The Antiangiogenic Property of Docetaxel Is Synergistic with a Recombinant Humanized Monoclonal Antibody against Vascular Endothelial Growth Factor or 2-Methoxyestradiol but Antagonized by Endothelial Growth Factors


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ABSTRACT

Numerous chemotherapeutic agents have been shown to have an inhibitory effect on endothelial cell proliferation and migration, and tubule formation. In this study, we examined the antiangiogenic activity of docetaxel. Docetaxel inhibited endothelial cell proliferation and tubule formation in vitro in a dose-dependent fashion. Docetaxel treatment also inhibited angiogenesis in an in vivo Matrigel plug assay. The endothelial stimulating factors, vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor; HUVEC, human umbilical venous endothelial cell; rhuMAb-VEGF, recombinant humanized monoclonal antibody directed against VEGF; 2ME2, 2 methoxyestradiol; CI, combination index.

INTRODUCTION

Angiogenesis (new blood vessel development) is vital to the growth and metastasis of human cancers, and as such it represents a potential therapeutic target. Numerous agents, both novel agents and traditional cytotoxics, have been shown to inhibit angiogenesis (1). Paclitaxel is a semisynthetic taxane that is cytotoxic by binding to tubulin and preventing microtubule disassembly. This results in the disruption of the dynamic equilibrium of mitosis and cell division and induces apoptosis (2). More recently, paclitaxel has been found to inhibit angiogenesis in both in vitro and in vivo models (3–5). Docetaxel is a related taxane, derived from Taxus brevifolia, with a similar mode of action (6). To date, there is little information regarding the antiangiogenic effects of docetaxel. This report examines the antiangiogenic effects of docetaxel in vitro.

If the antiangiogenic activity of the taxanes represents part of their antitumor activity, it is reasonable to ask why this activity has not led to the eradication of human solid tumors. This report examines the role of the proangiogenic factors VEGF and bFGF in inducing resistance to the antiangiogenic effects of docetaxel. It also examines means by which this novel form of drug resistance might be overcome.

MATERIALS AND METHODS

Materials. HUVECs (Clonetics, San Diego, CA) were cultured in EGM-2 medium (Clonetics) and harvested after having undergone no more than five passages. Docetaxel powder (provided by Aventis Pharmaceuticals, Parsippany, NJ) in 100% alcohol without polysorbate-80, rhuMAb-VEGF in PBS (provided by Genentech, South San Francisco, CA), and 2ME2 in DMSO (provided by EntreMed, Rockville, MD) were added at varying clinically relevant concentrations to a proliferation and a capillary formation assay. VEGF (Chemicon International, Temecula, CA), and bFGF (R&D Systems Minneapolis, MN) were added to the assays in doses that have been shown in vitro to induce the maximal amount of HUVEC endothelial cell proliferation (7, 8). Thrombin, bovine fibrinogen, and aprotinin (all from Sigma Chemical Co, St. Louis MO) were used for the formation of a fibrin clot. Microcarrier beads consisting of a thin layer of denatured collagen chemically coupled to a matrix of cross-linked dextran (175 k, Cytodex 3; Amersharm Pharmacia, Biotech AB, Uppsala, Sweden) were used as the base for the capillary formation. This assay has been shown to induce capillaries with identifiable lumens (9). The MTS/PMS system (Promega, Madison WI) was used to assess cell viability for the proliferation assay.

Proliferation. HUVECs were plated in a 96-well U-bottomed plate (Becton Dickinson Labware, Franklin Lakes, NJ) at a concentration of 10,000 cells/50 μl of medium and incubated in 5% CO2 at 37°C for 48 h. Varying drug concentrations in 50 μl of medium were added to the medium, and this mixture was added to each well within 1 h of the HUVECs being seeded. The proliferation experiments were performed with and without stimulation by the addition of VEGF (60 ng/ml) and bFGF (20 ng/ml). These factors in high doses were chosen to partially simulate the tumor microenvironment. Colorimetric readings were obtained using the MTS/PMS system and an ELISA plate reader. The readings obtained for each concentration tested were from an average of eight wells. Each experiment was expressed as a percentage of the solvent control and completed at least three times with consistent results. The results presented are an average of at least three experiments.

