Synergistic Antitumor Activity of Ixabepilone (BMS-247550) Plus Bevacizumab in Multiple *in vivo* Tumor Models

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Abstract

**Purpose:** Angiogenesis is a critical step in the establishment, growth, and metastasis of solid tumors, and combination of antiangiogenic agents with chemotherapy is an attractive therapeutic option. We investigated the potential of ixabepilone, the first in a new class of antineoplastic agents known as epothilones, to synergize with antiangiogenic agents to inhibit tumor growth.

**Experimental Design:** *In vitro* and *in vivo* cytotoxicity of ixabepilone as single agent and in combination with two targeted antiangiogenic agents, bevacizumab or sunitinib, were examined in preclinical tumor models. Direct effects of the agents against endothelial cells was also examined and compared with the effects of paclitaxel as single agent and in combination with bevacizumab.

**Results:** Ixabepilone showed robust synergistic antitumor activity in combination with bevacinumab and sunitinib in preclinical *in vivo* models derived from breast, colon, lung, and kidney cancers. The synergistic antitumor effect was greater with ixabepilone compared with paclitaxel. Furthermore, ixabepilone was more effective than paclitaxel at killing endothelial cells expressing P-glycoprotein *in vitro* and inhibiting endothelial cell proliferation and tumor angiogenesis *in vivo*.

**Conclusions:** Ixabepilone may enhance the antitumor effects of antiangiogenic therapy by direct cytotoxicity and also indirectly via the killing of tumor-associated endothelial cells. Given that ixabepilone has reduced susceptibility to drug efflux pumps compared with taxanes, these data may explain the increased antiangiogenic and antitumor activity of ixabepilone in combination with antiangiogenic agents. Phase II studies to assess the efficacy and safety of ixabepilone plus bevacizumab in locally recurrent or metastatic breast cancer are planned.

The epothilones and their analogues constitute a novel class of antineoplastic agents derived from the myxobacterium *Sorangium cellulosum*. Ixabepilone is the first epothilone B analogue in this new class, which potently bind and stabilize microtubules in dividing tumor cells. These antimicrotubule agents act in a similar manner to taxanes by stabilizing microtubules, resulting in arrested tumor cell division and apoptosis (1, 2). However, ixabepilone is structurally distinct from the taxanes and therefore has unique properties. For example, unlike the taxanes, which induce apoptosis via activation of caspase 9, ixabepilone affects multiple apoptotic pathways via caspase 2 and p53-mediated activation of Bax (3, 4). Importantly, ixabepilone has distinct tubulin-binding sites and reduced susceptibility to the drug efflux transporter P-glycoprotein (P-gp), which is frequently associated with multidrug resistance that limits the effectiveness of taxanes and other chemotherapeutic agents.

Ixabepilone is rationally designed for optimal *in vivo* efficacy, good metabolic stability, and low protein binding (5). Ixabepilone has broad antitumor activity in human preclinical models, with superior efficacy to taxanes in both taxane-resistant and taxane-sensitive models (6). The clinical activity of ixabepilone has been shown in phase II trials in a broad range of tumor types, including primary (7) and locally advanced or metastatic breast cancer (8), multidrug-resistant breast tumors (9), metastatic or recurrent pancreatic cancer (10), prostate cancer (11, 12), lymphoma (13, 14), non–small cell lung cancer (15), and renal cell cancer (16, 17). In addition, a recent study to identify markers that predict response to ixabepilone in breast cancer has shown promising efficacy in the neoadjuvant setting (7). Ixabepilone was efficacious in phase II trials of patients with metastatic breast cancer who failed frontline taxane-containing regimens (8, 18). A phase III study of ixabepilone in combination with capecitabine showed improved efficacy versus capecitabine monotherapy with significant prolongation of median progression-free survival (5.8 versus 4.2 months; *P* < 0.001) and increased response rate (35% versus 14%; *P* < 0.001; ref. 19). The activity seen in these patients is due, at least in part, to the reduced susceptibility of ixabepilone to several key tumor resistance mechanisms (6, 19, 20). For many patients, intrinsic and acquired tumor resistance, e.g., through overexpression of efflux pump proteins such as P-gp (21, 22) or alternative tubulin isoforms (23, 24),...
are major obstacles to successful cancer treatment. Ixabepilone could offer improved efficacy against drug-resistant tumors due to its noncross resistance with other antineoplastic agents.

Combination chemotherapy is the mainstay of current cancer treatment options. It is well-known that combining regimens with nonoverlapping mechanisms of action and toxicity profiles can produce a synergistic effect. Ixabepilone has shown combinability with previously approved agents. Ixabepilone in combination with capcetabine showed synergistic antitumor activity in patients with metastatic breast cancer pretreated with taxanes and anthracyclines in phase I/II trials (25), leading to phase II and III trials of this combination.

