Antiangiogenic and Antitumor Activity of 6-(2-Aminoethyl)Amino-5-Chlorouracil, a Novel Small-Molecule Inhibitor of Thymidine Phosphorylase, in Combination with the Vascular Endothelial Growth Factor-Trap

Haiyan Lu, Robert S. Klein, and Edward L. Schwartz

Abstract

Purpose: Tumors produce multiple proangiogenic factors, making it unlikely that agents targeting a single angiogenic pathway will be sufficient to treat the spectrum of tumors that occur clinically. Platelet-derived endothelial cell growth factor has angiogenic activity in vitro and in vivo and is overexpressed in most human cancers, where its expression has been correlated with increased microvessel density, more aggressive tumors, and poorer patient prognosis. Platelet-derived endothelial cell growth factor is identical to the enzyme thymidine phosphorylase (TP), and unlike other angiogenic factors, the proangiogenic actions of TP are dependent on its enzyme activity.

Experimental Design: A potent and specific small-molecule inhibitor of the catalytic activity of TP, 6-(2-aminoethyl)amino-5-chlorouracil (AEAC), was tested for antiangiogenic and antitumor activity in human cancer xenografts in vivo.

Results: Oral administration of AEAC caused 40% to 50% reductions in the growth of A549 non–small cell lung cancer and PANC-1 pancreatic cancer xenografts, but it was not active against a second pancreatic tumor, BxPC-3. AEAC reduced the microvessel density in the tumors, providing evidence for an antiangiogenic action. Equal or better activity was seen when the mice were treated with the vascular endothelial growth factor (VEGF)-Trap, a soluble VEGF decoy receptor, and the combination of AEAC and VEGF-Trap produced additive antitumor activity that was significantly greater than the VEGF-Trap alone. In the A549 tumors, the combination produced tumor regressions.

Conclusion: These studies show antitumor activity for a drug targeting TP and suggest that inhibitors of TP could be used to augment the clinical efficacy of drugs targeting the VEGF pathway. (Clin Cancer Res 2009;15(16):5136–44)

The number of drugs that target tumor angiogenesis is growing, and emerging clinical data indicate that these agents increase response rates and prolong survival in patients with advanced malignancies. Drugs targeting vascular endothelial growth factor (VEGF)–mediated pathways are the furthest advanced and have provided proof of concept for this class of antitumor agents (1). Thus, bevacizumab, a humanized variant of a mouse anti–VEGF-A antibody, increased response rates (in combination with cytotoxic drugs) and survival (or progression-free survival) in patients with metastatic colorectal cancer, non–small cell lung cancer (NSCLC), and metastatic breast cancer, and phase III studies of sunitinib and sorafenib, both small-molecule inhibitors of the kinases of the VEGF and platelet-derived growth factor-β (and other) receptors, showed that they had activity in renal cell carcinoma, one of the tumors most resistant to chemotherapy (1). These and other studies suggest that angiogenesis inhibitors are likely to have a broad spectrum of activity against solid tumors. Although these observations provide much promise, the percentage of patients showing significant responses in these trials is relatively small, and it is clear that the use of angiogenesis inhibitors that target a single angiogenic molecule or pathway will not be sufficient to treat the spectrum of heterogeneous tumors occurring clinically. The importance of targeting multiple angiogenesis pathways was recognized early in the consideration of antiangiogenesis therapy and supported by early animal tumor experiments (2, 3).
Our studies have focused on the angiogenic factor thymidine phosphorylase (TP) based on extensive evidence showing its role in experimental and human cancer (4, 5). TP was first identified as an enzyme involved in the cellular metabolism of thymidine, and recent studies have shown that it plays a critical role in maintaining steady-state plasma thymidine levels in humans (6, 7). A surprising observation was reported in 1992 when the sequencing of the cDNA of human TP revealed it to be identical to the factor platelet-derived endothelial cell growth factor (8). Platelet-derived endothelial cell growth factor is overexpressed in a wide range of human solid tumors, often at levels 10-fold higher than in adjacent uninvolved tissue, and high levels of TP have been correlated with increased tumor microvessel density, increased tumor invasion and metastasis, and shorter patient survival time. TP stimulates endothelial cell migration and is a key regulator of endothelial progenitor cells in culture. In this report, we show antiangiogenic and antitumor activities for a small-molecule inhibitor of TP in vivo and suggest that it or related compounds could be used clinically to complement the therapeutic activity of vascular endothelial growth factor inhibitors.

