 mean AI values of 4.35 (control), 3.40 (0.1 nM), 1.09 (0.5 nM), 0.64 (1 nM), 0.06 (5 nM), and 0.02 (10 nM) (Table 1, Figure 3). Over the course of the study, the control group demonstrated significant positive overall angiogenic growth of +52% Δ AI (Table 1, Figure 4). For this tissue, all 5 groups treated with epothilone B demonstrated an overall antiangiogenic effect over time. The 5 nM and 10 nM groups inhibited the angiogenesis at the initial time point to such an extent that the neovessel regression observed was not sufficient to be statistically significant. However, the lower-doses of 0.1 nM, 0.5 nM, and 1 nM epothilone B treatments caused significant overall time-dependent neovessel regression of -23% Δ AI, -94% Δ AI, and -97% Δ AI respectively (Table 1, Figure 4).

The most robust overall angiogenic growth of the four tissues was seen in the carcinoid seminal vesicle implant fragments (Figure 3, 5). Day 14 mean AI values were 12.00 (control), 10.84 (0.1 nM), 6.18 (0.5 nM), 4.15 (1 nM), 0.53 (5 nM), and 0.25 (10 nM) respectively, results that were, as with the other tissues, significant for an overall dose-dependent antiangiogenic effect (Table 1, Figure 4). Both the control and 0.1 nM treatment groups demonstrated statistically significant positive overall angiogenic growth of +12% Δ AI and +9% Δ AI, respectively over time. The 5 nM and 10 nM epothilone B treatments inhibited the initial angiogenic response to such a degree that the antiangiogenic effect observed over time was too small to be significant. The lower doses of 0.5 nM and 1 nM epothilone B treatments, however, did result in neovessel regression over time, as is evident

both by the respective -67% Δ AI and -67% Δ AI decreases in mean AI (Table 1, Figure 4), as well as the clear visual evidence of neovessel degeneration shown in Figure 5.

Discussion

The ongoing effort to develop effective solid tumor therapies has assumed a new direction in recent years. Antiangiogenic therapies have been demonstrated to be efficacious. This efficacy has resulted in the development of new therapeutic strategies that use antiangiogenic agents alone or in combination with traditional antineoplastic agents. For example, monoclonal antibodies (bevacizumab) and 5-fluorouracil or bevacizumab and other non-toxic antiangiogenic agents (cyclooxygenase-2 inhibitors) are currently being used in the treatment of a variety of tumor types (13,25–27). These antiangiogenic agents are typically used as adjuvants to more traditional therapeutic modalities, and require further investigation as to their overall role in primary treatment.

Metronomic dosing of chemotherapy is a relatively recent development in the therapeutic paradigm. This concept bridges the gap between traditional antiangiogenic and cytotoxic treatments. Traditionally, cytotoxic agents have been administered at maximum tolerable doses (MTD) aimed at rapid tumor cytoreduction. This strategy is often associated with the development of debilitating and painful side effects, and over time, the development of tumor clones that are refractory to the therapy. However these same agents when continuously administered at low doses over a protracted time course, act not as direct cytotoxic tumor treatments, but rather target the endothelial compartment of the tumor vasculature. This ultimately deprives the tumor of its nutrient supply (7,28,29). In several instances metronomic chemotherapy has produced beneficial clinical outcomes that appear to be sustainable over the long term (13–15). A principal advantage to the use of sub-toxic therapeutic drug concentrations is the elimination of the severe side effects typically associated with the traditional MTD use of these agents (5,6,12–15,25). It should be readily apparent that there are inherent benefits to an effective solid tumor therapy that circumvents the development of resistant tumor cell populations while effectively eliminating side effects

Epothilone B is a derivative of a naturally occurring microtubule stabilizer that binds tubulin in a manner that is competitive with the taxanes (16–19,30). A number of molecular derivatives of this compound are currently under investigation for use in the cytotoxic and antiangiogenic treatment of solid tumors (21,31,32). When dosed on a MTD schedule epothilone B has demonstrated greater anti-tumor efficacy relative to similarly acting drugs. These results have been maintained even when epothilone-B is used against multi-drug resistant cultured tumor cell lines (16,19,20). Additionally, clinical trials have revealed a decreased incidence of severe side effects associated with epothilone B therapy (21) as compared to other tubulin inhibitor therapy.

