Antineoplastic Effects of Chemotherapeutic Agents Are Potentiated by NM-3, an Inhibitor of Angiogenesis

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ABSTRACT

Antiangiogenic therapy, although effective in shrinking tumors, has not yet been established as a standalone treatment for cancer. This therapeutic limitation can be overcome by combining angiogenesis inhibitors with chemotherapeutic agents. NM-3, a small molecule isocoumarin, is a recently discovered angiogenesis inhibitor. Here we demonstrate that NM-3 inhibits the proliferation of human umbilical vein endothelial cells in vitro, at concentrations 10-fold less than those required to inhibit normal fibroblasts or tumor cells (HT29, MKN28, and MCF-7). NM-3 alone inhibits endothelial sprouting and tube formation in vitro. The results also show that synergistic antiproliferative activity is observed when human umbilical vein endothelial cells are treated with NM-3 in combination with 5-fluorouracil. The effects of treatment with NM-3 and various chemotherapeutic agents were also evaluated in tumor xenografts. The results demonstrate that combined treatment with NM-3 and chemotherapeutic agents significantly reduced mean tumor volume compared with either treatment alone, with no effects on body weight changes. Taken together, these findings demonstrate that NM-3 is a well-tolerated angiogenesis inhibitor that significantly increases the efficacy of existing antineoplastic agents.

INTRODUCTION

Angiogenesis, the formation of new blood vessels through sprouting and remodeling of preexisting vessels, is the primary process responsible for tumor neovascularization and is a tightly regulated event. The central role of angiogenesis in the development of numerous pathological conditions, including cancer, is now well appreciated (1–3). The assembly of endothelial cells into vascular structures requires specific signals from surface receptors that are activated by soluble and extra cellular matrix ligands. The regulation of angiogenesis is balanced by pro and antiangiogenic factors. VEGF2 and fibroblast growth factor-2 are the best characterized factors promoting angiogenesis (4–6). Inhibitors of angiogenesis include multiple classes of compounds with some of the most potent being proteolytic fragments of endogenous extra cellular matrix proteins (7–9).

Investigations of angiogenic agents have been conducted in preclinical studies and clinical trials. Multiple agents have been effective, such as neutralizing antibodies to angiogenic proteins, integrin molecules, and growth factor receptors (4, 10, 11). Moreover, kinase inhibitors, natural products, and antibiotic derivatives, such as minocycline, have been studied for their ability to inhibit angiogenesis (12–14). Another class of reagents, which has emerged as having antiangiogenic activity, contains extracellular matrix protein fragments. Endostatin, a Mf 20,000 COOH-terminal fragment of collagen XVIII, is the first such molecule undergoing Phase I clinical trials (15, 16). Recently, studies have shown that the NC1 domain of type IV collagen, which plays a crucial role in influencing basement membrane organization (17–19), has antiangiogenic and tumor growth delay properties in animal models (19–21).

Recent studies have demonstrated that radiation-induced tumor regression is enhanced by angiogenesis inhibitors (22–25). These include angiostatin, as well as antibodies to both VEGF and VEGFR2. In these studies, combined treatments produce greater than additive antitumor effects when compared with either treatment alone.

NM-3, 2-(8-hydroxy-6-methoxy-1-oxo-1H-2-benzopyran-3-yl) propionic acid is a synthetic derivative of cytogenin (26, 27). In vitro studies have demonstrated that endothelial cell growth is inhibited by NM-3 at concentrations 10-fold less than those required to inhibit normal fibroblasts or tumor cells (HT29, MKN28, and MCF7; Refs. 23, 28, 29). Moreover, antiangiogenic effects of NM-3 have been demonstrated in the mouse dorsal air sac model (27) and Matrigel plug angiogenesis model (29). NM-3 has a serum half-life of 3–10 h in preclinical models, a low toxicity profile, and, therefore, a potentially wide therapeutic window (29). The present studies demonstrate that when NM-3 is combined with various chemotherapeutic agents (paclitaxel, 5-FU, or CPA), a significant reduction in mouse xenograft tumor volumes was observed without an apparent increase in toxicity, as judged by serial weight changes. The results also show that NM-3 selectively inhibits endothelial cell proliferation, migration, sprouting, and tube formation with a minimal effect on tumor cell proliferation. Taken together, our results provide a rationale for the clinical development of NM-3 in ongoing Phase I clinical trials of NM-3 alone and in combination with existing chemotherapeutic agents.

