Antiangiogenesis Enhances Intratumoral Drug Retention

Jie Ma, Chong-Sheng Chen, Todd Blute, and David J. Waxman

Abstract

The tumor vasculature delivers nutrients, oxygen, and therapeutic agents to tumor cells. Unfortunately, the delivery of anticancer drugs through tumor blood vessels is often inefficient and can constitute an important barrier to cancer treatment. This barrier can sometimes be circumvented by antiangiogenesis-induced normalization of tumor vasculature. However, such normalizing effects are transient; moreover, they are not always achieved, as shown here, when 9L gliosarcoma xenografts were treated over a range of doses with the VEGF receptor-selective tyrosine kinase inhibitors axitinib and AG-028262. The suppression of tumor blood perfusion by antiangiogenesis agents can be turned to therapeutic advantage, however, through their effects on tumor drug retention. In 9L tumors expressing the cyclophosphamide-activating enzyme P450 2B11, neoadjuvant axitinib treatment combined with intratumoral cyclophosphamide administration significantly increased tumor retention of cyclophosphamide and its active metabolite, 4-hydroxycyclophosphamide. Similar increases were achieved using other angiogenesis inhibitors, indicating that increased drug retention is a general response to antiangiogenesis. This approach can be extended to include systemic delivery of an anticancer prodrug that is activated intratumorally, where antiangiogenesis-enhanced retention of the therapeutic metabolite counterbalances the decrease in drug uptake from systemic circulation, as exemplified for cyclophosphamide. Importantly, the increase in intratumoral drug retention induced by neoadjuvant antiangiogenic drug treatment is shown to increase tumor cell killing and substantially enhance therapeutic activity in vivo. Thus, antiangiogenic agents can be used to increase tumor drug exposure and improve therapeutic activity following intratumoral drug administration, or following systemic drug administration in the case of a therapeutic agent that is activated intratumorally. Cancer Res; 71(7); 2675–85. ©2011 AACR.

Introduction

The tumor vasculature is often characterized by low vascularity, poor organization, and abnormal morphology, which results in inefficient transport of oxygen and therapeutic agents into tumors and constitutes a substantial barrier to effective cancer treatment (1). Extensive efforts have been made to improve tumor drug uptake by developing new delivery vehicles or increasing tumor vascular permeability (2, 3). The growth and expansion of many tumors is associated with pathological angiogenesis stimulated by VEGF, which is a validated therapeutic target for cancer treatment. Bevacizumab, a neutralizing antibody that is activated intratumorally, or following systemic drug administration in the case of a therapeutic agent that is activated intratumorally.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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but are not optimal for vascular normalization. Supporting this possibility, increases in chemotherapeutic drug uptake are seen in some models at low but not standard doses of the antiangiogenic agent sunitinib (21,22), and improved antitumor responses are reported when bevacizumab is given to patients at low dose, but not high dose, in combination with conventional chemotherapy (23). An alternative reason why certain VEGF receptor RTKIs might not induce normalization may due to their co-inhibition of PDGFR–β, which promotes the close association between pericytes and endothelial cells that characterizes normal blood vessels (24), thereby destabilizing tumor blood vessels in a way that interferes with tumor vascular normalization induced by VEGF deprivation. These two possibilities are examined in the present study, where the impact of angiogenesis on tumor drug uptake is investigated over a range of doses for both axitinib and AG-028262, an antiangiogenic RTKI whose specificity for VEGFR-2 as compared with PDGFR-β is approximately 50-fold greater than that of axitinib (25).

As blood flow to the tumor decreases in response to antiangiogenesis, blood flow out of the tumor may also decrease. This could potentially increase the retention of therapeutic agents in tumors. Furthermore, for drugs that successfully extravasate into the tumor extravascular matrix, a decrease in tumor interstitial fluid pressure following angiogenesis inhibition may slow the leakage of drugs from the tumor periphery to the peritumoral space and could also lead to longer tumor drug retention. However, the inhibition of VEGF signaling reduces the permeability of blood vessels and can adversely affect the extravasation of drugs after they are delivered into the tumor through blood circulation (26). Thus, even if angiogenesis increase the retention of therapeutic agents in tumor vasculature, it is uncertain whether this can be translated into increased interstitial drug concentration and higher tumor cell drug exposure. These questions are presently investigated in a 9L gliosarcoma model that expresses cytchrome P450 2B11, which converts the anticancer prodrug CPA to its active, 4-hydroxy metabolite (27). Our findings demonstrate that pre-treatment of P450 2B11, which converts the anticancer prodrug CPA to its active, in a 9L gliosarcoma model that expresses cytochrome P450