Endothelial Cell Capillary Formation. Microcarrier beads (200 mg) suspended in PBS were autoclaved and then added to HUVECs at a concentration of 30 HUVECs per microcarrier bead. Microcarrier beads and cells were added to a siliconized Petri dish and rocked at 37°C in 5% CO2 for 48 h. The HUVEC-coated microcarrier beads were transferred to a fibrin clot solution. Fibrinogen was dissolved at 2.5 mg/ml in PBS with 0.15 units/ml of aprotinin. Approximately 20 HUVEC-coated microcarrier beads were added to each well of a 12-well plate, and then thrombin (0.625 units/ml) was added to form a gelatinous clot. Medium (1.5 ml) with 1% human serum and aprotinin (0.15 units/ml) was added to the top of each clot. The addition of VEGF (60 ng/ml) and bFGF (20 ng/ml) was required to ensure robust capillary formation. There was minimal capillary formation without stimulation; therefore, all results reported are with stimulation. The drugs to be tested were also added to the top layer. Capillary formation was then quantified after 4 days: every capillary greater than the radius of the bead was scored, and the average number of tubules for each bead per well was determined. The results were expressed as a fraction of the positive control. The experiments were repeated at least three times, and the results presented are the average of at least three experiments.

Assessment of Multiple Drug Effects. The Chou and Talalay (10) method for assessing the combined effect of multiple drugs based on the median effect principle was used in conjunction with the “Calcusyn” program (Biosoft, Cambridge, United Kingdom). Dose-response curves were generated for each
drug alone to determine the median effect plots. Variable ratios of drug concentrations and mutually exclusive equations were used to determine the CIs. This analysis allows drugs with different mechanisms of action to be assessed in combination and for different interactions at low and high doses to be observed if present. A CI \( \leq 1 \) denotes synergy (more than additive), a CI of \( 1 \) demonstrates summation (additive), and a CI \( >1 \) indicates antagonism (less than additive).

**In Vivo Analysis.** Low growth factor Matrigel (Becton Dickinson, Bedford, MA) was supplemented with \( 10^4 \) cancer cells/ml. The breast cancer cell line MCF-7 and a genetically modified MCF-7 variant with transfection of the VEGF gene (both a kind gift from Dr. Francis Kern, Molecular Oncology, Southern Research Institute, Birmingham, AL) were used. The MCF-7 contains neither VEGF receptor, and any effects noted are effects of the cytokine on the surrounding vasculature. Estrogen pellets were inserted 72 h prior to injection of 0.3 ml Matrigel into both flanks of nude mice. The right flank had the parental MCF-7 cell line, and the left had the VEGF transfected cell line. Treatment was commenced at day 7 after injection of the Matrigel. Treatment groups consisted of 10 mice that were treated in the following manner: (a) solvent control, 3 mg/kg of docetaxel given i.p. on days 7 and 10; (b) 0.25 mg/kg rhuMAb-VEGF given i.p. on days 7–14; (c) 20 mg/kg 2ME\(_2\) by oral gavage on days 7–14; (d) 3 mg/kg docetaxel plus 0.25 mg/kg rhuMAb-VEGF on days 7 and 10; (e) 3 mg/kg of docetaxel on days 7 and 10 plus 20 mg/kg 2ME\(_2\) on days 7–14. On the 14th day, each plug was resected, weighed, and immersed in 10 ml of distilled water per g of Matrigel plug. The mixture was placed in a shaking water bath for 24 h. After 24 h, 100 ml were mixed with 100 ml of Drabkin’s reagent (Sigma) for 15 min at room temperature and then placed in a 96-well plate. The hemoglobin concentration was determined by absorbance readings on a plate reader. Two absorbance readings were recorded per sample with the average reported for each. The result for each treatment group was reported as the average from the 10 mice in each cohort. The presence of statistical differences was evaluated by the two-sample two-sided \( t \) test without log transformation.

**RESULTS**

**In Vitro Analysis**

**Docetaxel as an Antiangiogenic**

Docetaxel inhibited HUVEC proliferation in a dose-dependent manner (Fig. 1). HUVECs incubated in the presence of stimulation required a higher concentration of docetaxel (38.0 ng/ml) to inhibit proliferation by 50% (IC\(_{50}\)) compared with the unstimulated cells (7.7 ng/ml). It was also noted that stimulation antagonized the effect of docetaxel at all doses \( >0.1 \) ng/ml because the CIs were \( >1 \) (Fig. 1). Much lower concentrations of docetaxel were required to inhibit capillary formation. When docetaxel was dosed as a single agent, it inhibited capillary formation in a dose-dependent fashion starting at 0.01 ng/ml with an IC\(_{50}\) of 0.27 ng/ml (Fig. 2).

**2ME\(_2\) as an Antiangiogenic**

In contrast to the findings with docetaxel, stimulation did not interfere with the ability of 2ME\(_2\) to inhibit HUVEC proliferation because the IC\(_{50}\) under both conditions was \( \sim 4 \) \( \mu \)M (Fig. 3). The doses of 2ME\(_2\) required to inhibit capillary formation (IC\(_{50}\), 0.71 ng/ml) were also much lower than those needed to inhibit proliferation (Fig. 4).