MATERIALS AND METHODS

Cell Culture and Reagents. HUVECs were obtained from Clonetics Corp. (San Diego, CA) and grown in complete EGM-2 media per manufacturer’s protocol. HT29 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. MCF-7, MKN28, and MDA-MB435 cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum and antibiotics. NM-3, CPA (Aldrich Chem.), and 5-FU (Sigma Chemical Co.) were prepared at a stock concentration of 1 mg/ml in PBS, filtered, and sterilized. Paclitaxel (Sigma Chemical Co.) was dissolved first in 50% Cremophor EL (Sigma Chemical Co.) in ethanol, then diluted further in PBS.

WST-1 Assays. Cells were seeded at a density of 2-3 × 10^3 for 24 h before treatment. MKN28, MCF7, and HT-29 cells were plated in 96-well culture plates and incubated with various concentrations of NM-3 for 72 h at 37°C in a CO2 incubator (5% CO2 and 95% air). At the end of the culture, cell proliferation was determined by the WST-1 colorimetric assay (Wako Pure Chemicals, Inc., Osaka, Japan) according to the manufacturer’s instructions. After treatment, the number of viable cells was determined by the trypan blue exclusion assay; cells stained were identified as nonviable, and cells unstained were identified as viable.

BrdUrd Labeling. Cells were plated onto 96-well plates and incubated overnight at 37°C in a 5% CO2 incubator. After the removal of the media, 100
μl of media containing the indicated compound were added to each well. Plates were incubated for an additional 48 and 72 h without subsequent medium changes, and BrdUrd incorporation was measured using an ELISA-based assay (Calbiochem).

**[3H]Thymidine Incorporation.** HUVECs (2 × 10^4 cells/well) were plated in gelatin-coated, 96-well culture plates in the presence or absence of NM-3 for 48 h at 37°C. Before harvesting (15 h), 1 μCi of [3H]thymidine was added to each well. At the end of the culture, cells were washed three times with PBS and harvested onto glass fiber filter paper. Incorporation of [3H]thymidine into cells was determined using a liquid scintillation counter.

**Migration Assay.** Migration assays were performed as described previously (30) with minor modifications. HUVECs were stimulated with 10 ng of VEGF, and 25,000 cells were used per well. All migration assays were performed using 48-well Boyden chambers.

**Matrigel Tube Formation Assay.** Plates (48 well) were coated with 150 μl of growth factor-reduced Matrigel (10 mg/ml; Collaborative Biomedical Products) and incubated at 37°C to promote gelling. HUVECs were suspended in EGM-2, and 300 μl of suspension containing 36,000 cells were seeded into each well of 24-well plates. NM-3 was added to the cells at concentrations ranging from 0 to 300 μg/ml. All test samples were performed in triplicate. After 18 h, images were captured, and tube formation was scored as one tube.

**Aortic Ring Assay.** Aortas were harvested from 6-week-old Sprague Dawley rats (Taconic Farms, Inc.) and flushed with EGM-2 media using a 21-gauge syringe. The aorta was cut into 1-mm slices, which were placed in 48-well plates containing 130 μl of ice cold Matrigel (10 mg/ml). The plates were incubated for 1 h at 37°C to promote gelling, after which the rings were fed with 200 μl of EGM-2 containing NM-3 at 0–200 μg/ml. Aortic rings were fed 3 days later and photographed on day 6. Degree of endothelial outgrowth/sprouting was scored from 0 (least positive) to 4 (most positive). A minimum of six rings were assayed for each data point.