Recent investigations demonstrate that epothilone B may also possess strong antiangiogenic properties. When architecturally intact human tumor fragments were cultured in a fibrin thrombin-based angiogenesis assay, epothilone B treatment significantly inhibited the initiation of an angiogenic response. In addition, this agent can cause the degeneration or regression of an existing neovessel network (23). When applied continuously over time, low doses of epothilone B inhibit the formation of angiogenic vessels and ultimately induce apoptosis in cultured human endothelial cells (3,22). The current study extends the observations on cultured endothelial cells using the human tumor angiogenesis model (HTAM) and confirms the antiangiogenic effects of epothilone B when used at very low doses for prolonged times.

In an ideal set of experiments metronomic dosing would result in a dose-dependent response to epothilone B treatment in the early time points, a continued increase in angiogenic growth over time in the control specimens and significant decreases in the angiogenic growth at later time points in the epothilone B treatment groups. Observations made at day 14 confirmed our results from previous studies, demonstrating that epothilone B has a dose-dependent antiangiogenic effect. The control groups for all four tissues demonstrated strong angiogenic growth over the course of the study. These data imply that neovessels in this fibrin-thrombin culture system are viable and robust in long term (56 day) culture. While the model used in this study has previously been established as a valid system for assessing short term tumor-derived angiogenic growth, the cultured tissue had not previously been maintained over such an extended time course (56 days) (23).

In evaluating the overall changes in the mean angiogenic index (AI) over the course of the study, our intent was to assess the antiangiogenic effect of prolonged exposure of epothilone B on tumor-derived neovessels. With one exception, the overall angiogenic growth seen in the 0.1 nM epothilone B treatment groups generally approximated the angiogenic growth seen in their respective control groups. At slightly higher epothilone B concentrations (0.5 nM) a significant time-dependent effect was noticed in three of the four tumor samples studied. The angiogenic degeneration observed in the carcinoid liver metastasis and the carcinoid seminal vesicle implant samples was statistically significant. All four tumor samples treated with 1 nM epothilone B experienced an overall antiangiogenic effect over time with significant neovessel regression in three tumors of these tumors. These results provide strong evidence of an antiangiogenic effect of low dose epothilone B treatment over an extended time course. It should also be noted that the 1 nM and 0.5 nM doses of epothilone B, representing 1/10th and 1/20th concentrations of the approximated MTD, caused the most apparent metronomic effects. Several investigations that used traditional cytotoxic agents on a metronomic schedule have demonstrated similar outcomes (5,11). These results suggest that these specific fractional doses may represent a strategic focal point for successful therapeutic outcomes associated with long term low dose treatment.

In this study, the 10 nM epothilone B treatment served as an *in vitro* approximation of the plasma levels associated with the MTD, and completely, or nearly completely, inhibited the initiation of an angiogenic response in all four tumor samples. The 5 nM epothilone B treatment effected a result similar to that seen with the 10 nM concentration.

There was also an important pattern noted with regard to the inter-tissue variation of the angiogenic response observed throughout the study. The two metastatic tissues (the carcinoid liver metastasis and the carcinoid seminal vesicle implant) demonstrated much higher rates of initiation of angiogenesis relative to the two primary tumor samples (GIST and carcinoid), a response that might be expected with more advanced and aggressive tumor subtypes. Additionally, it was those two tissues with the most robust angiogenic response that exhibited the greatest susceptibility to the effects of the metronomic therapy with epothilone B. The variability of the angiogenic responses are no doubt the result of inherent genetic and physiologic differences among the patients and their tumors. In the application of experimental results to real-world settings, this component of variation serves as an advantageous distinction between this model system and those that employ the use of genetically controlled tumor cell lines.

This study presents the first direct evidence that epothilone B inhibits tumor-derived angiogenesis when administered in low doses as part of a protracted metronomic regimen. However, more evidence of the antiangiogenic efficacy of low-dose epothilone B is needed, including more extensive testing within the experimental model presented here. Several recent efforts have focused on resolving the antiangiogenic mechanisms of protracted low-

dose therapy. Results from these studies suggest that the *in vivo* activity of low-dose epothilone B and cyclophosphamide may involve indirect mechanisms such as the stimulation of thrombospondin, or the inhibition of circulating endothelial cell progenitors, that contribute to an overall antiangiogenic effect (8,28). However, there is little evidence available regarding either the efficacy or mechanism of action of metronomic chemotherapy with any of the currently available cytotoxic anti-cancer compounds.

In a broader sense, low dose therapy with epothilone B (or other cytotoxic agents) should be considered as only one part of an overall antiangiogenic approach to the treatment of solid tumors. A growing body of evidence suggests that metronomic chemotherapy is far more efficacious when administered in combination with other antiangiogenic agents such as monoclonal anti-VEGF antibodies, PEX (a fragment of human metalloprotease-2), or COX-2 inhibitors, creating a multiple approach attack (2,5,6,9,11,13,25,26). With a better understanding of how this modality of treatment affects the antiangiogenic outcome subsequent efforts may be more focused and thus more efficacious.