Angiogenesis is a critical step in the establishment, growth, and metastasis of solid tumors by providing tumor cells with essential oxygen, nutrients, and growth factors required for sustained growth and metastasis, and inhibition of tumor angiogenesis is a potential means of retarding tumor growth. In these studies, we determined if the robust antitumor activity of ixabepilone in combination with bevacizumab and/or sunitinib, may provide efficacious regimens that maximize the antiangiogenic and cytotoxic effects of the individual agents. The potential utility of these combination regimens is particularly relevant to patients with drug resistant disease as ixabepilone has shown efficacy in tumors with overexpression of drug efflux transporters and/or βIII-tubulin isotype, two mechanisms that have been implicated in development of resistance to commonly used chemotherapeutic agents.

Translational Relevance

The experimental data presented in this article show synergistic antitumor activity of ixabepilone, a microtubule inhibitor, and two antiangiogenic agents, bevacizumab and sunitinib, in a number of preclinical models. Moreover, ixabepilone showed potent activity against endothelial cells, indicating that antiangiogenic activity of ixabepilone may form a basis for the observed synergy with agents such as bevacizumab. The results suggest that ixabepilone, in combination with bevacizumab and/or sunitinib, may provide efficacious regimens that maximize the antiangiogenic and cytotoxic effects of the individual agents. The potential utility of these combination regimens is particularly relevant to patients with drug resistant disease as ixabepilone has shown efficacy in tumors with overexpression of drug efflux transporters and/or βIII-tubulin isotype, two mechanisms that have been implicated in development of resistance to commonly used chemotherapeutic agents.

Materials and Methods

Study drugs and reagents. Ixabepilone and paclitaxel were synthesized in the Oncology Chemistry Department of Bristol-Myers Squibb Pharmaceutical Research Institute. Clinical vials of bevacizumab, sunitinib, and cisplatin were purchased. Sterile tissue cultures were obtained from Corning. Unless specified, chemicals and solutions used to maintain cell cultures were obtained from Life Technologies, Inc. All other reagents were obtained from Sigma Company at the highest grade available.

Cell lines and in vitro cytotoxic assays. In vitro cytotoxicity was assessed in a panel of cells (including tumor cells and primary endothelial cells) by a tetrazolium-based colorimetric assay, which takes advantage of the metabolic conversion of 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-[4-sulphenyl]-2H-tetrazolium (MTS; inner salt) to a reduced form that absorbs light at 492 nm (31). Cells were seeded 24 h before drug addition. After a 72-h incubation at 37°C with serially diluted compound, MTS was added to the cells, in combination with the electron-coupling agent phenazine methosulfate. The incubation was continued for 3 h, after which the absorbance of the medium at 492 nm was measured with a spectrophotometer to obtain the number of surviving cells relative to control populations.

The in vitro cytotoxicity of ixabepilone and paclitaxel was evaluated in primary endothelial and established tumor cell lines of diverse histologic type by determining the concentration of drug required to kill 50% (IC50) of the cells. Aortic bovine endothelial cells (ABAE) were used to investigate the cytotoxic activity of ixabepilone against endothelial cells in vitro. The tumor cell lines used in this study were as follows: human prostate (LNCAP and PC3); human squamous carcinoma (A431); human breast (MCF7, SKBR-3); human colon [parental HCT116, HCT116/VN46 (MDR1-expressing), HCT116/VP35 (etoposide-resistant, CACO-2, macrophage inflammatory protein, LS147T)]; human ovarian [A2780/DDP-S (parental), A2780/DDP-R (cisplatin-resistant), A2780/Tax-S (parental), A2780/Tax-R (paclitaxel-resistant due to tubulin mutation), OVCAR-3]; human lung (A549, LX-1); human leukemia (CCRF-CEM, HL60, K562); human foreskin fibroblast (HS27); mouse lung fibroblast; and mouse lung carcinoma (M109). All of the cell lines used in this study were purchased from American Type Culture Collection except for the paclitaxel-resistant lines that were developed and described previously (6).

Antitumor in vivo models. Antitumor activity was evaluated in human xenograft models, including colon (GEO, WiDr, HCT116/ VN46), breast (Pat-21 and KPL4), lung (L2987), and renal (151b) carcinoma models. S.c. xenografts were purchased from American Type Culture Collection with the exception of Pat-21, which was derived from a patient who failed paclitaxel, and was obtained from the Cancer Institute of New Jersey. The human tumors were maintained in female BALB/c, nu/nu nude, or Beige-severe combined immunodeficient mice. 16/C and 16C/ADR tumors were maintained in C3H mice. All mice were purchased from Harlan Sprague-Dawley. Tumors were propagated as s.c. transplants in the appropriate mouse strain using tumor fragments taken from donor mice. Eight female mice were used for each experimental test condition.