Translational Relevance

Although angiogenesis inhibitors that target the vascular endothelial growth factor pathway have a broad spectrum of activity against human solid tumors, the percentage of patients showing significant responses to these drugs in clinical trials has been relatively small. It is likely that angiogenesis inhibitors that target a single angiogenic molecule or pathway will not be sufficient to treat the spectrum of heterogeneous tumors occurring clinically. The angiogenic factor thymidine phosphorylase (TP) is overexpressed in a wide range of human solid tumors, often at levels 10-fold higher than in adjacent uninvolved tissue, and high levels of TP have been correlated with increased tumor microvessel density, increased tumor invasion and metastasis, and shorter patient survival time. TP stimulates endothelial cell migration and is a key regulator of endothelial progenitor cells in culture. In this report, we show antiangiogenic and antitumor activities for a small-molecule inhibitor of TP in vivo and suggest that it or related compounds could be used clinically to complement the therapeutic activity of vascular endothelial growth factor inhibitors.

Materials and Methods

Cell lines, drugs, and animals. Human umbilical vein endothelial cells (HIVEC, GlycoTech, by arrangement with the Developmental Therapeutics Program Angiogenesis Resource Center, National Cancer Institute) were grown in MCDB131, 2% fetal bovine serum (FBS), 10 ng/mL epidermal growth factor, 12 µg/mL endothelial cell growth supplement, 1 µg/mL hydrocortisone, 10 units/mL heparin, 2 mmol/L L-glutamine, penicillin G, and streptomycin sulfate. A549 human NSCLC cells (American Type Culture Collection) were maintained in RPMI 1640 with 10% FBS; PANC-1 and BxPC-3 human pancreatic carcinoma cells (American Type Culture Collection) were maintained in DMEM and RPMI 1640, respectively, with 10% FBS. Cells were maintained at 37°C with 5% CO2. AEAC was provided by the Developmental Therapeutics Program, National Cancer Institute. The VEGF-Trap was from Regeneron Pharmaceuticals, Inc., and was provided by the Cancer Treatment Evaluation Program, National Cancer Institute. Pathogen-free female NCR nude mice (6-8 wk old, 19-24 g) were purchased from Taconic Laboratories. All mice were maintained in a pathogen-free animal facility for at least 1 wk before each experiment. The animal use committees of the Albert Einstein College of Medicine and Montefiore Medical Center approved all animal study protocols described in this publication, and experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals.

In vitro proliferation assays. A549, PANC-1, and BxPC-3 cells were plated at a density of 4,000 per well in 96-well plates in 100 µL of culture medium supplemented with 10% FBS. After 24 h of incubation, 100 µL of complete medium containing AEAC were added to each well. At 72-h time points, the number of viable cells was detected using the MTT method.

Xenograft tumor models and treatment. Nude mice were inoculated s.c. in their right flank with 2 × 106 (A549, PANC-1, and BxPC-3) or 12 × 106 (A549) cells in 100 µL PBS. When tumors became palpable (1-3 wk), mice were randomly assigned into treatment and control groups. AEAC was dissolved in H2O containing 0.5% (w/v) methylcellulose and administered via oral gavage 5 d per week; mice in the control group received an equal volume of vehicle (0.5% methylcellulose). VEGF-Trap was administered via s.c. injection twice per week. Mice were

![Fig. 1. Structure of AEAC.](image-url)
were divided into groups of five mice, and therapy was begun. Mice were treated with vehicle (○, significantly different (P < 0.01, ANOVA; all time points between days 7 and 28 were significantly different). Drug therapy was begun on day 1 and continued for the time indicated by the horizontal bars. Tumor size was measured twice weekly. Tumor volumes were calculated as (l × w²) / 2 and are expressed relative to the tumor size at the start of therapy. The data in A and B are from the same experiments; note that the y axis has been changed in B to better illustrate the difference between the VEGF-Trap alone–treated (✈) and AEAC + VEGF-Trap–treated (◊) mice. A and B, points, mean of two experiments (n = 10); bars, SE. C, points, mean of a single experiment (n = 5); bars, SE. *p < 0.05 was considered significant.