**Human Tumor Xenograft Models.** NCRNU nude mice (Taconic Farms, Inc.), 5–6 weeks old and weighing approximately 20–25 grams, were implanted with 2 × 10^6 MDA-MB435 cells into the subcutaneous mammary fat pad of female mice; 2 × 10^6 PC3 cells were implanted into the dorsal subcutis of male mice. When the tumors were between 50 and 100 mm³, the animals were pair matched into treatment and control groups. Each group contained nine mice, each of which was ear tagged and followed individually throughout the experiment. Initial doses were given on the day of pair matching (day 0). NM-3 was administered via i.p. injection daily at doses ranging from 10 to 100 mg/kg; paclitaxel, 5-FU, and CPA were administered via i.p. at doses indicated. Mice were weighed twice weekly, and tumor measurements were taken by calipers twice weekly, starting on day 0. These tumor measurements were converted to tumor volume (V) using the formula (V = W² × L/2), where W and L are the smaller and larger diameters, respectively, and plotted against time. On termination, the mice were weighed and sacrificed, and their tumors were excised. The mean tumor weight per group was calculated, and the mean treated tumor weight/mean control tumor weight × 100% was subtracted from 100% to give the TGI for each group. Treatment-related toxicity was evaluated by means of serial weight measurements.

**Tumor Histopathology.** Mice were sacrificed and then perfused with saline, followed by phosphate-buffered formalin via cardiac perfusion. Tumors were biopsied, immersion fixed for 4 h, dehydrated through a graded series of ethanols, and processed for routine paraffin embedding. Tumors were evaluated histologically for microvessel density (CD31-positive cells) and proliferation index (PCNA staining).

**CD31 Staining.** Sections were cut, deparaffinized, and treated with 0.25% trypsin for 30 min at room temperature to enhance antigen availability to CD31 monoclonal antibody (clone MECl3; PharMingen), used at 25 μg/ml. Nonspecific rat IgG (25 μg/ml) was used as a control. Immunoperoxidase staining was carried out using the Vectastain Elite ABC Kit (Vector) following the manufacturer’s protocol. Sections were counterstained with Methyl green.

**PCNA Staining.** Sections were cut, deparaffinized, and stained using the DAKO EnVision+ System (3,3′-diaminobenzidine) following the manufacturer’s protocol. PCNA (Ab-1; Oncogene) was used at 1.25 μg/ml. Sections were counterstained with Methyl green.

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![Fig. 1. Effects of NM-3 on proliferation of cells.](image)

In A, HUVECs were treated with various concentrations of NM-3 for the indicated times. The number of viable cells was determined by the trypan blue exclusion assay. Values are the mean ± SE of four experiments. * and **, significantly different from the control at P < 0.05 and P < 0.01, respectively. In B, HUVECs were cultured in the presence or absence of NM-3 for 48 h at 37°C and assayed for [3H]thymidine incorporation as described in “Materials and Methods.” In C, MKN28, MCF7, and HT-29 cells were plated in 96-well culture plates and incubated with various concentrations of NM-3 for 72 h at 37°C. Cell proliferation was determined by the WST-1 colorimetric assay. In D, HUVECs and MDA-MB435 cells were plated in 96-well culture plates and incubated with various concentrations of NM-3 for 48 h at 37°C. Cell proliferation was determined by the BrdUrd labeling assay.
antiangiogenic properties of NM-3.

NM-3 Selectively Inhibits Endothelial Cell Migration. To further establish the antiangiogenic properties of NM-3, we performed the HUVEC migration assay. Migration of endothelial cells is considered important for the induction of angiogenesis. HUVECs were stimulated by VEGF in a Boyden chamber system and evaluated for the capacity of NM-3 to inhibit migration. NM-3 inhibited migration of HUVECs in a dose-dependent manner, with maximal inhibition observed at 200 μg/ml (Fig. 2). These findings reveal that NM-3 selectively inhibits the growth of endothelial cells.

