Combining Agents that Target the Tumor Microenvironment Improves the Efficacy of Anticancer Therapy

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Abstract Purpose: Over the past 60 years, cytotoxic chemotherapy has targeted the cancer cell. Despite this, there have been few cancer cures. A new approach to cancer therapy is to target the multicellular biological entity of the tumor microenvironment.

Experimental Design: Lenalidomide, an immunomodulatory drug, sunitinib, a tyrosine kinase inhibitor, and low-dose metronomic cyclophosphamide, were tested alone and in combination for their abilities to inhibit endothelial cell tube formation, rat aortic ring growth, tumor growth, and metastatic development in mice. In addition, ectopic tumor lysates were evaluated for the presence of proangiogenic proteins.

Results: The three agents alone were shown to significantly inhibit endothelial cells’ ability to form tubes and significantly inhibit the multicellular microenvironment in the rat aortic ring assay (P < 0.01 and P < 0.001). This effect was also significantly augmented when the agents were combined. Furthermore, the three-drug combination was able halt the progression of tumor growth almost completely in xenograft models of ocular melanoma, colon cancer, pancreatic cancer, and cutaneous melanoma. These agents significantly decrease the number of proliferating cells in tumors, significantly increase the number of cells undergoing active cell death in tumors, and significantly decrease the number of blood vessels in treated tumors (P < 0.05). Combination therapy shows a decrease in the compensatory up-regulation of proangiogenic proteins after treatment when compared with single-agent therapy.

Conclusions: This combination of agents causes an inhospitable microenvironment for tumor cells and shows great promise for use in the clinic.

Cytotoxic chemotherapy to combat cancer was originally introduced almost 60 years ago when the first patient was treated with aminopterin for leukemia (1). Since then, chemotherapeutics have been designed almost exclusively to target the cancer cell itself. Despite the exponential growth of chemotherapeutics used in the treatment of cancer, there have been relatively few cures for solid tumors from these methods. One hypothesis to this ineffectiveness is that neoplastic cells are rapidly dividing and, as a byproduct, are genetically unstable and mutate rapidly (2). This instability leads to the high rates of drug resistance from the treatment of cancer, there have been relatively few cures for solid tumors from these methods. One hypothesis to this ineffectiveness is that neoplastic cells are rapidly dividing and, as a byproduct, are genetically unstable and mutate rapidly (2). This instability leads to the high rates of drug resistance from chemotherapies (2). In addition, the doses of chemotherapy necessary to achieve complete tumor remission are often extremely toxic to the patient.

Because of the relatively few cures from chemotherapeutics, we need to rethink our treatment strategy and address the complex network of multiple cell types within the tumor microenvironment instead of focusing on the tumor cells alone. This network is made up of a heterogeneous group of cells, including, epithelium, fibroblasts, endothelial cells, pericytes, recruited circulating endothelial cells, and tumor-infiltrating lymphoid cells. Further complexity in the network comes from the scaffolding of the extracellular matrix. In the tumor, all of these cell types, as well as the extracellular matrix, take part in a dynamic and bidirectional feedback process, which allows the tumor to grow, invade, and ultimately metastasize. This concept was initially termed the Seed and Soil Hypothesis by Paget more than 100 years ago (3).

With the tumor microenvironment as our ultimate target, we designed a treatment strategy that used drugs with mechanisms of action that attacked distinct elements within the tumor. Along with the inherent faults of targeting only tumor cells, targeting only endothelial cells may also have limitations. Antiangiogenic agents have always been considered immune to drug resistance due to the stable genetic make-up of endothelial cells (4, 5). However, if resistance is defined as a therapy’s inability to cause tumor regression, tumors are indeed resistant to antiangiogenic agents. This may be due to the microenvironment’s ability to compensate by redundancies in the complex pathways available (6). By combining agents that target...
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Different pathways, compensatory up-regulation is dampened, which leads to improved inhibition of angiogenesis in the tumor (6).

We evaluated three compounds to test our hypothesis that targeting various components of the microenvironment would improve anticancer therapy. Each compound targeted distinct pathways and were chosen to block multiple levels of this redundant system. Specifically, lenalidomide, an immunomodulatory agent, sunitinib, a tyrosine kinase inhibitor, and metronomic cyclophosphamide, a directly cytotoxic chemotherapeutic, were used. The goal was to show that combinatory therapy can lead to inhibition of endothelial cell function, progression of endothelial cells and other supporting cells in the microenvironment in an ex vivo model, and ultimately inhibit tumor growth in mouse xenograft models.