Results

Both VEGF and TP inhibition are required to block carcinoma cell–mediated HUVEC migration in vitro. The migration of endothelial cells in vitro can be stimulated by purified angiogenic factors and by carcinoma cells that are producing these factors. HUVEC migration in a Boyden chamber was stimulated by VEGF, purified human TP, or by HT29 carcinoma cells that expressed both TP and VEGF (Supplementary Fig. S1). Under these conditions, neither a VEGF inhibitor nor a TP inhibitor, when used alone, was able to fully block HT29-mediated HUVEC migration, but the combination of the two inhibitors reduced HUVEC migration to that of control wells (no angiogenic factors or tumor cells; Supplementary Fig. S1).

AEAC and VEGF-Trap inhibited A549 NSCLC and PANC-1 pancreatic cancer xenograft growth in nude mice. The antitumor activity of AEAC was assessed in nude mice bearing xenografts of a human NSCLC cell line, A549, or two human pancreatic cancer cell lines, PANC-1 and BxPC-3. AEAC was administered by oral gavage at 50 mg/kg once daily for 5 days each week, a dose and schedule that was chosen arbitrarily, because AEAC has not been previously tested in animals. In preliminary experiments, AEAC at doses up to 100 mg/kg had no effect on the body weight of mice with chronic administration, nor did it have any apparent acute toxic effects (data not shown). Because these cancer cells also express VEGF (20), we tested AEAC in combination with VEGF-Trap. The VEGF-Trap binds to all forms of VEGF, purified human TP, or by HT29 carcinoma cells that expressed both TP and VEGF (Supplementary Fig. S1). Under these conditions, neither a VEGF inhibitor nor a TP inhibitor, when used alone, was able to fully block HT29-mediated HUVEC migration, but the combination of the two inhibitors reduced HUVEC migration to that of control wells (no angiogenic factors or tumor cells; Supplementary Fig. S1).

Immunohistochemical staining for blood vessels, macrophages, and TP. Tumor tissue was fixed in 4% buffered formalin, embedded in paraffin, sectioned, and stained with H&E using standard histologic techniques. In addition, slides were immunostained for TP (Oncogene), CD34 (Cedarlane Labs), and macrophages (rat monoclonal antibody against isolated mouse macrophages; RM0029-11H3; Abcam). Antigen retrieval was done by heating the slides at 95°C to 97°C in 0.01 mol/L sodium citrate (pH 6.0) for 10 min. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide in methanol/water (1:1) for 10 min at room temperature; sections were then incubated with 10% normal serum in PBS for 40 min at room temperature followed by incubation with the primary antibody overnight at 4°C. Following incubation with the secondary antibody, the Vectastain avidin-biotin complex method kit (Vector Laboratories) was used for detection with diaminobenzidine tetrahydrochloride (Vector) as the substrate. Slides were counterstained with hematoxylin. Negative control sections were incubated with preimmune serum.

To determine the density of the vessels and macrophage in tumors, three “hot” spots of each section were photographed and the diaminobenzidine tetrahydrochloride pixel area in the lesion was quantified using the ImageJ program. Five mice per group were analyzed.