NM-3 Is Antiangiogenic in Matrigel Tube Formation and Rat Aortic Ring-sprouting Assays. Previous studies have shown that treatment of VEGF secreting malignant tumor cells (Sarcoma 180 mouse sarcoma cells) with different doses of NM-3 is associated with inhibition of angiogenesis as measured by the dorsal air sac model (27). To further assess the angiogenic response of NM-3 in HUVECs, we first examined the ability of NM-3 to inhibit the formation of capillary-like structures in Matrigel. This in vitro assay measures the ability of endothelial cells to form tube networks in a Matrigel basement membrane preparation. In forming tubes, the cells must migrate and organize in a manner similar to the steps that occur during angiogenesis. The results demonstrate formation of tubes after 18 h in wells that contain untreated cells in EGM (Fig. 3, A and B). More importantly, treatment with NM-3 resulted in a dose-dependent inhibition of tube formation (Fig. 3, A and B). To further confirm the effect of NM-3 on angiogenesis, an aortic ring-sprouting assay was performed. This ex vivo assay mimics several stages in angiogenesis, i.e., endothelial cell sprouting, migration, and tube formation. Aortic endothelial cells were induced to sprout from the aorta in the presence of Matrigel and EGM containing angiogenic cytokines, such as VEGF and basic fibroblast growth factor. Aortic rings were placed in wells containing Matrigel and fed with EGM in the presence or absence of NM-3 for 6 days. The effect of NM-3 on angiogenesis was measured microscopically by scoring the degree of endothelial cell sprouting from the aorta. Extensive endothelial cell outgrowth from rat aorta ring explants was observed when grown in the absence of NM-3 (Fig. 4, A and B). However, aortic rings grown in the presence of NM-3 (200 μg/ml) resulted in a significant (~3-fold) reduction of endothelial outgrowth and sprouting (Fig. 4, A and B). Taken together with the studies using the dorsal air sac and Matrigel tube formation assays, these findings demonstrate that NM-3 has significant antiangiogenic activity.

Statistical Methods. Significance was determined using the Student t test based on comparisons with control samples tested at the same time. Ps < 0.05 were considered significant.

RESULTS

NM-3 Selectively Inhibits Endothelial Cell Proliferation. HUVECs were treated with different concentrations of NM-3 for varying intervals of time. After treatment, viability of cells was determined by trypan blue exclusion. The results demonstrate that treatment of HUVECs with NM-3 is associated with a significant reduction in cell viability (Fig. 1A). To assess whether the antiproliferative effect of NM-3 is selective to endothelial cells, several different tumor cell lines were treated with various doses of NM-3, and proliferation was measured using three different assays ([3 H] thymidine uptake, BrdUrd labeling, and WST-1). In contrast to MKN28, HT29, MDA-MB435, and MCF-7 cells, treatment of HUVECs with NM-3 (10–200 μg/ml) was associated with a significant decrease in cell growth (Fig. 1, B–D). No effect on cell proliferation was observed when these tumor cells were treated with high doses (100–200 μg/ml) of NM-3 (Fig. 1, C and D). These findings reveal that NM-3 selectively inhibits the growth of endothelial cells.

Fig. 2. Effect of NM-3 on migration of VEGF-stimulated HUVECs. Increasing concentrations of NM-3 were added in the top chamber to assess inhibitory capacity. Maximal inhibition was observed at a concentration of 200 μg/ml. Each experiment had 12 replicates, and all assays were performed at least three times. Error bars, SD between experiments.

Fig. 3. Inhibition of angiogenesis by NM-3. HUVECs were seeded onto Matrigel-coated, 96-well plates. The indicated concentrations of NM-3 were added to the cells, and after 18 h, images were captured, and tube formation (A) was scored in one X4 microscopic field as follows: one tube was designated as a three branch point event. In B, data are shown as the mean ± SE of two independent experiments with all of the samples in triplicate. *, **, and ***, significantly different from the control at P < 0.05 and P < 0.005, respectively.
NM-3 Enhances the Effects of Chemotherapeutic Agents in Inhibiting Cell Proliferation in Vitro. Experiments were conducted to determine the effects of NM-3 in combination with chemotherapeutic agents on the growth of HUVECs and tumor cells. Combination studies were carried out using the BrdUrd proliferation ELISA assay. These studies compared the inhibition of NM-3 at the IC50 dose to a combination of NM-3 plus 5-FU or paclitaxel. This was accomplished by mixing the drugs in different ratios with respect to their individual IC50 dose. To determine the IC50s, HUVECs and MDA-MB435 tumor cells were treated with different concentrations of NM-3, paclitaxel, or 5-FU for 48 h. The IC50s (mean ± SD of four independent experiments) for HUVECs were calculated as: NM-3, 192.5 ± 78.9 μg/ml; 5-FU, 0.317 ± 0.06 ng/ml; and paclitaxel, 1.03 ± 0.67 ng/ml. The IC50s for MDA-MB435 tumor cells were calculated as: NM-3 > 200 μg/ml; 5-FU, 3.04 ± 0.06 ng/ml; and paclitaxel, 2.75 ± 0.21 ng/ml. The combination treatments were then compared with the IC50 doses of the individual drugs. Isobologram analysis was used to determine whether the interaction between the two drugs exhibited synergistic effects in vitro. In this study, the IC50 of each drug was determined, and fractions of the IC50 of each drug were applied to cells in vitro, either alone or in combination, using a checkerboard titration as shown in Fig. 5A. Simultaneous exposure of NM-3 and 5-FU results in a greater inhibitory effect than drugs exposed separately. When plotted, the drugs in combination produce a concave-up isobole, indicating a synergistic effect (Fig. 5A). Similar results were obtained when MDA-MB435 cells were treated with NM-3 and paclitaxel (Fig. 5B).