**Combination Antiangiogenic Therapy**

**Cytostatic Agent (rhuMAb-VEGF) Plus Proapoptotic Agent (Docetaxel).** The rhuMAb-VEGF had minimal activity as a single agent in both the proliferation and capillary formation assays (data not shown).
shown). However, the addition of 10 ng/ml rhuMab-VEGF to docetaxel abrogated the protective effect induced by stimulation. The IC50s generated from the graphs comparing docetaxel plus stimulation to docetaxel plus stimulation plus rhuMab-VEGF revealed synergy for 10 ng/ml rhuMab-VEGF plus docetaxel doses between 0.1 and 10 ng/ml and summation at 100 ng/ml (Fig. 1). In the capillary formation assay, rhuMab-VEGF was synergistic with docetaxel at all doses (Fig. 2).

**Combination Proapoptotic Agents (Docetaxel Plus 2ME2)**

We checked for the presence of synergy with docetaxel and 2ME2 in the presence of stimulation. Synergy was noted for the combination of 1 ng/ml docetaxel plus 0.1, 0.5, and 1 µM 2ME2 (Fig. 3). However, slight antagonism was observed when 2ME2 was dosed at 5 and 10 µM with 1 ng/ml docetaxel. In the capillary formation assay, the combination of low dose (0.1 ng/ml) docetaxel was synergistic with 0.1, 0.5, 1, 2.5, and 5 µM 2ME2 (Fig. 4).

**In Vivo Correlation**

The *in vitro* findings were confirmed in the *in vivo* experiments. Angiogenesis was assessed by measuring the amount of hemoglobin in the Matrigel plugs and recorded as an absorbance reading. The control cohort (parental MCF-7) gave an absorbance reading of 4.43 (Table 1). The amount of angiogenesis in the VEGF-transfected cohort was 1.7-fold greater (*P* = 0.001) with an absorbance reading of 7.56. The amount of angiogenesis in the docetaxel treated cohort was 54% of control (*P* = 0.0048). The amount of angiogenesis in the mice with the VEGF-transfected MCF-7 cells decreased to 57% of that in the untreated mice with the transfected cells (*P* = 0.0004). It is of note that the amount of angiogenesis in the mice with the VEGF-transfected cell line treated with docetaxel (absorbance reading of 4.36) did not differ statistically (*P* = 0.921) from the observations in the untreated mice with the parental MCF-7 cell line (absorbance reading of 4.42). This confirms the *in vitro* findings that showed that excess VEGF protects the endothelial compartment from docetaxel.

The amount of angiogenesis in the rhuMab-VEGF-treated mice with parental MCF-7 was 75% of control (*P* = 0.148). The efficacy of rhuMab-VEGF in the mice with parental MCF-7 did not statistically differ from the results in the rhuMab-VEGF-treated mice with transfected MCF-7 cells (*P* = 0.42). The amount of angiogenesis in the 2ME2-treated mice was 37% of control (*P* = 0.0006). Even in the presence of the VEGF-transfected cells, 2ME2 decreased angiogenesis to 54% of the control (*P* = 0.0053). In contrast to the *in vitro* findings, the excess VEGF appeared to slightly impair the efficacy of 2ME2 (*P* = 0.053).

Combination therapy was also evaluated. The rhuMab-VEGF plus docetaxel combination was able to impact on the amount of angiogenesis in the mice with VEGF-transfected cells as effectively as if not slightly better than docetaxel alone given to mice with parental MCF-7 (*P* = 0.07). In other words, rhuMab-VEGF was able to overcome the resistance to docetaxel induced by VEGF. Docetaxel plus 2ME2 was very effective therapy, with the amount of angiogenesis being 17% of control in the mice with parental MCF-7 (*P* = 0.0009) and 27% of control when the same therapy was given to mice with VEGF-transfected MCF-7 cells (*P* = 0.0006).

**DISCUSSION**

Docetaxel has *in vitro* antiangiogenic properties that are dose dependent and achievable at plasma concentrations routinely achieved in humans. This statement is based on the fact that docetaxel dosed at 100 ng/ml every 3 weeks in humans results in a serum concentration >50 ng/ml for ~10 h (11). It is also of note that *in vitro* cytotoxic doses of docetaxel against cancer cells are in the same range as the doses required to inhibit endothelial cell proliferation. Specifically, the IC50 of docetaxel for a variety of cancer cell lines has been reported to be <10 ng/ml (12). Docetaxel has also been shown to inhibit endothelial cell (HUVEC) migration in a modified Boyden chamber assay with an IC50 of 0.01 nm (13). Therefore, docetaxel has *in vitro* antiangiogenic properties against three endothelial cell functions (proliferation, migration, and capillary formation). Moreover, the antiangiogenic IC50s are below the serum levels achieved in humans and are similar to the *in vitro* cytotoxic IC50s against cancer cells.