Finally, Woltering et al have discovered that epothilone B will also inhibit angiogenesis arising from benign adenomatous and hyperplastic parathyroid glands. Unlike the use of epothilones in cancer treatment, the chronic use of these compounds to control the angiogenic response in a benign disorder may require a significant reduction in their side effect profile. We have previously shown that 10^{-8} M epothilone B will significantly reduce the angiogenic response in hyperparathyroidism (data not shown). We would propose that a reduction of 10–100 fold in the plasma concentrations of epothilone B might allow for the continuous use of this drug for the treatment of primary hyperparathyroidism. Clearly methods to deliver continuous low systemic doses of epothilone B need to be developed.

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Abbreviations

AI	angiogenic index
ANOVA	analysis of variance
SEM	standard error of the mean
%I	percent of wells developing an angiogenic response
FBS	fetal bovine serum

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Figure 1. Angiogenesis Assay Methodology

The left side of the diagram summarizes the experimental method used in this study. Surgically excised tissue samples were cut into 1 mm³ fragments, and embedded in a fibrin-thrombin matrix in a 96-well plate for individual culture. Fragments were overlain with nutrient medium alone, or nutrient medium containing one of five different concentrations of Epothilone B (Epo B), and maintained for 56 days. Fresh medium and Epo B were applied weekly. The right side of the diagram above is a representation of the scoring system used to assess the angiogenic growth of individual tumor fragments. Using an inverted microscope, individual culture wells were visually divided into four quadrants, each of which was assigned a numeric score from 0 to 4 based on the number of angiogenic vessels (density), the length of vessels, and the percentage of the quadrant's circumference involved with the angiogenic response. Scores from the four quadrants were summed to give the angiogenic index (AI), a numerical rating from 0–16. A score of 0 indicated no vessel growth in any of the four quadrants, while a score of 16 indicated long, dense angiogenic vessel growth in all four quadrants. AI scores were recorded for each tissue fragment every seven days, starting at day 14 of culture and ending at day 56.

Figure 2. Angiogenic Initiation in Tumors

All tumor fragments were enmeshed within a fibrin/thrombin matrix, and were maintained over a 56 day course with weekly administration of nutrient medium containing the designated concentrations of Epothilone B (EpoB). Angiogenic assessments were obtained using an inverted microscopy at the time of weekly treatment application. Each graph illustrates changes that occurred over the 56-day experimental period for the different specimens. For each Epo B dose, the percentage of wells with fragments demonstrating an angiogenic response (%I) is indicated.

Figure 3. Angiogenic Index in Tumors

All tumor fragments were enmeshed within a fibrin/thrombin matrix, and were maintained over a 56 day course with weekly administrations of nutrient medium containing the designated concentrations of Epothilone B (Epo B). Angiogenic assessments were obtained using an inverted microscopy at the time of weekly treatment application, and the mean values shown include wells with positive AI values, and also wells showing no angiogenic growth (represented by a 0 score). Each graph illustrates changes that occurred over the 56-day experimental period for the different specimens. For each Epo B dose, the mean angiogenic index (AI $\pm$ SEM) of fragments is indicated. The values marked with * represent observations that are statistically significant at a value of $P < 0.05$.

Figure 4. Change in Angiogenic Response in Tumors

The graphs demonstrate the percent change in the angiogenic index for each Epothilone B (Epo B) treatment over the course of the experiment $\% \Delta \text{AI}$ ((mean AI day 56 – mean AI day 14)/mean AI day 14) for each of the four tumors examined. The values marked with * represent observations that are statistically significant at a value of $P < 0.05$.

Figure 5. Photomicrographs of angiogenesis

These images are visual representations of the overall angiogenic growth, and antiangiogenic effect of Epothilone B (Epo B), observed in three individual tumor fragments at the conclusion of the study. Shown are tumor fragments from the seminal vesicle implant carcinoid tissue sample. Figure A (40 $\times$) is an image of one fragment treated only with nutrient medium and 20% fetal bovine serum throughout the course of the experiment, and is representative of the robust angiogenic growth observed in the control group for this tissue sample. The tumor fragment shown in Figure B (100X) was treated with nutrient medium plus 0.5 nM Epo B, and the fragment in Figure C (100X) was treated with nutrient medium plus 1.0 nM Epo B, both through the course of the experiment. The latter two images are representative of the degeneration of angiogenic vessels observed in these respective treatment groups. These pictures were taken on day 61 of the experiment.