Statistical analysis. Statistical analysis was done with one-way ANOVA, and pairwise comparison procedures including calculation of P values were done using the Tukey’s multiple comparison test. P < 0.05 was considered significant.
In the first set of experiments, nude mice were inoculated s.c. with $2 \times 10^6$ A549 cells, and tumors were allowed to grow until they reached an average size of 75 mm$^3$, at which point they were divided into groups of five mice and therapy was begun. Mice were treated with vehicle only (control), AEAC, VEGF-Trap, or a combination of AEAC plus VEGF-Trap. AEAC alone significantly inhibited tumor growth by 39 ± 6.5% ($P < 0.05$) compared with control, and a larger antitumor effect was observed with the VEGF-Trap alone (91 ± 0.9% inhibition) in these xenografts (Fig. 2A). A higher dose of AEAC (75 mg/kg) did not noticeably increase its antitumor efficacy. Although VEGF-Trap has substantial activity when used alone, a further significant reduction in tumor size was seen in the AEAC + VEGF-Trap group when compared with either the AEAC-alone or the VEGF-Trap-alone groups (Fig. 2B). The combination notably also produced tumor regressions in 10 of the 10 mice versus in 3 of 10 mice treated with the VEGF-Trap alone. The tumors in the combination group rapidly regressed with treatment to an average of 52 ± 6.3% of their original size, and tumor size remained stable for the first 3 weeks of drug therapy. At that point, tumor growth resumed and seemed to parallel an observed increase in growth rate in the VEGF-Trap-alone group. However, it cannot be ascertained from these experiments whether the renewed tumor growth resulted from a loss of sensitivity to AEAC, VEGF-Trap, or both.

The drugs were also tested in mice bearing substantially larger A549 tumors, in which therapy was initiated 7 days before the untreated mice had to be euthanized due to the size of their tumors and/or evidence of morbidity (Fig. 2C). Under these conditions, VEGF-Trap was somewhat less effective, producing a 25% reduction in mean tumor growth relative to control, compared with a 50% reduction in the AEAC-treated mice (Fig. 2C; Table 1A). Evidence for an approximately additive combination for selected xenograft tumor growth, and importantly, it also had an additive inhibitory effect on these models when coadministered with VEGF-Trap.

**AEAC had no effect on tumor cell growth in vitro.** To evaluate the possibility that the antitumor activity of AEAC could be due to a direct effect on the tumor cells, the growth of A549, PANC-1, and BxPC-3 cells was assessed in vitro after drug treatment for 72 hours. AEAC had no effect on cell proliferation; at the highest concentration tested, 100 μmol/L, inhibition was <10% (data not shown).

**Changes in TP expression in human tumor xenografts.** TP expression was examined in the excised tumors by immunohistochemistry and by Western blotting (Fig. 4). TP was most prominently expressed in the tumor cells, with expression also significant in the tumor stroma, with expression also significantly different from control ($P < 0.05$).

### Table 1. Antitumor activity of the TP inhibitor AEAC

<table>
<thead>
<tr>
<th>Tumor xenograft</th>
<th>Mean tumor growth inhibition (% reduction compared with control)</th>
<th>VEGF-Trap</th>
<th>AEAC + VEGF-Trap</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANC-1 (pancreatic cancer)</td>
<td>38 ± 4.7%*</td>
<td>31 ± 8.1%*</td>
<td>80 ± 4.5%*</td>
</tr>
<tr>
<td>BxPC-3 (pancreatic cancer)</td>
<td>-4.4 ± 8.0%</td>
<td>57 ± 1.8%*</td>
<td>79 ± 2.5%*</td>
</tr>
<tr>
<td>A549 (NSCLC)</td>
<td>50 ± 5.4%*</td>
<td>25 ± 18%*</td>
<td>86 ± 8.2%*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tumor xenograft</th>
<th>Control</th>
<th>AEAC</th>
<th>VEGF-Trap</th>
<th>AEAC + VEGF-Trap</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANC-1</td>
<td>186 ± 20</td>
<td>85 ± 29*</td>
<td>102 ± 16*</td>
<td>68 ± 13*</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>294 ± 40</td>
<td>304 ± 139</td>
<td>79 ± 15</td>
<td>57 ± 20*</td>
</tr>
<tr>
<td>A549</td>
<td>527 ± 77</td>
<td>329 ± 24*</td>
<td>114 ± 14*</td>
<td>45 ± 24*</td>
</tr>
</tbody>
</table>