Lenalidomide (Revlimid Celgene) is an oral immunomodulatory drug which is a derivative of thalidomide. It also has a multifunctional profile and has been shown to cause caspase-dependent apoptosis of tumor cells lines, inhibit basic fibroblast growth factor and vascular endothelial growth factor (VEGF)–induced neovascularization in vivo, abrogate AKT/PKB phosphorylation required for endothelial cell migration, inhibit proangiogenic tumor necrosis factor-α production, and activate and stimulate proliferation of cytotoxic T-cell lymphocyes (7–14).

Sunitinib (Sutent, Pfizer) is an oral multitargeted tyrosine kinase inhibitor which inhibits a variety of tyrosine kinases involved in tumor growth and metastasis. Specifically, sunitinib blocks the phosphorylation of VEGF receptor 1 (VEGFR1), VEGFR2, and VEGFR3, as well as platelet-derived growth factor receptor-β and c-kit (15). This multifunctional profile causes inhibition of tumor proliferation via the c-kit pathway, endothelial cell activation and proliferation via VEGFR-2, and recruitment of pericytes via platelet-derived growth factor receptor-β (required for vessel stabilization and maturity; refs. 16–21).

Metronomic chemotherapy is a newer method of scheduling for chemotherapeutic agents. In the 1960s, multiple dosing schedules were compared in preclinical models and maximal tolerated dose chemotherapy was found to lead to the highest percentage of cure rates (22). Therefore, maximal tolerated dose dosing became the standard of care for cancer patients. Maximal tolerated dose dosing uses high doses of chemotherapy given with extended treatment intervals. This type of dosing is designed to kill the maximum number of cancer cells per dose; however, the dosing causes normal host cell injury from the chemotherapy. Extended treatment-free intervals are needed to allow the normal host cells to recover from the cytotoxic insult. This extended drug-free interval can lead to increased resistance from the tumor cells and tumor regrowth (23).

Observations of this dosing schema noted that endothelial cells are susceptible to chemotherapeutics (24, 25). The extended treatment-free interval with maximal tolerated dose chemotherapy leads to regrowth of endothelial cells, which in turn supports regrowth of tumors (23). This led to the use of lower doses of chemotherapy given more frequently in an attempt to combat this problem. This type of dosing has been termed “antiangiogenic chemotherapy” or “metronomic dosing” (23, 26).

There is evidence that the effects of metronomic dosing of cyclophosphamide can be enhanced when it is combined with an antiangiogenic small molecule inhibitor, such as TNP-470 (23). The combination of these compounds addresses multiple levels of the tumor microenvironment as it pertains to tumor cell proliferation and apoptosis, vascular induction and stabilization, immunomodulation, and stromal support.

Our hypothesis is that modulation of the tumor’s microenvironment with multidirected therapy, in the form of combinatory treatment, will have improved efficacy in angiogenesis assays and in xenograft models. We feel that, by using agents that target different pathways in the microenvironment, we will be able to reduce compensation from other proangiogenic pathways which will enhance the antitumor effect seen by single-agent therapy. We therefore studied these agents alone and in combination in our in vitro, ex vivo, and in vivo models. Our goal is to develop more effective agents for the treatment of patients with cancer.

Materials and Methods

Compound preparation
Sunitinib (Pfizer) and Cyclophosphamide (Bristol-Myers Squibb) were obtained from NIH Pharmacy. Cyclophosphamide is an inactive prodrug, which is converted to 4-hydroxylation in vivo by cytochrome P450 (27). For our in vitro studies, freshly reconstituted 4-hydroperoxycyclophosphamide (Niomech Company) was added to medium. This compound spontaneously converts to 4-HC in an aqueous solution.

Lenalidomide was obtained from Celgene Corporation. For in vitro and ex vivo studies, lenalidomide, sunitinib, and fumagillin (Sigma) were solubilized in 100% DMSO on the day of treatment. Fumagillin (100 μmol/L) was used as a positive control in these studies, secondary to its known inhibition of endothelial cells. For combinatory treatments, sunitinib and lenalidomide were kept in 1:1 concentration ratios when solubilized in DMSO, so that the final treatment medium was 0.1% DMSO. For in vivo studies, cyclophosphamide, sunitinib, and/or lenalidomide were suspended in an aqueous solution of 0.5% carboxymethylcellulose and 0.25% Tween 80 (Sigma).

Cell proliferation assays
Human umbilical vascular endothelial cells (HUVEC; American Type Culture Collection) were grown in culture with EBM-2 (Cambrex) complete medium and, on the day of each assay, were trypsinized, counted, and plated in treatment medium. Treatment medium consisted of EBM-2 with 1% fetal bovine serum and different concentrations of experimental compound. All groups were kept in a 37°C incubator. Negative control was 0.1% DMSO.