NM-3 and Taxol Significantly Enhance Tumor Growth Delay Against 50-mm3 Preexisting MDA-MB435 Tumor Xenografts. Initial studies were conducted with paclitaxel to determine the optimal dose of this agent when administered in combination with NM-3. Treatment was initiated when tumor volumes reached 50 mm3. As a single agent, paclitaxel showed minimal effects at 10 mg/kg, given every 7 days via i.p. injection, whereas 20 mg/kg had a significant inhibitory effect on tumor growth (data not shown). Therefore, paclitaxel was administered at 10 mg/kg i.p. q7d for all combination regimens with NM-3. Fig. 6A shows MTVs plotted against time, and the results of this study are summarized in Table 1. NM-3 administered as a single agent at 100 mg/kg i.p. daily resulted in a TGI of 15.29%, with a final mean tumor weight of 180 mg, compared with 212.5 mg in the vehicle control group. This effect by NM-3 was not significantly different from that found for the vehicle control group. When mice were treated with paclitaxel at 10 mg/kg i.p. weekly, no significant TGI occurred. The combination of paclitaxel at 10 mg/kg q7d and NM-3 at 100 mg/kg i.p. daily resulted in a TGI of 72.35% (final tumor weight of 58.75 mg). There were five complete responses. No substantial weight loss was observed in any of the groups throughout the protocol. Mean vessel density of tumors from mice treated with combination therapy showed a significant reduction (Fig. 6B, bottom panel), whereas mice treated with NM-3 or paclitaxel...
fewer vessels. Combination therapy (bottom panel) have significantly fewer vessels.

alone did not show any reduction in tumor vessel density (Fig. 6, top panel and data not shown).

**NM-3 Enhances 5-FU-induced Tumor Growth Delay in MDA-MB435 Breast Carcinoma Xenografts.** Initial studies were conducted with 5-FU to determine a nontoxic dose of this agent for combination studies with NM-3. 5-FU administered alone at 10 mg/kg, q14d, did not show a significant TGI (P = 0.12) and was therefore used in the combination protocol. Table 2 summarizes the in vivo data on TGI. Animals treated with NM-3 administered as a single agent at 100 mg/kg i.p. daily had a final mean tumor weight of 259.2 ± 46.99 mg, not significantly different from the control group (mean tumor weight of 341.5 ± 28.70 mg; P = 0.17). Although the addition of 5-FU at 10 mg/kg i.p. q14d resulted in a TGI of 24.66%, it was not significantly different from the control (P = 0.08). 5-FU in combination with NM-3 at the dose of 100 mg/kg i.p. daily resulted in a TGI of 50.8%, with a P < 0.01. The final mean tumor weight of treated animals was 168.0 ± 45.6 mg, compared with the controls, which had a final MTV of 341.5 ± 28.70 mg. No substantial weight loss was observed in any of the groups throughout the experimental period. Tumor sections from mice in the various groups were analyzed for vessel density and proliferation index (Table 2). The mean vessel density as determined by CD31-positive cell counts of tumors treated with NM-3 alone or 5-FU alone was not significantly different from the control group; however, a significant reduction in CD31-positive cells was observed in the tumors treated with NM-3 in combination with 5-FU (Table 2, P < 0.05). Tumor cell proliferation was analyzed using PCNA-positive cell counts. Tumors treated with 5-FU alone showed a reduction in the proliferation index (120 compared with 189.75 in the control group), and tumors treated with 5-FU combined with NM-3 showed the largest reduction in the proliferation index (51.75 compared with 189.75).