**NOTE:** A. Subcutaneous tumors were measured twice weekly, and tumor volumes were calculated as ($l \times w^2$) / 2. Mean tumor growth inhibition was calculated from tumor volume measurements determined at time points during drug treatment using the formula (50) \% inhibition = [1 - (Vt / Vct) / 1 - Vct] × 100, where Vt equals the tumor volume in drug-treated mice on day x expressed as ratios of the tumor volumes of the same mice on day 0, and Vct equals the tumor volumes in control mice on day x expressed as ratios of the tumor volumes of the same mice on day 0. B. Tumors were excised at the completion of the experiments, dissected from surrounding connective tissue, and weighed. Values are mean ± SE ($n = 5$).

*Significantly different from control ($P < 0.05$).

*Significantly different from control and from single-agent AEAC or VEGF-Trap ($P < 0.05$, ANOVA with Tukey’s multiple comparisons test).
occasionally noted in stromal cells (Fig. 4A). Shown in Fig. 4B are whole-cell protein extracts from the cancer cells grown in tissue culture (lane 1) or from individual tumors (lane 1, 2, or 3) from mice treated as in Fig. 3 with vehicle, AEAC, VEGF-Trap, or the combination of AEAC + VEGF-Trap. TP expression was equal to or higher in all the in vivo tumors compared with the same tumor cells in tissue culture. This may have been due to the up-regulation of TP expression that we and others have shown to occur in response to a variety of cytokines, including tumor necrosis factor-α, IFN, and interleukin-8 (reviewed in 5). However, there was a large degree of heterogeneity observed in TP expression among the individual tumors, preventing any conclusions from being drawn about the relationship between the level of TP expression and response to therapy.

Using an in vitro assay, AEAC was found to inhibit TP activity in extracts from the A549 tumors (Supplementary Fig. S2).

**AEAC and VEGF-Trap reduced tumor microvessel density.** The effect of AEAC and VEGF-Trap on microvessel density (MVD) was examined in the tumors, as a measure of their angiogenic actions (Fig. 5A). Blood vessels in 10-μm-thick histologic sections were immunohistochemically stained with CD34, an endothelial cell marker commonly used for microvessel quantification, and blood vessel density was quantitated by analysis with ImageJ (Supplementary Fig. S3). Both AEAC and VEGF-Trap had antiangiogenic activity, which correlated with their antitumor actions (Fig. 4; Supplementary Fig. S3). Thus, AEAC at 50 mg/kg significantly reduced MVD in the A549 and PANC-1 tumors but not in the BxPC-3 tumors, whereas VEGF-Trap was active in all three xenografts. More importantly, the combination therapy of AEAC and VEGF-Trap significantly reduced the tumor MVD compared with control in all three models, and there was also a significant difference for the combination when compared with the VEGF-Trap alone for the PANC-1 tumors (Fig. 5A).

**VEGF-Trap reduced macrophage infiltration into xenograft tumors.** TP expression seemed to be lower in tumors from VEGF-Trap–treated mice (i.e., both the VEGF-Trap–alone and the AEAC + VEGF-Trap groups) when compared with the control and AEAC-alone groups (Fig. 4B). Because TP is highly expressed in macrophages as well as in tumor epithelial cells, a reduction in tumor-associated macrophages could have contributed to a reduction in tumor TP. As shown in Supplementary Figs. S4 and S5B, for the A549 and PANC-1 tumors, both the VEGF-Trap alone and combination of AEAC and VEGF-Trap significantly inhibited macrophage infiltration into the tumor tissues. Interestingly, VEGF-Trap did not cause a reduction in macrophages in the BxPC-3 tumors. Although tumors from AEAC-alone–treated mice had a reduction in macrophage infiltration, it was not statistically significant, and in fact, a significant increase in macrophage infiltration was seen in the BxPC-3 tumors.