Cells were plated onto 96-well plates at 3,000 cells per well in complete medium and allowed to grow for 24 h. At 24 h, medium was removed, and treatment medium was added. After 24 h in treatment medium, viability was assessed by WST-1 assay, which quantifies mitochondrial metabolic activity, as per the manufacturer’s instructions (Roche).

Angiogenesis assays
Tube formation assay. Matrigel (BD Biosciences) was plated at 200 μL/well in 24-well plates and allowed to reach the solid phase. HUVECs were then suspended in treatment medium and plated on top of the Matrigel at a density of 50,000 cells per well. After 6 h in an incubator, the wells were imaged on an inverted phase contrast microscope (Zeiss, Axiovision). HUVECs normally form a branching plexus of tubes on artificial extracellular matrices, such as Matrigel. We defined a tube forming node as one which had three or more branches coming from a common center. Quantization was blinded and done by counting each nodal branch point that had three or more branches.
Branch point counts per image constituted the raw data for statistical analysis. There were six replicates per treatment group.

**Rat aortic ring assay.** The rat aortic ring assay was done as previously described (28). Briefly, Matrigel was plated on CultureSlides (BD). Six-week-old Sprague-Dawley rats were sacrificed, and their thoracoabdominal aortas were procured. The aortas were dissected free of any fibroadipose tissue and sectioned into 0.5-mm rings. Rings were placed on Matrigel, one ring per chamber. Each ring was then embedded in Matrigel. Complete medium was added, and the rings were incubated for another 24 h. The next day, medium was exchanged for basal medium containing various concentrations of drug in 0.1% DMSO. The rings were incubated in treatment medium for 7 days, with medium and drug compound being refreshed every other day; after 7 days, they were imaged on an inverted phase contrast microscope. The images were imported into Adobe Photoshop CS2. Blinded quantization was done as previously described (28) by using Photoshop to quantify pixel counts that represented the outgrowth. These pixel counts served as raw data for analysis.

**Xenograft models**

All animal studies were in accord with the NIH Animal Care and Use Committee Guidelines. Female NCr-nu/nu mice (Taconic Farms, NCI, Animal Production Program) were used for tumor challenge experiments with human cell lines. Female C57/Black 6 mice (Taconic Farms) were used with the MC-38 murine colon cancer cell line. The 92.1 human ocular melanoma cell line (gifted by Bruce R. Ksander, Harvard Medical School), HT-29 human colon cancer cell line (American Type Culture Collection), MiaPaCa2 human pancreatic cancer cell line (American Type Culture Collection), and M-21 human cutaneous melanoma cell line were maintained in culture using RPMI 1640 (Life Technologies) with 10% fetal bovine serum. The MC-38 murine colon cancer cell line (American Type Culture Collection) was maintained in culture using DMEM (Life Technologies) with 10% fetal bovine serum. Mice were dorsally injected s.c. with 100 μL of cell suspension, using a 27-g needle. When mice had evidence of a palpable mass, they were randomized to treatment groups. Therapy involved daily oral gavage of 100 mg/kg lenalidomide, 40 mg/kg sunitinib, and/or 25 mg/kg cyclophosphamide in 50 μL doses through a 22-g oral gavage needle. Therapy was given 7 days a week throughout treatment. Combination therapy was given as a cocktail to keep the amount of vehicle constant between groups. Doses of compounds were chosen from previously published mouse studies that showed efficacy with little to no toxicity and the stated doses tested (15, 23, 29). Mice were followed daily for signs of toxicity to the treatment, such as subjective weight loss, inability to eat, or changes in behavior. S.c. lesions were measured in three dimensions on a twice a week schedule. To count visceral surface metastases in the mice inoculated with 92.1 tumors, the tracheobronchopulmonary tree was resected en bloc lungs insufflated with 4% formalin. The counts were blindly done with the use of a dissecting microscope. There were 7 to 10 mice in each treatment group.