**NM-3 Enhances CPA-induced Tumor Growth Delay in PC-3 Prostate Carcinoma Xenografts.** Mice bearing 100-mm³ PC-3 tumors were injected with CPA daily (25 mg/kg, i.p.) for 2 consecutive days. On day 3, mice were injected i.p. once daily with various doses of NM-3 for the duration of the study. As shown in Fig. 7A, mice treated with 20 mg/kg/day of NM-3 in combination with CPA achieved a treated versus control of 154%, whereas CPA alone showed only a treated versus control of 123%. Doses of NM-3 of 2, 20, and 100 mg/kg/day i.p. in combination with CPA delayed tumor growth of PC-3-bearing mice, compared with CPA alone as shown in Fig. 7B. In the 20 mg/kg NM-3 dose level, two of six mice had no detectable tumors at 60 days.

**Table 1 Summary of in vivo effects of paclitaxel and NM-3 treatment on tumor growth delay, tumor cell proliferation, and tumor angiogenesis**

<table>
<thead>
<tr>
<th>Paclitaxel</th>
<th>NM-3 (mg/kg; i.p. daily)</th>
<th>Final tumor wt. (mg) ± SE</th>
<th>% Tumor growth inhibition</th>
<th>Mice with complete regression</th>
<th>CD31 counts (mean ± SD)</th>
<th>PCNA counts (mean ± SD)</th>
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<tr>
<td>0</td>
<td>0</td>
<td>212.5 ± 30.92</td>
<td>0</td>
<td>12.58 ± 1.44</td>
<td>180.81 ± 15.85</td>
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</tr>
<tr>
<td>10mpk q7d</td>
<td>10</td>
<td>228.8 ± 26.35</td>
<td>5</td>
<td>7.54 ± 4.85</td>
<td>107.81 ± 17.27</td>
<td></td>
</tr>
<tr>
<td>10mpk q7d</td>
<td>100</td>
<td>58.75 ± 29.91</td>
<td>72.35*</td>
<td>12.79 ± 7.70</td>
<td>103.19 ± 21.63</td>
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<tr>
<td>0</td>
<td>100</td>
<td>180.0 ± 18.13</td>
<td>15.29</td>
<td>0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>10mpk q7d</td>
<td>0</td>
<td>215.6 ± 19.73</td>
<td>0</td>
<td>129.56 ± 13.06</td>
<td>129.56 ± 13.06</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.01.  
* P < 0.05.

**Table 2 Summary of in vivo effects of 5-FU and NM-3 treatment on tumor growth delay, tumor cell proliferation, and tumor angiogenesis**

<table>
<thead>
<tr>
<th>5-FU</th>
<th>NM-3 (mg/kg)</th>
<th>Final tumor wt. (mg) ± SE</th>
<th>% Tumor growth inhibition</th>
<th>CD31 counts (mean ± SD)</th>
<th>PCNA counts (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>341.5 ± 28.70</td>
<td>0</td>
<td>27.30 ± 4.82</td>
<td>189.75 ± 8.81</td>
</tr>
<tr>
<td>10mpk q14d, 0.14</td>
<td>10</td>
<td>257.3 ± 31.06</td>
<td>24.66</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10mpk q14d, 0.14</td>
<td>100</td>
<td>168.0 ± 45.6</td>
<td>50.80*</td>
<td>16.39 ± 3.44</td>
<td>51.75 ± 17.97</td>
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<tr>
<td>0</td>
<td>100</td>
<td>259.2 ± 46.99</td>
<td>24.1</td>
<td>26.45 ± 3.27</td>
<td>167.75 ± 40.06</td>
</tr>
<tr>
<td>10mpk q14d, 0.14</td>
<td>0</td>
<td>259.6 ± 36.22</td>
<td>23.98</td>
<td>30.45 ± 1.95</td>
<td>120.00 ± 15.43</td>
</tr>
</tbody>
</table>

* P < 0.01.  
* P < 0.05.