Compounds were administered and evaluated at the maximum tolerated dose (MTD), which is defined as the dose level immediately below which excessive toxicity (i.e., more than one death) occurred. The MTDs in this study were determined to be as follows: ixabepilone (6 mg/kg i.v. every 4 d for 3 doses), bevacizumab (4 mg/kg i.p. every 4 d for 3 doses), paclitaxel (24 mg/kg i.v. every other day for 5 doses), and sunitinib (40 mg/kg i.v. every day for 14 doses). The compounds were evaluated for tumor response as single agent therapy or in combination to assess synergy.

Tumor response was determined by measurement of tumors with a caliper twice a week, until the tumors reached a predetermined “target” size. Tumor weights (mg) were estimated from the formula:

\[
\text{Tumor weight (mg)} = \frac{\text{Tumor volume (mm}^3\text{)}}{\text{Density}}
\]

where the density is estimated at 1.05 mg/mm^3. Tumor size was measured with a digital caliper with a resolution of 0.01 mm.

Tumor volume was calculated with the formula:

\[
\text{Tumor volume (mm}^3\text{)} = \frac{\text{Length (mm)} \times \text{Width (mm)} \times \text{Height (mm)}}{2}
\]
Synergistic Antitumor Activity: Ixabepilone and Bevacizumab

Tumor weight = (length × width²) ÷ 2(6)

Tumor response end points were reported in terms of tumor volume change [i.e., partial regression, 50% tumor volume reduction; or complete regression = disappearance of measurable tumor] and in terms of delay of tumor growth or progression (T-C value), which is defined as the difference in time (days) required for the treated tumors (T) to reach a predetermined target size compared with those of the control group (C). Because different tumor types can have vastly different exponential growth rates, delays in tumor growth were "normalized" by converting them to gross log cell kill values (LCK), which was accomplished by dividing the T-C value by the tumor volume doubling time multiplied by the exponential function 3.32 [i.e., T-C value/tumor volume doubling time] × 3.32. Compounds were considered active and tumor models were deemed sensitive if the LCK was >1. Conversely, if the LCK value was <1, compounds were considered inactive and tumor models deemed resistant.

Potential drug toxicity interaction affecting treatment tolerability is an important consideration in combination chemotherapy trials. Therefore, interpretation of combination therapeutic results must be based on comparison of antitumor activity of the best possible response for the single agents versus the combination at comparably tolerated doses. Therefore, therapeutic synergism was defined as a therapeutic effect achieved with a tolerated regimen of the combined agents that exceeded the optimal effect achieved at any tolerated dose of monotherapy (32–34). Statistical evaluations of data were done using Gehan’s generalized Wilcoxon test, and P values of <0.05 were considered statistically significant (35).

Determination of MDR1 mRNA levels by quantitative PCR. Bovine tissue RNAs were obtained commercially (Rockland Immunochensicals). Total RNA for other samples was isolated directly from cells using the RNeasy system (Qiagen). cDNA templates for real-time PCR were generated using a SuperScript First-Strand Synthesis kit (Invitrogen). Total RNA for other samples was isolated directly from cells using the RNeasy system (Qiagen). cDNA templates for real-time PCR were generated using a SuperScript First-Strand Synthesis kit (Invitrogen). Real-time PCR was done on a 7900HT real-time PCR machine (Applied Biosystems) using SYBR green–based detection (Eurogentec). Sequences for oligonucleotides used in quantitative PCR were MDR1: GGGACA-GAAAGCTCATGTC AGAA, ACTGGAATGCTGTGTTGACAG and GAPDH: AGCCGAGCCACATCGGT GTGACCAGGCGCCCAATAC. Relative quantities of cDNA were determined using ΔΔCt analysis. Samples were normalized to glyceraldehyde-3-phosphate dehydrogenase unless otherwise stated.

Immunocytochemistry of ABAE cells. ABAE cells in 24-well plates were fixed in methanol for 10 min, blocked with 0.3% hydrogen peroxide in PBS, and rinsed with HIC Wash buffer (Dako). Cells were incubated at room temperature with primary anti-P-gp antibody (rabbit polyclonal SC-8313; Santa Cruz Biotechnology) diluted 1:100 in blocking solution (Dako) for 1 h, then with biotinylated anti-rabbit secondary antibody (RA1000; Vector Laboratories), diluted 1:200 in PBS, for 30 min. Cells were then rinsed and incubated with avidin-biotin complex (ABC Elite; Vector Labs) for 30 min at room temperature. The chromagen 3,3′-diaminobenzidine (Biocare Medical) was used to visualize the staining. All image analyses in this study used a SCCD camera attached to an Olympus BX-60 microscope and Image-Pro Plus software.