**Immunofluorescence studies**

Mice were injected with 92.1 tumor cells as previously described, and when mice had evidence of a palpable mass, they were randomized to treatment groups. Therapy involved daily oral gavage as previously described and was carried out for 4 days. After treatment, animals were euthanized, and their s.c. lesions were resected and immediately snap frozen in liquid nitrogen. Frozen tumors were sectioned at 10-μm thickness, placed onto slides, and subsequently fixed with 4% paraformaldehyde (USBweb.com) for 1 min. Slides were washed in PBS twice. Cells were blocked (1 h) and incubated (1 h) with monoclonal antibodies (ki-67, 1:200 rabbit monoclonal antibody, Labvision Corp.; activated caspase-3, 1:500 rabbit monoclonal antibody, BD PharMingen; CD31, 1:50, rat monoclonal antibody, BD PharMingen) in blocking buffer (antibody diluent, Zymed). Cells were then washed thrice with PBS and 1% Tween 20 (Sigma) and incubated with Alexa-Fluor 488 or Alexa-Fluor 594 secondary antibody (ki-67, goat anti-rabbit Alexa-594, 1:200; caspase-3, goat anti-rabbit Alexa-488, 1:500; CD31, goat anti-rat Alexa-488, 1:400) in blocking buffer for 30 min. Cells were washed thrice with PBS and 1% Tween 20, mounted in Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories), and visualized by epifluorescent microscopy. All procedures were done at room temperature. All experiments consisted of tumors from three mice for each treatment group. Three sections from each tumor were evaluated, and two areas per section were imaged. Images were taken randomly at low powered fields, as well as a mosaic image consisting of a quilt of nine consecutive low powered fields stitched together using imaging software. Quantification was done using the Zeiss Axiovision quantification software.

**Angiogenesis protein array**

Mice were injected with the 92.1 cell line as previously described, and daily oral gavage of treatment were given once tumors reached ~200 mm3. Treatment began on day 0 and ended on day 2. After treatment on day 1 and day 2, animals were euthanized and their s.c. lesions were resected and immediately snap frozen in liquid nitrogen. Two mice were euthanized from each group for each day. Tumors were then ground using a small tissue grinder (Kendall) in cell lysis buffer with protease inhibitors (RayBiotech). Fifty micrograms of total protein lysate was hybridized to a glass chip angiogenesis antibody array (RayBiotech) according to manufacturer’s guidelines. Tumor lysate was tested from two different mice from each different treatment group in separate experiments (duplicate spots for each array = 4 replicates total). Fluorescence intensity was analyzed using GenesPix 4000B microarray scanner (Molecular Devices) and its software, which calculated the mean intensity of each spot minus background.

**Statistical method**

Statistical analysis was done with the use of GraphPad InStat v.3.05, GraphPad Prism v.4.02, and Excel 2002. Statistical analysis involved one-way ANOVA followed by Tukey-Kramer multiple comparisons testing. Data from the xenograft model were evaluated with one-way ANOVA with multiple comparisons testing.

**Results**

4-HC and sunitinib inhibit the proliferation of endothelial cells in culture. In vitro assays, done with increasing concentrations of sunitinib alone and in a 1:1 ratio with lenalidomide, tested for the ability to inhibit the proliferation of HUVECs in a WST-1 assay. Sunitinib inhibits the proliferation of HUVEC in a statistically significant dose-dependent fashion beginning at 0.01 μmol/L (P < 0.01; Fig. 1A). The inhibitory capacity increases substantially above 1.0 μmol/L. The addition of lenalidomide does not improve the inhibition of HUVEC proliferation (Fig. 1A). 4-HC almost completely inhibits the proliferation of HUVECs at concentrations of >5 μg/mL (Fig. 1B).

4-HC, sunitinib, and lenalidomide are able to affect the function of endothelial cells and the interaction of cells with the extracellular matrix in an in vitro model of the microenvironment. Sunitinib, lenalidomide, and 4-HC were tested for their ability to affect the phenotype of endothelial cells and their interaction with the extracellular matrix in the tube formation assay. When HUVECs are placed onto Matrigel, they form tube-like structures which can be quantified. Matrigel (BD Bioscience) is a mouse sarcoma-solubilized basement membrane preparation, which includes many components of the extracellular matrix (30), as well as growth factors which are known to potentiate angiogenesis, including basic fibroblast growth factor and platelet-derived growth factor.
In the presence of higher concentrations of lenalidomide, there is inhibition of tube formation beginning at a concentration of 0.01 μmol/L (data not shown). In the presence of sunitinib, there is inhibition of HUVECs’ ability to form tubes in a statistically significant fashion beginning at a concentration of 0.1 μmol/L (P < 0.01; Fig. 1C). In the presence of 4-HC alone, there is a statistically significant increased inhibition of tube formation (Fig. 1C). When the two compounds, 4-HC and sunitinib, are combined, there is an additive effect. Specifically, at a sunitinib concentration of 0.1 μmol/L when higher concentrations of 4-HC are added, there is an additive inhibition of tube formation (Fig. 1C) in a statistically significant fashion (P < 0.05 for sunitinib 0.1 μmol/L/no 4-HC compared with sunitinib 0.1 μmol/L/4-HC 1.0 μg/mL). When all three agents are combined, there is more marked inhibition of tube formation compared with single-agent or doublet therapy (n = 6 for each treatment group). Bars, SE.