Results

Mice bearing bilateral 9L/2B11 tumors were randomized into five groups on the day of initial drug treatment, when the average tumor volume reached 800 to 900 mm³ (5–8 mice per group), and treated as follows: (i) vehicle injection [5 μL per g body weight, i.p., once a day (sid) for 4 days], followed by 120 μL PBS sid for 2 days, administered at 3 intratumoral injection sites per tumor at 20 μL per injection and 2 tumors/mouse beginning 24 hours after the last vehicle injection; (ii) axitinib (25 mg/kg body weight, i.p., sid for 4 days); (iii) CPA [150 mg CPA/kg body weight in a total vol of 120 μL, administered at 3 intratumoral (i.t.) injection sites per tumor at ~20 μL per injection site and 2 tumors/mouse], sid for 2 days; (iv) CPA, followed by axitinib given 24 hours after the second CPA injection; and (v) axitinib, followed by CPA given 24 hour after the fourth axitinib injection. For groups (i) and (ii), the mice were terminated when the tumor volume reached the limit set by institutional guidelines. For groups (iii), (iv), and (v), a second drug treatment cycle was initiated on day 21, as shown in Figure 6, below, after which the mice were maintained drug-free. Tumor volume and mouse body weight were monitored throughout the study.

Materials and Methods

Additional details on chemicals and analytical procedures, including drug treatments, 4-OH-CPA and CPA analysis, pharmacokinetic data analysis, endothelial cell chemosensitivity to 4-OH-CPA, and analysis of CPA-induced apoptosis are provided in Supplementary Materials

Tumor cell lines and xenograft models

9L rat gliosarcoma cells infected with a retroviral vector encoding P450 2B11 in combination with P450 reductase (9L/2B11 cells), and 9L tumor cells infected with the empty retroviral vector pBabe (9L cells) were described previously (28). Cells were grown in DMEM culture medium containing 10% FBS at 37°C in a humidified, 5% CO₂ atmosphere. Immune-deficient male Fox Chase ICR scid mice were purchased from Taconic, Inc. and housed in the Boston University Laboratory of Animal Care Facility in accordance with approved protocols and federal guidelines. Autoclaved cages containing food and water were changed once a week. Mouse body weight was monitored every 3 to 4 days. On the day of tumor cell inoculation, 9L or 9L/2B11 cells at 70% to 80% confluence were trypsinized and resuspended in FBS-free DMEM medium. Cells (4 × 10⁴) in a volume of 0.2 mL were injected subcutaneously (s.c.) into each flank of a 6-week-old male scid mouse. Tumor sizes were measured every 3 to 4 days using digital calipers (VWR International) and volumes were calculated as (3.14/6) × (L × W)³/2.

Tumor growth delay study

Mice bearing bilateral 9L/2B11 tumors were randomized into five groups on the day of initial drug treatment, when the average tumor volume reached 800 to 900 mm³ (5–8 mice per group), and treated as follows: (i) vehicle injection [5 μL per g body weight, i.p., once a day (sid) for 4 days], followed by 120 μL PBS sid for 2 days, administered at 3 intratumoral injection sites per tumor at 20 μL per injection and 2 tumors/mouse beginning 24 hours after the last vehicle injection; (ii) axitinib (25 mg/kg body weight, i.p., sid for 4 days); (iii) CPA [150 mg CPA/kg body weight in a total vol of 120 μL, administered at 3 intratumoral (i.t.) injection sites per tumor at ~20 μL per injection site and 2 tumors/mouse], sid for 2 days; (iv) CPA, followed by axitinib given 24 hours after the second CPA injection; and (v) axitinib, followed by CPA given 24 hour after the fourth axitinib injection. For groups (i) and (ii), the mice were terminated when the tumor volume reached the limit set by institutional guidelines. For groups (iii), (iv), and (v), a second drug treatment cycle was initiated on day 21, as shown in Figure 6, below, after which the mice were maintained drug-free. Tumor volume and mouse body weight were monitored throughout the study.

Statistical analysis

Results are expressed as mean ± SE based on the indicated number of tumors or tissue samples per group. Statistical significance of differences was assessed by 2-tailed Student’s t test using GraphPad Prism software, and is indicated by *, P < 0.05; **, P < 0.01; and ***, P < 0.001. All drug-treated samples were compared with controls, unless indicated otherwise.