Matrilg slug assays. The in vivo effects of each drug on angiogenesis were analyzed in mice using a VEGF/basal fibroblast growth factor–driven endothelial cell infiltration and proliferation Matrilg (BD Biosciences) plug assay. Matrilg was mixed with 75 ng/mL VEGF (recombinant human VEGF165; Peprotech, Inc.) and 200 ng/mL basic fibroblast growth factor (recombinant human basic fibroblast growth factor; Peprotech, Inc.). Because cisplatin belongs to a class of drugs with a different mechanism of action than taxanes and epothilones, its antiangiogenic activity was also evaluated in this model. LX1-0 tumor fragments with Matrilg were implanted s.c. into nude mice on day 0 (n = 8 for each experimental condition). Effects were assessed at various dose levels up to the MTD for each agent. Ixabepilone was administered i.v. at 6.3, 3.15, 1.58, or 0.79 mg/kg every 4 d for 3 doses. Paclitaxel was administered i.v. at 36, 18, 9.0, or 4.5 mg/kg every other day for 5 doses. Cisplatin was administered i.p.

### Table 1. Ixabepilone synergizes with bevacizumab and sunitinib

<table>
<thead>
<tr>
<th>Tumor types</th>
<th>Ixabepilone</th>
<th>Bevacizumab</th>
<th>Combination</th>
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<tr>
<td></td>
<td>Antitumor activity</td>
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<tr>
<td></td>
<td>LCK PR (%) CR (%)</td>
<td>LCK PR (%) CR (%)</td>
<td>LCK PR (%) CR (%)</td>
</tr>
<tr>
<td>Lung L2987</td>
<td>3 38 0 2 0 0</td>
<td>&gt;5.9</td>
<td>88 50</td>
</tr>
<tr>
<td>Breast KPL4</td>
<td>0.5 29 0 2.2 58 14</td>
<td>&gt;3.2</td>
<td>100 86</td>
</tr>
<tr>
<td>Pat-21</td>
<td>1.6 88 25 0.3 0 0</td>
<td>2.3</td>
<td>100 63</td>
</tr>
<tr>
<td>Kidney 151b</td>
<td>0.5 0 0 0.6 10 10</td>
<td>1.5</td>
<td>30 20</td>
</tr>
<tr>
<td>Colon GEO</td>
<td>0.4 0 0 1.1 0 0</td>
<td>5.1</td>
<td>0 0</td>
</tr>
<tr>
<td>WiDr</td>
<td>1.9 38 0 0.2 0 0</td>
<td>&gt;2.2</td>
<td>100 63</td>
</tr>
<tr>
<td>HCT116/VM46</td>
<td>1.2 25 13 0.6 0 0</td>
<td>2.6</td>
<td>88 25</td>
</tr>
<tr>
<td>Kidney 151b</td>
<td>0.6 0 0 0.5 10 10</td>
<td>1.33</td>
<td>20 20</td>
</tr>
</tbody>
</table>

*Abbreviations: PR, partial regression; CR, complete regression.
*MTD of 6 mg/kg, i.v., every 4 d for 3 doses.
*Optimal dose, 4 mg/kg, i.p. every 4 d for 3 doses.
+Synergy is statistically superior efficacy shown in combination compared with single agents alone.
Antitumor activity is reported as LCK, a value that is proportional to the delay of tumor growth to a predetermined target tumor size (1000 mg for GEO and 500 mg for KPL4, Pat-21, 151b, WiDr and HCTVM46); LCK>1 is active.

P < 0.01.

P < 0.05.

**MTD, 40 mg/kg, PO every day for 14 doses.
at 8.0, 4.0, or 2.0 mg/kg every 4 d for 3 doses. After 30 d, or when the tumor reached 1,000 mm³, Matrigel plugs were fixed overnight in 10% neutral-buffered formalin. Plugs were paraffin-embedded, sectioned at 5 μm, and stained with H&E.

Migrating endothelial cell counts and images were obtained for 50 fields per mouse. Antiangiogenic efficacy of each dose was expressed as the percentage of inhibition relative to untreated control. Student’s t tests to detect differences between means were done using SAS JMP software.