4-HC, sunitinib, and lenalidomide inhibit the microenvironment cells in an ex vivo model. The rat aortic ring model incorporates multiple cell types to mimic the microenvironment. When sunitinib is tested alone for its ability to inhibit outgrowth from the rat aortic ring (Fig. 2B), it begins to inhibit outgrowth at a concentration of 0.01 μmol/L (P < 0.001) and then almost completely inhibits outgrowth at 0.1 μmol/L. When 4-HC is applied to rat aortic rings alone at a concentration of 10 μg/mL, there is complete inhibition of outgrowth from the rat aortic ring (Fig. 2D). When sunitinib and lenalidomide are added to the treatment group, there is an additive effect. At a sunitinib concentration of 0.1 μmol/L with higher concentrations of 4-HC, there is more inhibition of tube formation. When all three agents are combined, there is more marked inhibition of tube formation compared with single-agent or doublet therapy (Fig. 1C and D).
additive inhibition compared with single agent alone (Fig. 2B and D). At sunitinib/lenalidomide doses of 0.01 μmol/L, there is a trend toward greater inhibition with higher doses of 4-HC.

Combining agents that target multiple pathways in the tumor microenvironment halts the growth of primary tumors and inhibits the development of surface lung metastases in a model of ocular melanoma. When mice are treated with single-agent therapy in a xenograft model of human ocular melanoma, there is a statistically significant inhibition in tumor growth over time when compared with mice treated with vehicle alone (Fig. 3A). Sunitinib, when given as a single agent, has the greatest inhibition of any of the three agents alone. When therapies are combined as doublets, there is an additive increase in the inhibition of primary tumor growth, but when all three agents are combined, there is an even greater inhibition of the primary tumor growth. This inhibition with triple therapy (sunitinib, lenalidomide, and metronomic doses of cyclophosphamide) is statistically greater than doublet therapy ($P < 0.05$).

In this highly aggressive xenograft model, spontaneous surface lung metastases were counted for each treatment group. Cyclophosphamide was able to decrease the number of surface lung metastases from a median of 18 to a median of 9. Sunitinib was able to lower the number of lung metastases to a median of 1. The triple combination therapy completely eliminated surface lung metastases in treated mice ($n = 7$).

Combining different agents that target different pathways in the tumor microenvironment can halt the growth of tumors in a histologically independent fashion. Sunitinib, lenalidomide, metronomic cyclophosphamide, and the combination of the three agents were used to treat xenograft models of human ocular melanoma.

**Fig. 2.** Effects of sunitinib, lenalidomide, and metronomic cyclophosphamide on the tube formation model and rat aortic ring model of the tumor microenvironment. **A,** representative sections which illustrate the ability of the compounds to inhibit tube formation. The first column of photographs represent tube formation without compounds, the second column represents tube formation at sunitinib and lenalidomide concentrations of 0.01 μmol/L and 4-HC concentration of 0.1 μg/mL, and the third column represents tube formation at sunitinib and lenalidomide concentrations of 1.0 μmol/L and 4-HC concentration of 0.1 μg/mL. **B,** sunitinib is able to inhibit outgrowth from a rat aortic ring when used as a single agent in a statistically significant manner beginning at doses of 0.1 μmol/L. **C,** representative rat aortic rings are shown from treatment experiments. These rings were treated with vehicle alone, sunitinib at 0.1 μmol/L, and fumagillin, which was used as a known angiogenic inhibitor control. **D,** when 4-HC, lenalidomide, and sunitinib are combined, there is an improvement in the inhibition of microvessel outgrowth when compared with single-agent alone (B). At sunitinib and lenalidomide doses of 0.01 μmol/L, when 4-HC doses are increased, there is more inhibition of microvessel outgrowth. This trend does not reach statistical significance. Eight aortic rings were tested at each drug concentration for each experiment. Bars, SE.
colon cancer, murine colon cancer, human pancreatic cancer, and human cutaneous melanoma.

In the colon cancer xenograft model, single-agent therapy slowed the growth of the primary tumor grown s.c. compared with vehicle alone (Fig. 4A). This inhibition can be augmented by combination of the three agents, sunitinib, lenalidomide, and metronomic cyclophosphamide, in an additive fashion. At day 15 of treatment, the triple drug combination inhibits the growth of tumors greater than lenalidomide or cyclophosphamide in a statistically significant fashion ($P < 0.001$ and $P < 0.01$ respectively; Fig. 4A). By day 22 of treatment, the triple drug regimen significantly improves the inhibition of tumor growth over any single agent alone ($P < 0.001$; Fig. 4A).