**Discussion**

The angiogenic factor TP is overexpressed in a wide range of human solid tumors, often at levels 10-fold higher than in adjacent uninvolved tissue (5, 23). The degree to which TP expression is elevated varies widely, however, even among those tumors of the same histologic type; thus, overexpression of TP is not an inevitable characteristic of solid tumor formation. Rather, most studies have found a correlation between a high level of TP expression and increased tumor MVD, increased tumor invasion and metastasis, and shorter patient survival time (5). TP, VEGF, and basic fibroblast growth factor expression were examined in the same specimens in some of these studies. In a study of 96 patients with colorectal cancer, TP was expressed in 83% of the patients’ tumors, and the level of expression was significantly correlated with vessel counts but not with the level of VEGF expression (24). In a study of 104 human NSCLC patients, high TP expression was seen in 32% of the patient tumors and was associated with increased vascular density (25). VEGF was highly expressed in 47% of the NSCLC patient tumors and was likewise correlated with vessel counts. Linear regression analysis revealed only a weak positive correlation between TP and VEGF expression in the lung cancers. TP was overexpressed in 67% of 384 pancreatic cancer specimens when compared either with adjacent uninvolved pancreatic tissue or with tissue from benign pancreatic diseases. Overexpression of VEGF and basic fibroblast growth factor was observed in 54% and 58%, respectively, of the pancreatic cancer specimens, and there was a significant correlation reported for the coexpression of TP and VEGF (26–29). In addition to elevated expression in the pancreatic tumor cells, 40% to 50% of patient specimens had elevated TP expression in adjacent stromal cells (macrophages, lymphocytes, and/or fibroblasts; refs. 26, 28, 30). Overall, the conclusion from clinical studies in which both TP and VEGF have been evaluated was that TP is an independent prognostic marker of tumor aggressiveness and may be the predominant angiogenic factor in tumors that express low or no detectable VEGF.

TP-expressing cells mediate endothelial cell migration via the intracellular metabolism of thymidine to thymine and dR-1-P, the intracellular conversion of dR-1-P to 2dR, and the subsequent extracellular release of 2dR, which then forms a concentration and chemotactic gradient (31). We have previously shown
that 2dR has direct effects on cell signaling pathways and cell migration in endothelial cells (32). State-of-the-art proteomics also recently identified TP as a key regulator of the angiogenic potential of endothelial progenitor cells in vitro (33). These critical studies provided the basis for the hypothesis that small-molecule inhibitors directed at the catalytic site of TP would have antangiogenic and antitumor therapeutic activities.

A large number of TP inhibitors have been evaluated in vitro since the first compounds were synthesized over 40 years ago. A majority of these were analogues of uracil, deoxyuridine, thymidine, or related acyclonucleosides (34). Few, however, have been found to inhibit TP activity with IC_{50}s of <1 μmol/L, the exceptions being an aminoimidazolyl-methyluracil analogue, an iminopyrrolidinyl-methyluracil derivative called TPI, and AEAC (19, 35, 36). Using a different strategy, a bicyclic pyrimidine nucleoside multisubstrate inhibitor (i.e., binds to both the nucleoside and phosphate binding sites on the enzyme) has also been reported to inhibit human TP with nanomolar activity (37). Thus, there are only a few TP inhibitors with sufficient potency to be considered for use in vivo, and to date, only TPI has been reported to have antitumor activity in preclinical models (15, 38). In those studies, TPI was shown to decrease the growth rate, increase the apoptotic index, reduce the MVD, and suppress liver metastases of KB (HeLa) cells in mouse xenografts. Interestingly, activity was seen only with tumors from KB cells that had been transfected with a TP cDNA and that overexpressed TP protein by 100-fold. Thus, our studies are the first to show activity of a TP inhibitor in tumors from cells that have not been manipulated to overexpress TP and also in tumors that are representative of human cancers that are often refractory to other chemotherapeutic agents.