**Immunohistochemistry.** The following antibodies were used for immunohistochemistry: MDR (goat polyclonal SC-1517; Santa Cruz Biotechnology), von Willebrand Factor (goat polyclonal SC-8068; Santa Cruz Biotechnology), and CD34 (rat monoclonal 553731; Pharmingen). Excised tumors were fixed, paraffin-embedded, and sectioned to 5 μm. After blocking, heat-induced antigen retrieval was done using a Biocare Neducak decloaking chamber and Citra Plus (BioGenex) solution at 95°C. Sections were incubated overnight at 4°C with Factor VIII primary antibody (SC-8068; Santa Cruz Biotechnology) diluted 1:100 in blocking solution, then incubated with biotinylated anti-goat secondary antibody (BA5000; Vector Laboratories), diluted 1:200 in PBS for 30 min at room temperature. Sections were rinsed and incubated with avidin-biotin complex for 30 min at room temperature, and vascular staining were visualized with 3,3'-diaminobenzidine. Sections were counterstained with Gill 2 hematoxylin and dehydrated through graded ethanol to xylene. Image analysis was done for 50 fields per slide.

**Results**

**Ixabepilone synergizes with antiangiogenic therapy bevacizumab (Avastin) in vivo.** The ability of ixabepilone to synergize with bevacizumab in vivo was assessed in a range of human tumor xenograft models, including colon, breast, lung, and renal carcinoma models. Compounds were always evaluated at a range of doses including the MTD and/or optimal dose as single-agent therapy and in combination. Activity was assessed as tumor growth delay quantified as LCK value. For LCK values of >1, compounds were considered active and tumor models were deemed sensitive. Conversely, for LCK values of <1, compounds were considered inactive and tumor models resistant. Synergism was defined as the combination treatment attaining statistically significant better antitumor activity than the best response attained by the single agent administered at its MTD or optimal dose.

Among the 7 tumors tested, single-agent ixabepilone was active in terms of LCK in 4 of 7 models, whereas bevacizumab was active in 3 of 7 (Table 1). Ixabepilone achieved more tumor regression (6 of 7) than bevacizumab (2 of 7). In the L2987 lung carcinoma model, single-agent ixabepilone and bevacizumab were active with LCK values of 3 (P = 0.005) and 2 (P = 0.002), respectively (Table 1; Fig. 1A). However, the combination of ixabepilone with bevacizumab resulted in even greater antitumor activity (LCK > 5.9) and, most impressively, resulted in cures, which was defined as the absence of any tumor over a period of 10 tumor volume doublings (Fig. 1A). The combination was significantly more effective than single agent ixabepilone (P = 0.003) and bevacizumab (P = 0.0008) alone, thus demonstrating synergy between the two agents (Table 1; Fig. 1A).

The ability of ixabepilone to synergize with bevacizumab was then tested in six other tumor models, including the KPL4 breast, Pat-21 breast, GEO colon, WiDr colon, HCT116/VM46 colon, and 151b renal carcinoma models. In all 6 models, the combination of ixabepilone and bevacizumab resulted in significantly greater antitumor activity compared with the single agents (Table 1). Of interest, synergy was observed regardless of the tumor model sensitivity or resistance to the single-agent therapy of ixabepilone or bevacizumab. The L2987 lung model was sensitive to both ixabepilone and bevacizumab single-agent therapy (Table 1). The 151b renal model was resistant to both ixabepilone and bevacizumab alone (Table 1). The Pat-21 breast, GEO, WiDr, and HCT/VM46 colon models were sensitive to ixabepilone but resistant to bevacizumab (Table 1). The KPL4 breast model was resistant to ixabepilone but sensitive to bevacizumab (Table 1). However, in all of these models, the combination of ixabepilone and bevacizumab resulted in greater antitumor effects and synergy. It is interesting to note that the improved antitumor activity of the combination applied to both the growth delay (LCK) and tumor volume reduction measures (Table 1).

**Ixabepilone synergizes with antiangiogenic therapy sunitinib (Sutent) in vivo.** We next wanted to determine if ixabepilone enhancement of antitumor activity was specific to the monoclonal antibody bevacizumab or if ixabepilone could enhance the antitumor effects of other antiangiogenic therapy, including the small molecular inhibitor sunitinib (Sutent; Pfizer). The ability of these agents to synergize was assessed in the 151b renal lung carcinoma model. Both single-agent ixabepilone and sunitinib were inactive in delaying tumor growth in this model, with respective LCK values of 0.6 and 0.5 (Table 1; Fig. 1B). However, the combination of ixabepilone
with sunitinib resulted in significant tumor delay (LCK 1.33). The combination of ixabepilone and sunitinib was significantly more effective than single-agent ixabepilone \((P = 0.003)\) and bevacizumab \((P = 0.002)\) alone, thus demonstrating synergy between the two agents (Table 1; Fig. 1B). Taken together, these data suggest that ixabepilone can enhance the ability of 2 antiangiogenic therapies to inhibit tumor progression \textit{in vivo}.