In a model of colon cancer in mice with a competent immune system, single-agent sunitinib, lenalidomide, and metronomic cyclophosphamide slowed the growth of primary tumors compared with vehicle alone. The combination of the three drugs significantly enhances this inhibition by day 14 of treatment over any single agent alone ($P < 0.001$; Fig. 4B).

In a xenograft model of pancreatic cancer, the combination of sunitinib, lenalidomide, and metronomic cyclophosphamide can inhibit the growth of primary tumors greater than any single agent alone in a statistically significant fashion ($P < 0.001$ comparing triple therapy to any single agent at day 28 of treatment; Fig. 5C). Furthermore, in a xenograft model of cutaneous melanoma, the three-drug combination can inhibit the growth of a primary tumor greater than any single agent alone (at day 18 and day 21 comparing sunitinib alone to triple therapy, $P < 0.05$; Fig. 4D).
We found no evidence of treatment-related toxicity in the mice treated with single-agent or combination therapy. This was true of all the mice through all histologies.

**Triple therapy inhibits proliferation of cells of the tumor microenvironment, causes an increase in apoptosis in cells of the microenvironment, and decreases the amount of blood vessels in the tumor microenvironment.**

To examine the effects of these agents alone and in combination, immunofluorescence imaging was used to quantify levels of cell proliferation and blood vessel density in the tumor microenvironment. After mice had been treated for 4 days with single-agent therapy with sunitinib, lenalidomide, metronomic cyclophosphamide or the combination of three agents, tumors were harvested, and tumor sections were stained for proliferation markers, apoptotic markers, and for the quantification of blood vessels. Day 4 tumors were used to minimize the effect of size on the observations because at this point all tumors were of equal size.

For ki67, a marker of cellular proliferation, representative sections for each cohort are shown in Fig. 5 (column 1). For single-agent therapy, when compared with vehicle

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**Fig. 4.** Effects of sunitinib, lenalidomide, metronomic cyclophosphamide alone, and triple combination therapy on xenograft models of colon cancer, pancreatic cancer, and cutaneous melanoma. A, in a human colon cancer xenograft model, single-agent therapy can significantly slow the growth of a primary tumor compared with vehicle-treated tumors ($P < 0.001$), and triple combination therapy can significantly slow the progression of the primary tumor compared with single agents alone ($P < 0.001$). B, in a murine colon cancer xenograft model, single-agent therapy can significantly slow the growth of a primary tumor compared with vehicle-treated tumors ($P < 0.001$), and triple combination therapy can significantly slow the progression of the primary tumor compared with single agents alone ($P < 0.001$). C, single-agent therapy can inhibit the growth of a pancreatic primary tumor in mice when compared with tumors in mice treated with vehicle alone. Triple therapy further inhibits the tumor growth over single-agent therapy in an additive fashion (both $P < 0.001$). D, triple-agent therapy can statistically significantly inhibit the growth of a primary human cutaneous melanoma tumor when compared with tumors grown in mice treated with single agents alone ($P < 0.05$). *, $P < 0.001$; **, $P < 0.05$ ($n = 7$ to 10 for each treatment group). Bars, SE.

**Fig. 5.** Representative images from tumors treated with either single-agent or triple combination therapy daily for four consecutive days. A, column 1, representative tumors stained for ki67, a marker of cell proliferation after four days of treatment; column 2, representative tumors stained for activated caspase-3 after treatment; column 3, representative tumors stained for CD31, a marker for endothelial cells in treated tumors. B, the percentage of cells in the tumors staining positive for ki67, caspase-3, and CD31 were quantified using Zeiss Axiowission software to gauge therapeutic effect in the tumors. The percentage of ki67-positive cells in the vehicle alone – treated tumors is significantly higher than single-agent sunitinib – treated tumors. Tumors treated with three-drug combination treatment showed a significant decrease in the percentage of ki67-positive cells compared with single-agent treatment. The percentage of cells undergoing active cell death was quantified by looking at levels of caspase-3. The vehicle-treated tumors had very few cells undergoing active cell death compared with a statistically higher percentage of cells in the single-agent and an even higher percentage of cells in the combination therapy group. Vessel densities were lower in the single agent – treated groups when compared with control tumors as shown when CD31 percentages were quantified in tumor sections. Triple therapy further lowered blood vessel density compared with single-agent therapy. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $n = 3$ mice per treatment group. Bars, SE.
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A