Fig. 6. A, effect of combination of paclitaxel and NM-3 treatment on 50-mm³ preexisting MDA MB435 human tumor xenografts. MTV ± SE is plotted against time. NM-3 was administered daily at 100 mg/kg, whereas paclitaxel was administered q7d at 10 mg/kg. In B, CD31 staining of tumor sections from control mice (top panel) shows abundant vessels, whereas tumor sections from mice treated with NM-3 + paclitaxel combination therapy (bottom panel) have significantly fewer vessels.
DISCUSSION

Our findings demonstrate that a new isocoumarin, NM-3, increases the antitumor effects of various existing chemotherapeutic drugs in breast and prostate tumor models, as measured by TGI. The increase in the antitumor effects of chemotherapy in combination with NM-3 was accomplished without any apparent increase in toxicity. Although the mechanisms by which NM-3 exerts antitumor effects are not completely understood, this favorable therapeutic index is attributable to the selective effects of NM-3 on the tumor vasculature. Our data clearly demonstrate that NM-3 specifically inhibits several stages of angiogenesis, in vitro and in vivo, including endothelial cell proliferation, migration, tube formation, sprouting, and neovascularization in the Matrigel plug model. Although direct effects on tumor cells cannot be ruled out, this indicates that NM-3 acts indirectly on delaying tumor growth in vivo by inhibiting tumor angiogenesis.

Antiangiogenic therapy, although effective in inhibiting tumor growth, has not been shown to be tumoricidal in most studies. This therapeutic limitation can be overcome by combining angiogenesis inhibitors with cytotoxic therapies. Prior studies have demonstrated that the combination of an angiogenic agent with chemotherapy can enhance TGI in a greater than additive manner. The combination of TNP-470, an angiogenic agent, with a conventional schedule of CPA resulted in a 40–50% cure rate in drug-sensitive Lewis lung carcinoma xenografts (31). Browder et al. (32) subsequently demonstrated that CPA given in an angiogenic schedule, characterized by lower doses given on a more frequent schedule, eradicated similar tumor burdens of drug-sensitive Lewis lung carcinoma. Furthermore, they revealed that TNP-470 at one-seventh the dose used by Teicher et al. combined with an angiogenic regimen of CPA eradicated drug-resistant Lewis lung carcinoma. Similarly, vinblinostat, when given on an angiogenic schedule, combined with a VEGF receptor-2 antibody (33) caused sustained tumor regression.

The results of the present study support a new paradigm in which an agent at nontoxic doses potentiates the effects of chemotherapeutic drugs. Our findings demonstrate that NM-3, a small molecule inhibitor of angiogenesis, significantly enhances TGI at nontoxic doses when combined across a broad spectrum of chemotherapeutic agents given at subtherapeutic doses in a variety of schedules in human tumor xenograft models. These effects were particularly marked when NM-3 was combined with CPA and resulted in two of six mice having no detectable tumors at 60 days. Additionally, a highly significant inhibition of tumor growth was noted when doses of paclitaxel and NM-3, which individually did not cause significant TGI, were combined in mice with low-volume (50 mm³) MDA-MB435 breast carcinoma xenografts. Such a schedule of paclitaxel administration is commonly used in treating patients with advanced solid tumors, and our results provide a basis for enhancing the efficacy of this regimen.

Results of in vitro assays of angiogenesis suggest that the effects of combining NM-3 with chemotherapeutic agents in causing TGI are mediated through decreased proliferation of endothelial cells. Our findings reveal that NM-3 is a well-tolerated, antiangiogenic agent that significantly increases the efficacy of a broad spectrum of chemotherapeutic agents. NM-3 is currently being evaluated in Phase I clinical trials in patients with advanced solid tumors. The results presented in the current study provide a compelling rationale for evaluating NM-3 in combination with chemotherapeutic agents in future trials.

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