\textit{Ixabepilone is directly cytotoxic to endothelial cells \textit{in vitro} and \textit{in vivo}.} \textit{In vitro} cytotoxicity (measured by IC\textsubscript{50}) of ixabepilone (A) and paclitaxel (B) against tumor and normal cell lines. Note that different scales have been used for the ixabepilone and paclitaxel graphs to accommodate all IC\textsubscript{50} values. C and D, in \textit{vivo} antiangiogenic activity of ixabepilone, paclitaxel, and cisplatin in the VEGF/basal fibroblast growth factor – driven endothelial cell infiltration and proliferation Matrigel plug assay in mice represented graphically and by immunohistochemical staining. Paclitaxel was administered i.v. every other day for 5 doses and ixabepilone and cisplatin were administered every 4 d for 3 doses at a range of doses up to and including the MTD. MTDs for ixabepilone, paclitaxel, and cisplatin were 6.3, 36, and 8 mg/kg, respectively. Magnification, ×100. E and F, effects of ixabepilone and paclitaxel, alone and in combination with bevacizumab on tumor vascular density with representative microscopy images. Magnification, ×200. MTDs for ixabepilone and paclitaxel were 6.3 and 36 mg/kg, respectively. Columns, mean; bars, SD. CPT, cisplatin; PTX, paclitaxel; *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).
The direct effect of ixabepilone on endothelial cells was tested \emph{in vivo}. In these experiments, Matrigel plugs supplemented with VEGF/FGF were implanted s.c. into mice. Animals were treated with ixabepilone, paclitaxel, or cisplatin at their respective MTD and proportional fractions (1/2, 1/4, and 1/8) of each MTD. After 30 days of allowing host endothelial cells to migrate, proliferate, and form vascular-like tubes within the Matrigel, the plugs were collected and stained to visualize the surviving endothelial cells after treatment with ixabepilone or paclitaxel. There was a marked reduction (up to 85%) in the number of endothelial cells that migrated into and survived within the Matrigel plugs from mice treated with ixabepilone (Fig. 2C and D). The reduction in endothelial cell number was dose dependent and still exhibited ~60% less endothelial cells at a relatively low dose (1/8 of the MTD), suggesting that ixabepilone does exhibit antiangiogenic effects (Fig. 2C and D).

Furthermore, ixabepilone was significantly more active than paclitaxel at inhibiting endothelial cell infiltration and proliferation in this assay. At 1/8 of the MTD, compared with ixabepilone, which led to 57% reduction of endothelial cells, paclitaxel treatment only caused 27% reduction of endothelial cells (P < 0.0001; Fig. 2C and D). The unrelated heavy metal alkylating–like chemotherapy agent cisplatin was used as a negative control and was significantly less effective at inhibiting endothelial cell infiltration and proliferation than either ixabepilone or paclitaxel at all dose levels evaluated (Fig. 2C and D). Taken together with the cytotoxic \emph{in vitro} assays, these data suggest that ixabepilone has some intrinsic antiangiogenic activity and can potently and directly affect the endothelial cells.

Combination of ixabepilone with bevacizumab results in reduced tumor vessel density. Next, we examined if ixabepilone alone and in combination with bevacizumab showed inhibitory effects on endothelial cells and tumor vessel density in the context of a tumor. Immunohistochemistry using von Willebrand Factor to stain tumor-associated endothelial cells was done on size-matched GEO colon carcinoma tumors treated with bevacizumab, ixabepilone, or the combination of the two agents. Both ixabepilone and bevacizumab as single agents decreased the overall tumor vascular density ~50% compared with untreated controls (P < 0.05; Fig. 2E and F). This supports the \emph{in vitro} finding that ixabepilone was cytotoxic to ABAE cells, and further suggests that ixabepilone may have intrinsic antiangiogenic effects in tumors. Moreover, ixabepilone combined with bevacizumab produced an ~75% reduction in tumor vessel density that was significantly greater than either single-agent ixabepilone or bevacizumab (P < 0.05; Fig. 2E and F). Unlike ixabepilone, paclitaxel at its MTD did not significantly reduce vessel density (P = 0.61). Furthermore, the combination of ixabepilone and bevacizumab had a much more inhibitory effect (~75%) on tumor vessel density compared with the combination of paclitaxel and bevacizumab (~15%; P < 0.001; Fig. 2E and F).