- Ki67
- Caspase-3
- CD31

Vehicle

CTX

Lenalidomide

Sunitinib

Triple

200 μm

B

- % Area with Ki67

Vehicle

Lenalidomide

Sunitinib

CTX

Combo

200 μm

Percentage of Area with Positive Staining for Caspase-3

Vehicle

Lenalidomide

Sunitinib

CTX

Combo

200 μm

% area with Positive Staining for CD31

Vehicle

Lenalidomide

Sunitinib

CTX

Combo

200 μm
alone–treated tumors, there is a trend toward less proliferation in the tumor microenvironment, and this reaches statistical significance in the sunitinib-treated tumors ($P < 0.05$; Fig. 5, column 2). The tumors treated with triple drug combination show a much lower percentage of proliferating cells compared with single agent–treated tumors ($P < 0.001$ compared with cyclophosphamide alone).

Activated caspase-3 staining was done in tumor sections to observe apoptotic cells within the tumor microenvironment. For single-agent therapy, when compared with vehicle alone–treated tumors, there are more cells undergoing apoptosis (Fig. 5, column 2) in the tumor microenvironment. This increase is significant for all single agents with $P$ of $<0.001$. The tumors treated with triple drug combination show an additive effect on apoptosis compared with single agent–treated tumors ($P < 0.001$).

CD31 staining was completed to assess the relative density of blood vessels in these highly vascular tumors. For single-agent therapy, there is a significant decrease in the number of blood vessels compared with vehicle-treated tumors ($P < 0.01$; Fig. 5, column 3). When the three agents are combined, there is an additive effect of inhibiting blood vessels ($P < 0.05$ when comparing tumors in mice treated with sunitinib alone versus triple therapy; Fig. 5).

Single-agent therapy leads to up-regulation of compensatory cytokines and receptors in the tumor microenvironment, and combination therapy dampens this compensation. Single-agent therapy to treat tumors may fail because of significant up-regulation of compensatory mechanisms in the tumor microenvironment. This may lead to continued growth of tumors in the presence of antitumor therapy. Combination therapy may be more effective than single-agent treatment by blocking this compensatory activity (6). To test this theory, protein levels were studied in tumor cell lysates at 24 and 48 h after treatment to look for changes due to therapy. Single-agent therapy caused a significant up-regulation in angiopoietin-1, G-CSF, MCP-3, MMP-1, IL-1β, and VEGFR2 protein levels when compared with vehicle-treated tumors (see Fig. 6). Mice treated with combination therapy had a statistically significant less dramatic increase in protein levels when compared with control.

**Discussion**

A treatment strategy targeting the tumor microenvironment as a whole and not just a specific component was designed and evaluated. We chose three compounds, each working through multiple, distinct mechanisms. This approach leads to maximal inhibition of the complex network of pathways of the microenvironment. Specifically, lenalidomide was chosen because it has known antiangiogenic properties, which make it an excellent inhibitor of the tumor microenvironment (7–14). Sunitinib targets multiple tyrosine kinases known to be involved in angiogenesis (15). It works through distinct mechanisms than lenalidomide and, therefore, complements lenalidomide. Furthermore, metronomic cyclophosphamide is directly cytotoxic to endothelial cells, as well as multiple tumor cell types, which make it an excellent addition (23, 26). Because these three compounds are able to inhibit multiple pathways involved in angiogenesis, the combination of the three allows for the greatest tumor inhibition.

Our goal was to show that this combinatory approach could lead to inhibition of endothelial cell function, inhibition of progression of endothelial cells and other supporting cells in the microenvironment in an *ex vivo* model, and, ultimately, inhibition of tumor growth in the mouse xenograft model.

We were able to show that in *in vitro* models of angiogenesis, these drugs can inhibit the proliferation of endothelial cells. Not only is there a direct inhibition of endothelial cell proliferation, but these compounds inhibit the function of the endothelial cell. Specifically, the compounds inhibit the capacity of the endothelial cell to form tubes when cells are placed into a microenvironment with an extracellular matrix. The combination of the three drugs is able to inhibit tube formation in an additive manner.

These drugs are able to inhibit outgrowth in the rat aortic ring model. The model uses not just endothelial cells in culture but a truer set of cells that would be present in a tumor growing in *vivo*. The microvessels formed from the rat aortic ring contains endothelial cells, fibroblasts, and pericytes, which all function together to form the outgrowth (31, 32). These cells interact with the Matrigel extracellular matrix so that a true microenvironment is present. In addition, cytokines and growth factors that the extracellular matrix secretes as well as the cytokines that each cell secretes due to the interactions among cell types are present. Each drug plays a role in inhibiting ring outgrowths. When the drugs are combined, there is enhanced inhibition of the outgrowth possibly due to blocking different cell types in the microenvironment.