Table 1
EFFECT OF EPOTHILONE B TREATMENT ON TUMOR-DERIVED ANGIOGENESIS

Tissue	Epo B Concentration	N	%I Day 14 [#]	%I Day 56 [#]	AI ± SEM Day 14 [†]	AI ± SEM Day 56 [†]	ΔAI ± SEM [‡]	%ΔAI [€]
Carcinoid	0	50	62.00	70.00	3.96 ± 0.62	5.84 ± 0.67	1.88 ± 0.63[*]	+47.47[*]
	0.1nM	60	65.00	66.67	3.30 ± 0.47	5.23 ± 0.59	1.93 ± 0.43[*]	+58.59[*]
	0.5nM	53	30.19	16.98	0.58 ± 0.17	0.45 ± 0.20	-0.13 ± 0.11	-22.58
	1nM	59	11.86	3.39	0.12 ± 0.04	0.03 ± 0.02	-0.08 ± 0.04	-71.43
	5nM	58	0.00	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00
	10nM	58	0.00	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00
GIST	0	54	48.15	55.56	3.26 ± 0.58	3.87 ± 0.63	0.61 ± 0.39	+18.75
	0.1nM	56	48.21	46.43	2.71 ± 0.50	3.43 ± 0.55	0.71 ± 0.20[*]	+26.32[*]
	0.5nM	57	35.09	35.09	0.88 ± 0.21	1.02 ± 0.24	0.14 ± 0.13	+16.00
	1nM	55	36.36	27.27	0.71 ± 0.18	0.67 ± 0.17	-0.24 ± 0.10[*]	-35.14[*]
	5nM	58	13.79	8.62	0.17 ± 0.07	0.12 ± 0.06	-0.05 ± 0.06	-30.00
	10nM	54	9.26	0.00	0.00 ± 0.00	0.00 ± 0.00	-0.09 ± 0.04	-100
Carcinoid liver metastasis	0	52	90.38	92.31	4.35 ± 0.33	6.62 ± 0.45	2.27 ± 0.44[*]	+52.21[*]
	0.1nM	47	89.36	57.45	3.40 ± 0.27	2.60 ± 0.43	-0.81 ± 0.46[*]	-23.75[*]
	0.5nM	46	56.52	4.37	1.09 ± 0.18	0.07 ± 0.05	-1.02 ± 0.17[*]	-94.00[*]
	1nM	45	28.89	2.22	0.64 ± 0.22	0.02 ± 0.02	-0.62 ± 0.22[*]	-96.55[*]
	5nM	51	5.88	1.96	0.06 ± 0.03	0.02 ± 0.02	-0.04 ± 0.03	-66.67
	10nM	41	2.44	0.00	0.02 ± 0.02	0.00 ± 0.00	-0.02 ± 0.02	-100
seminal vesicle Implant Carcinoid	0	36	100	97.22	12.00 ± 0.40	13.44 ± 0.53	1.44 ± 0.44[*]	+12.04[*]
	0.1nM	31	96.77	96.77	10.84 ± 0.59	11.81 ± 0.56	0.97 ± 0.33[*]	+8.93[*]
	0.5nM	40	100	50.00	6.18 ± 0.49	2.05 ± 0.49	-4.13 ± 0.38[*]	-66.80[*]
	1nM	40	97.50	60.00	4.15 ± 0.36	1.35 ± 0.24	-2.80 ± 0.33[*]	-67.47[*]
	5nM	40	35.00	25.00	0.53 ± 0.13	0.35 ± 0.10	-0.18 ± 0.19	-33.33
	10nM	40	20.00	10.00	0.25 ± 0.09	0.18 ± 0.09	-0.08 ± 0.14	-30.00

[#] %I Day 14 and %I Day 56 represent the percent initiation of an angiogenic response at the first and final time-points at which data was recorded, respectively.

[†] AI ± SEM Day 14 and AI ± SEM Day 56 represent the mean angiogenic index ± the standard error of the mean at the first and final time-points at which data was recorded.

Δ AI $\pm$ SEM represents the change in mean angiogenic index $\pm$ the standard error of the mean from Day 14 (initial data recording) through Day 56 (final data recording).
 ϵ % Δ AI represents the percent change in mean angiogenic index $\pm$ the standard error of the mean from Day 14 (initial data recording) through Day 56 (final data recording).
 * Represents observations that are considered statistically significant at a value of $P < 0.05$.