There is also clinical evidence supporting the notion that taxanes are antiangiogenic. Namely, low weekly doses of taxanes have been shown to induce tumor regression in some patients with tumors refractory to paclitaxel administered every 3 weeks (14). These responses to low, frequent taxane exposure may occur as result of inhibition of tumor angiogenesis. However, the fact that cancers are not eradicated by this schedule of taxanes implies that the vascular compartment may be resistant to the antiangiogenic properties of taxanes.

Although low doses of docetaxel inhibit HUVEC proliferation and capillary formation *in vitro*, cancers are able to grow and divide in patients with ample vasculature. The disconnect between the *in vitro* and the clinical data suggests that the vasculature in the tumor microenvironment may be “protected” or that regrowth is not dependent on the development of new vasculature. The data presented here infer that VEGF and bFGF, two substances secreted by tumor cells and present in the tumor microenvironment, are able to alter the phenotype of HUVECs and protect them from docetaxel. Taken together, these data support the findings that intracellular signals are “switched” on by growth factors and that these signals can be blocked by rhuMab-VEGF. Specifically, VEGF has been shown to prevent apoptosis by activating p54 mitogen-activated protein kinase, c-jun-NH2-kinase, phosphoinositide 3-OH kinase, and Akt pathways as well as by inducing Bcl-2 and inhibitors of apoptosis (7, 15, 16). Fibroblast growth factor has also been shown to induce Bcl-2 and survivin in endothelial cells (7, 17). It has also been observed that endothelial cells derived from the tumor microenvironment express P-glycoprotein, whereas HUVECs do not and that the latter were markedly more sensitive to the cytotoxic effects of paclitaxel (18).

The data presented here also suggest that the survival properties induced by growth factors can be overcome with either an agent that directly blocks these survival factors (rhuMab-VEGF) or with low doses of an antiangiogenic agent with proapoptotic capabilities (2ME2; Ref. 19). 2ME2 is an estrogen metabolite with very little estrogenic activity that has been observed in only a few systems. It is also antiproliferative in tumor and endothelial cell lines in both *in vitro* and *in vivo* assays (20, 21). The observation that stimulation affected the antiproliferative effects of docetaxel but not those of 2ME2 suggests that these agents induce apoptosis by different mechanisms. The fact that synergy was observed at low doses of both docetaxel and 2ME2 suggests that apoptosis is induced most effi-

<table>
<thead>
<tr>
<th>Group</th>
<th>Parental MCF-7</th>
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<td></td>
<td><em>A</em> (SD)</td>
<td><em>A</em> (SD)</td>
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<td>2.39 (0.83)</td>
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<td>rhuMab-VEGF</td>
<td>3.38 (1.27)</td>
<td>3.85 (1.21)</td>
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<td>1.65 (0.95)</td>
</tr>
<tr>
<td>Docetaxel + 2ME2</td>
<td>0.76 (0.51)</td>
<td>1.23 (0.42)</td>
</tr>
</tbody>
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* A, absorbance.
ciently at low doses. In contrast, the antagonism noted at higher doses of 2ME₂ with 1 ng/ml docetaxel suggests that these drugs may interfere with the apoptotic capabilities of one another. This may be relevant when determining the doses of drugs to use when designing clinical trials.

The in vivo data presented correlate with the in vitro findings. It must be pointed out that the model used does not differentiate well between an antitumor and a direct antiangiogenic effect. However, it does evaluate the effect of treatment on the tumor microenvironment. The model also evaluates the effect of the paracrine release of VEGF on the efficacy of treatment in inhibiting angiogenesis. Moreover, because MCF-7 cells have neither VEGF receptor 1 nor 2, the changes induced by this cytokine are more likely the result of its effect on the vasculature. With this understanding, we concluded that docetaxel can inhibit angiogenesis in vivo and that excess VEGF can impair this capability. This “resistance” phenomenon was less apparent with the other proapoptotic agent, 2ME₂. It was also noted that rhuMAb-VEGF or 2ME₂ was able to enhance the ability of docetaxel to decrease the amount of angiogenesis in the tumor microenvironment. The augmentation of the effect was seen in both the unstimulated and stimulated environments.

Three recent reports have shown in vivo that a combination of low, frequent dose chemotherapy plus an agent that specifically targets the endothelial cell compartment is able to eradicate or control tumor growth much more effectively than the cytotoxic alone. The agents used were low-dose cyclophosphamide plus TNP-470 (22), vinblastine plus an antibody against VEGF receptor-2 (23), and carboplatin plus rhuMAb-VEGF (24). The data presented here provide rationale for combining low doses of docetaxel with rhuMAb-VEGF or 2ME₂.

In conclusion, there is an increasing amount of data suggesting that a combination of a low, frequent dose of a cytotoxic agent that targets the tumor cell and the vascular compartment in combination with an agent that inhibits angiogenesis may be a fruitful area for future clinical research.

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REFERENCES


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