Perhaps, more importantly, this combination of agents is able to almost completely inhibit the growth of primary tumors in mice. This is in contrast to single-agent therapy, which can only slow the progression of tumor growth in mice. In addition, the combination of the three drugs completely eliminates the development of surface lung metastases in a highly aggressive xenograft model. At present, it is not known why the combination of agents inhibits the development of lung metastases. The agents may work in concert to cause an inhospitable microenvironment in these mice. This may be due to the therapy inhibiting neovascularization in metastases as described by other groups (33). Further studies are necessary to elucidate the exact mechanism of action.

This therapy works in a histologically independent fashion. In our models, we have shown that single-agent therapy and combination therapy are able to inhibit endothelial cell function and can also inhibit microenvironment function in the rat aortic ring model. These models lack tumor cells, and thus, the observed effects of the agents are on the endothelial cells in a simulated microenvironment. Therefore, tumor growth inhibition is not caused solely on effects of therapy on tumor cells but rather on the therapy's ability to cause an inhospitable tumor microenvironment. Some of the effects of treatment may be due to direct cytotoxic tumor effects from the compounds. This makes the therapy a potentially beneficial one to use in the clinic due to its possible widespread applicability.

The mechanism of action of this therapy is beginning to be understood. There is a reduction in the number of blood vessels in the treated tumors, suggesting that the agents are antiangiogenic. Both sunitinib and metronomic cyclophosphamide are directly cytotoxic to endothelial cells. This could be a major cause of the lower vessel density that is seen in single-agent therapy, as well as in combination therapy. Lenalidomide is
known to inhibit VEGF and basic fibroblast growth factor angiogenesis and, therefore, would decrease the blood vessel density. Furthermore, sunitinib is known to inhibit downstream signaling from VEGFR2 and platelet-derived growth factor receptor-β, both of which are necessary for angiogenesis. Specifically, VEGFR2 is involved in endothelial cell proliferation and survival (34). Platelet-derived growth factor receptor-β is expressed on the supporting cells of the microenvironment, pericytes and vascular smooth muscle cells, to grow. Platelet-derived growth factor receptor-β is a key target of sunitinib therapy (35, 36). By blocking pericyte and smooth muscle cell proliferation and function, two of the key supporting cells for angiogenesis and stabilization of the microenvironment are lost, leading to enhanced effects on the microenvironment and ultimately on limiting tumor growth.

After treatment, there is an increase in the number of apoptotic cells and a decrease in the number of proliferating cells. Single-agent therapy leads to up-regulation in compensatory proangiogenic proteins in the tumor after treatment when compared with vehicle alone–treated tumors. When protein levels are quantified at day 1 and day 2 after treatment, there is a statistically significant increase in proangiogenic proteins in the tumor cell lysate in mice treated with single-agent therapy. Triple combination therapy reduced this compensation, although it does not completely inhibit the up-regulation of these proteins (*, P < 0.05; **, P < 0.001; n = 2 mice per treatment, 2 replicates per mouse = 4 replicates total per group). Bars, SE.

![Graphs showing changes in protein levels after treatment](image)
cells, suggesting that these agents can cause cells to undergo active cell death. This is due to either a direct tumoricidal effect or an indirect effect caused by therapy changing cues in the tumor microenvironment or a combination of the two effects. Because of this slow down in cell growth, we must conclude that these agents target all aspects of the tumor microenvironment.

The benefits of combining agents are illustrated by the dramatic reduction in the growth of primary tumors and complete elimination of surface lung metastases in mice when compared with single-agent treatment. Single-agent therapy fails because it is unable to inhibit the multiple angiogenic pathways and multiple cell types found in the microenvironment. This has been shown by other groups and now has been shown by illustrating elevated protein levels in the tumor microenvironment of treated tumors when compared with vehicle-treated tumors (6). By using three agents with different mechanisms of action, there should be an improvement in the overall response to treatment because these agents should inhibit these different pathways (6). Furthermore, combination therapy has been shown to limit the compensatory up-regulation in the microenvironment, which further supports combination therapy to target the microenvironment (6). By combining three agents to target the tumor microenvironment, there is less compensation in angiogenic protein levels in the microenvironment, which may account for the reduction in tumor volume seen in the triple therapy combination—treated animals.

As stated previously, our ultimate goal is to have a therapy which can cause tumor regression in cancer patients. We are currently actively pursuing this three-drug regimen in the clinic in a phase II trial, which is enrolling patients with metastatic ocular melanoma.

References
Combining Agents that Target the Tumor Microenvironment Improves the Efficacy of Anticancer Therapy


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