\textbf{Ixabepilone may be more effective against endothelial cells due to expression of P-gp.} A number of studies suggest that endothelial cells express P-gp and multidrug resistance protein (36–42). Because ixabepilone has reduced susceptibility to drug transporters frequently associated with multidrug resistance to taxanes and other chemotherapy, we next investigated if the models in which ixabepilone exhibited antiangiogenic activity also expressed drug efflux pump proteins such as P-gp.

If so, this could provide a potential explanation of why ixabepilone showed superior effects compared with paclitaxel as paclitaxel is more susceptible to these drug efflux pumps. \emph{In vitro}, endothelial cells express high levels of \textit{MDR1} mRNA, which encodes for P-gp as determined by quantitative real-time PCR (Fig. 3A), and they also express P-gp protein as shown by immunocytochemistry (Fig. 3B). Importantly, we confirmed this in tumor-associated endothelial cells \emph{in vivo}. P-gp was expressed on endothelial cells of tumor-associated vessels in the L2987 lung tumor model (Fig. 3C). Taken together, these data suggest that the reduced susceptibility of ixabepilone to P-gp–mediated efflux could allow ixabepilone to show better activity against endothelial cells compared with paclitaxel.

\emph{Compared with paclitaxel, ixabepilone produces greater therapeutic synergism with bevacizumab.} Thus far, our data suggested that ixabepilone was able to synergize with antiangiogenic

\section*{References}

Fig. 3. Tumor-associated endothelial cells express P-gp. \textbf{A}, \textit{MDR1} gene expression levels in ABAE cells and bovine tissues as determined by quantitative real-time PCR. ABAE cells expressed high levels of \textit{MDR1} mRNA relative to other bovine tissues. Data were normalized to total input of RNA and expressed as the total amount of mRNA. \textit{MDR1}, multidrug resistant protein 1. \textbf{B}, P-gp protein expression in ABAE cells, as assessed by immunocytochemistry. Magnification, ×100. \textbf{C}, immunohistochemical staining of P-gp protein in tumor-associated blood vessel endothelial cells in the L2987 lung carcinoma model. Magnification, >200.
Synergistic Antitumor Activity: Ixabepilone and Bevacizumab

Table 2. Ixabepilone produces greater therapeutic synergism with bevacizumab compared with paclitaxel

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antitumor activity. LCK (PR) [CR]</th>
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<tbody>
<tr>
<td></td>
<td>Ixabepilone †</td>
</tr>
<tr>
<td>Colon</td>
<td>0.4 (25) [25]</td>
</tr>
<tr>
<td>GEO</td>
<td>1.2 (25) [13]</td>
</tr>
<tr>
<td>HCT116/VM46</td>
<td>1.2 (25) [13]</td>
</tr>
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</table>

Abbreviations: Ixa, ixabepilone; bev, bevacizumab; PTX, paclitaxel.
*Antitumor activity is reported as LCK, a value that is proportional to the delay of tumor growth to a predetermined target.
† MTD of 6 mg/kg, i.v., every 4 d for 3 doses.
‡ Optimal dose, 4 mg/kg, i.p. every 4 d for 3 doses.
§ MTD, 24 mg/kg, i.v. every other d for 5 doses.
¶ Synergy is statistically superior efficacy shown in combination compared with single agents alone.

Discussion

Angiogenesis is a critical step in the establishment, growth, and metastasis of solid tumors. Inhibition of angiogenesis with the biological agent bevacizumab in combination with chemotherapy is now approved for the treatment of metastatic colon cancer. Bevacizumab has shown preliminary clinical efficacy in breast, pancreatic, non–small cell lung cancer, pancreatic, renal, and head-and-neck cancers (43). Development of combination regimens with bevacizumab could enhance antitumor activity in a wide range of tumor types; this has been seen in recent clinical studies in which the outcomes of metastatic breast cancer and non–small cell lung cancer patients were improved by the addition of bevacizumab to pacltaxel-containing regimens (28, 29). The primary goal of our study was to test the ability of ixabepilone to synergize with bevacizumab. Robust synergistic antitumor activity of ixabepilone in combination with bevacizumab was shown in six different tumor xenograft models in this study, which strongly supports the rationale to test combinations in clinical trials.