Results

VEGFR-selective inhibitors suppress 9L tumor uptake of CPA and 4-OH-CPA without vascular normalization

The anticancer prodrug CPA is activated by hepatic P450 enzymes and then delivered to the tumor via the bloodstream (29). Axitinib treatment for 4 days (25 mg/kg/day, i.p.) significantly reduced the number of blood vessels in 9L tumors grown s.c. in scid mice (Fig. 1A), consistent with our earlier
findings (14). Axitinib also decreased tumor uptake of CPA and of liver-derived 4-OH-CPA when assayed at the $T_{\text{max}}$, 15 minutes after i.p. injection of CPA (140 mg/kg). A, immunohistochemical staining of CD31-positive blood vessels in 9L tumors before and after axitinib treatment. Intratumoral CPA (B) and 4-OH-CPA (C) concentrations were assayed 6 or 15 minutes after CPA treatment. Mean $\pm$ SE, $n$ = 6 tumors per time point. D, 4-OH-CPA in 9L tumors treated with axitinib (2–25 mg/kg, p.o., bid), or AG-028262 (5–40 mg/kg, p.o., bid) for 4 days were assayed in tumors excised 15 minutes after i.p. injection of CPA. Numbers along x-axis indicate doses of axitinib or AG-028262 for each group in mg/kg. Mean $\pm$ SE, $n$ = 4 to 6 tumors per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with controls.

Figure 1. Axitinib and AG-028262 inhibit tumor uptake of CPA and 4-OH-CPA — Scid mice bearing s.c. 9L tumor xenografts with no pretreatment or pretreated with axitinib (25 mg/kg, i.p., sid for 4 days) were given a single i.p. injection of CPA (140 mg/kg). A, immunohistochemical staining of CD31-positive blood vessels in 9L tumors before and after axitinib treatment. Intratumoral CPA (B) and 4-OH-CPA (C) concentrations were assayed 6 or 15 minutes after CPA treatment. Mean $\pm$ SE, $n$ = 6 tumors per time point. D, 4-OH-CPA in 9L tumors treated with axitinib (2–25 mg/kg, p.o., bid), or AG-028262 (5–40 mg/kg, p.o., bid) for 4 days were assayed in tumors excised 15 minutes after i.p. injection of CPA. Numbers along x-axis indicate doses of axitinib or AG-028262 for each group in mg/kg. Mean $\pm$ SE, $n$ = 4 to 6 tumors per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with controls.

Antiangiogenesis increases exposure of 9L/2B11 tumors to CPA and 4-OH-CPA by increasing drug retention

Next, we investigated whether antiangiogenic drugs might be used to increase intratumoral drug retention. CPA was
injected intratumorally into 9L/2B11 tumors, where CYP2B11, a cytochrome P450 enzyme, catalyzes intratumoral metabolism of CPA to its active metabolite, 4-OH-CPA. CPA that exits the tumor intact can be converted by liver P450 enzymes to 4-OH-CPA, a portion of which may then reenter the tumor (27). Five minutes after CPA injection, intratumoral CPA levels were 580 μmol/L in untreated tumors as compared with 1840 μmol/L in axitinib-pretreated tumors (Fig. 2A). However, the initial tumor level of 4-OH-CPA was not significantly higher in the axitinib-pretreated tumors (Fig. 2B), indicating that axitinib does not increase the intrinsic rate of intratumoral CPA 4-hydroxylation, despite the 3.2-fold increase in initial intratumoral CPA concentration. This finding can be explained by CYP2B11 being already saturated at the concentration of 580 μmol/L CPA that is reached when CPA is injected i.t. without prior axitinib treatment (c.f., Km (CPA) = 70 μmol/L in 9L/2B11 cells; ref 28). As the intratumoral CPA levels declined with time due to a combination of drug efflux and intratumoral conversion to 4-OH-CPA, we observed an increase in overall intratumoral 4-OH-CPA exposure with axitinib treatment (Fig. 2B). Total intratumoral exposure to CPA, and 4-OH-CPA was 76% to 77% higher in axitinib-pretreated mice, as indicated by the area under the curve (AUC; Table 1). This finding is consistent with axitinib slowing drug efflux from the tumors. After 30 minutes, intratumoral 4-OH-CPA levels declined both with and without axitinib-pretreatment, reflecting depletion of the intratumoral pool of CPA available for metabolism to 4-OH-CPA. The Cmax of tumor exposure to 4-OH-CPA was also increased with axitinib pretreatment, by 42% (Table 1B). CPA and 4-OH-CPA levels were substantially lower in plasma and liver than in the tumor (Fig. 2C–F), as is expected for an intratumoral route of drug delivery. Moreover, the 3-minute time point, plasma and liver 4-OH-CPA levels were significantly lower in axitinib-pretreated mice than in controls (P < 0.05), indicating that the greater retention of CPA and 4-OH-CPA by the axitinib-pretreated tumor renders CPA less available for hepatic metabolism and also reduces the efflux of tumor-derived 4-OH-CPA during this initial time period. By 30 minutes, however, plasma and liver levels of CPA and 4-OH-CPA were higher in the axitinib-pretreated mice, reflecting the delay in drug release from the tumors.