Our experiments combined AEAC with VEGF-Trap, a novel, soluble VEGF decoy receptor protein, which is composed of fragments of the VEGF receptors VEGFR-1 (flt1) and VEGFR-2 (flk1, KDR; refs. 39, 40). The VEGF-Trap has shown substantial antiangiogenic and antitumor activity in preclinical models (including in pancreatic cancer) and is currently in phase III clinical trials for patients with NSCLC, pancreatic, prostate, and colorectal cancers (39, 40). In our studies, we found that AEAC had antitumor activity in pancreatic cancer and NSCLC xenografts,
and more importantly, it significantly increased the antitumor activity of VEGF-Trap in two different pancreatic cancers and in a lung cancer xenograft. For these studies, AEAC was administered at 50 mg/kg/d. In other experiments, daily administration of AEAC at doses up to 100 mg/kg had no effect on body weight nor did it produce any apparent acute toxic effects on mice (data not shown). Because VEGF inhibitors are now approved for use in some human solid tumors and are being widely studied in others, it is likely that there will be future opportunities to conduct clinical trials that add a relatively nontoxic, orally available inhibitor of a second angiogenic pathway. Although the present studies do not directly show that AEAC is acting via a effect on TP, we have previously shown that AEAC did not inhibit VEGF-induced endothelial cell migration at concentrations that completely inhibited TP-induced endothelial cell migration (19). Because it is possible that AEAC could have additional actions in vivo, mechanisms of AEAC action that are not directly due to TP inhibition cannot be ruled out by the current studies.

In human colon and other gastrointestinal tumors, TP overexpression occurred more often in tumor-associated macrophages and other stromal cells when compared with expression in the colon cancer epithelial cells (24). High levels of expression of TP in tumor-associated macrophages have also been observed in human breast, prostate, lung, and brain tumors (41–45). These findings suggest that TP in tumor-associated macrophages may play a more direct role in tumor angiogenesis, and it has been hypothesized that tumor cells can amplify their own angiogenic activity by recruiting or activating macrophages, which then express high levels of angiogenic factors (46). The possibility that overexpression of TP in certain malignancies is a consequence of a host response suggests that TP-directed therapy may have a broad applicability. Thus, anti-TP therapy could be useful even in instances where the tumor epithelial cells themselves are not overexpressing TP. Interestingly, we found that macrophage infiltration was substantially reduced in tumors from mice treated with VEGF-Trap. This confirms a recent report that showed that tumor-associated macrophages express VEGFR-2 and that selective inhibition of VEGFR-2 inhibits recruitment of macrophages into pancreatic tumors in vivo (47). The reduced number of macrophages in the tumors from the VEGF-Trap–treated mice could also have contributed to the reduced levels of TP expression observed in these tumors.

**Fig. 5.** AEAC and VEGF-Trap reduce MVD and macrophage infiltration in tumors. Histologic sections in tumors from mice treated as described in Figs. 2 and 3 were immunohistochemically stained for endothelial cells (A) or macrophages (B). For each tumor, images in three high-powered microscope fields were analyzed for total vessel area [pixels/high-power field (hpf)] using ImageJ. Columns, mean of five mice per group; bars, SE. *, significantly different from control (P < 0.05, ANOVA with Tukey’s multiple comparison test or Dunnett’s test); **, significantly different from both control and VEGF-Trap alone (P < 0.05).
In addition to contributing to angiogenesis in tumors, TP may play a proangiogenic role in inflammatory and other non-neoplastic pathologies as well as in normal physiology (5). Studies have also shown that carcinoma cells that were transplanted with and overexpressed TP were more invasive and metastatic in vitro and in vivo than were control-transfected cells (16, 48, 49). Although our studies with AEC in s.c. tumor xenografts were not designed to detect drug effects on tumor invasion and metastasis, data showing that this compound also had direct effects on cancer cells in vivo would support the continuing evaluation of this novel drug.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


6. Nishino I, Spinazzola A, Hirano M. Thymidine phosphorylase activity associated with neoplastic pathologies as well as in normal physiology (5).


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