There are many possible reasons for the synergy seen between ixabepilone and bevacizumab. It is possible that bevacizumab is able to normalize tumor vasculature, increasing delivery of coadministered drugs. This is based on the hypothesis that tumoral interstitial hypertension caused by irregular and leaky vasculature can limit drug delivery (44). It is possible that normalization of tumor vasculature allows bevacizumab to reduce tumoral interstitial hypertension enough to increase the penetration of ixabepilone. An alternative (but not mutually exclusive) explanation is that ixabepilone complements the antiangiogenic activity of bevacizumab by killing both endothelial and tumor cells directly possibly by interfering with endothelial cell migration and invasion. Ixabepilone, therefore, might enhance the effects of bevacizumab, not only through its complementary antitumor activity, but also through direct antiangiogenic effects on tumor-associated endothelial cells, which is consistent with the data presented in this report. Ixabepilone showed robust cytotoxic activity in a wide range of tumor cell lines in vitro but was also cytotoxic to endothelial cells in this assay. Our in vitro observations are supported by in vivo data demonstrating significant reduction in endothelial cell infiltration and proliferation. The precedent for the antiangiogenic effect of a tubulin-targeting agent was set in a study...
conducted by Pasquier and colleagues that showed the ability of paclitaxel to inhibit endothelial cell proliferation through C2-M arrest and apoptosis, as seen in tumor cells (45). A more recent study found that the antimicrotubule agents docetaxel, epothilone B, and vinblastine significantly inhibited endothelial cell migration, invasion, and tube formation, which reduced Rho GTPase activity and disturbances in the actin cytoskeleton (30). Our findings suggest that ixabepilone also has direct antiangiogenic activity, and that this activity is greater than that seen with paclitaxel.

It is noteworthy that bevacizumab had greater in vivo antitumor activity in combination with ixabepilone than with paclitaxel in the two tumor models tested—GEO and HCT116/VM46. The increased in vivo antitumor activity of the bevacizumab-ixabepilone combination could be due to the higher antiangiogenic activity of ixabepilone both in vitro and in vivo compared with paclitaxel. Therefore, any direct effects on tumor-associated endothelial cells might have been greater with the ixabepilone-containing combination. Additionally, the reduced susceptibility of ixabepilone to drug efflux pumps such as P-gp (19) could have allowed higher concentrations of ixabepilone than paclitaxel to be maintained in the tumor cells (e.g., MDR HCT116/VM46 model) and tumor-associated endothelial cells (e.g., HCT116/VM46 and non-MDR GEO models). P-gp and other drug efflux proteins are expressed on a wide range of tumors (21, 22), and a number of studies have identified expression of P-gp and multidrug resistance protein by tumor-associated vasculature (36–42). Consistent with other preclinical findings, our immunohistochemistry data suggest that P-gp is expressed on tumor vasculature associated with the human GEO xenograft used in this study (46). Thus, the higher antiangiogenic activity of ixabepilone coupled with the expression of drug efflux proteins by tumors and their associated vasculature could contribute to an explanation of the greater antitumor efficacy of the ixabepilone plus bevacizumab combination.

Previous studies have shown that paclitaxel treatment can induce expression of VEGF in tumor cells in vitro through production of reactive oxygen species (47). Because VEGF is an important survival factor, it has been postulated that this could be a potential mechanism of paclitaxel resistance. Accordingly, the ability of bevacizumab to counteract the effects of VEGF expression may contribute to the synergistic interaction between paclitaxel and bevacizumab. It is not known whether ixabepilone also induces VEGF expression and if it does, whether to a greater or lesser extent in comparison with paclitaxel. Both paclitaxel and ixabepilone cause apoptosis, during which reactive oxygen species are generated (48). However the two agents differ in the pathway of apoptosis induction—paclitaxel induces apoptosis through up-regulation of caspase-9 activity and Bax (3), whereas ixabepilone affects multiple apoptotic pathways (4), including enhancement of caspase-2 activity and causes p53 to activate the death effector Bax through induction of expression of the BH3-only protein PUMA (4). Based on these differences, the abilities of the two agents to induce reactive oxygen species may also differ resulting in varying extent of positive interaction with bevacizumab. Further evaluation of this mechanism is clearly warranted, as this has important implications for identifying the optimal cytotoxic combination partner for bevacizumab therapy.

In summary, the data presented here support the rationale for combining ixabepilone and bevacizumab when administered at MTD. Clinical studies of this combination, including trials in drug-resistant populations, are warranted. A phase II efficacy and safety study of ixabepilone plus bevacizumab versus paclitaxel plus bevacizumab as first-line therapy for locally recurrent or metastatic breast cancer is currently recruiting patients.

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References


Disclosure of Potential Conflicts of Interest

All authors, Bristol-Myers Squibb, employees. C. R. Fairchild, Bristol-Myers Squibb, ownership interest.

Appendix A

Synergistic Antitumor Activity: Ixabepilone and Bevacizumab


Synergistic Antitumor Activity of Ixabepilone (BMS-247550) Plus Bevacizumab in Multiple In vivo Tumor Models


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