To confirm the role of intratumoral P450 metabolism in the observed retention of 4-OH-CPA, we used mice bearing wild-type 9L tumors to investigate the impact of axitinib pretreatment on the intratumoral pharmacokinetics of CPA and 4-OH-following i.t. CPA injection. Wild-type 9L tumors do not express significant levels of P450 enzymes and cannot metabolize CPA to 4-OH-CPA. As anticipated, we observed higher levels of intratumoral CPA in the axitinib-pretreated tumors at both 6 and 15 minutes, reflecting increased tumor drug retention (Fig. 2G), however, intratumoral 4-OH-CPA levels were lower through 30 minutes, reflecting the inhibition of tumor uptake of 4-OH-CPA formed in the liver (Fig. 2H).

Two other antiangiogenic drugs, AG-028262 and SU5416, were investigated for their effects on intratumoral drug retention in mice bearing 9L/2B11 tumors (Fig. 3). Four days of pretreatment with AG-028262 or SU5416 had no significant effect on intratumoral CPA or 4-OH-CPA levels 15 minutes after intratumoral CPA injection, although a trend of increasing drug retention was apparent for CPA (Fig. 3A). However, 30 minutes after CPA injection, when intratumoral CPA concentrations decreased well below the Km for metabolism by CYP2B11 (c.f.; Fig. 2A), both antiangiogenic drugs significantly increased intratumoral 4-OH-CPA compared with controls (P < 0.05; Fig. 3B). Thus, prolonged tumor drug retention may represent a common response to antiangiogenesis treatments involving these RTKIs.

Although axitinib decreased tumor levels of both CPA and 4-OH-CPA in mice bearing wild-type 9L tumors following i.p. CPA administration (Fig. 4A and B, left set of bars; also see Fig. 1B and C; refs. 11, 14), axitinib affected no such decrease in tumor 4-OH-CPA levels in mice bearing 9L/2B11 tumors either 15 or 30 minutes after i.p. injection of CPA (Fig. 4B). This can be explained by the increased retention of CPA that enters the axitinib-pretreated tumors, which makes CPA more available for metabolism by the tumor cell-expressed P450 2B11 enzyme, and by the increased retention of tumor cell-derived 4-OH-CPA. This increased retention of CPA and 4-OH-CPA in part compensates for the decreased tumor uptake of 4-OH-CPA that is formed in the liver. Thus, antiangiogenesis not only increases the exposure of tumor cells to drugs delivered intratumorally, but can also increase intratumoral metabolism, and exposure, to the activated form of a prodrug that is administered systemically.

In control experiments, we observed that the CPA 4-hydroxylase activity of 9L/2B11 tumor-derived microsomes was not altered by axitinib pretreatment (Supplementary Fig. S2). Hepatic cytochrome P450-catalyzed CPA 4-hydroxylation is also unaffected by axitinib treatment (14). Thus, the increase in intratumoral concentration of 4-OH-CPA seen following axitinib treatment cannot be explained by an increase in the intrinsic CPA 4-hydroxylase activity of either the liver or tumor.

**Axitinib enhances CPA-induced tumor cell apoptosis**

The impact of the enhanced drug retention following axitinib treatment on tumor cell apoptosis was investigated by measuring caspase activity in tumor samples excised 24 hours after the last drug treatment. Basal 9L/2B11 tumor caspase activity was unaffected by axitinib treatment (Fig. 5A). Intratumoral administration of CPA (50 mg/kg, 2 injections 24 hours apart) significantly increased caspase activity. However, the highest caspase activity was observed when mice bearing the intratumoral CPA-treated 9L/2B11 tumors were pretreated with axitinib (Fig. 5A). Thus, the prolonged exposure of tumor cells to 4-OH-CPA translates into a significant increase in tumor cell apoptosis. Axitinib had no effect on the intrinsic sensitivity of endothelial cells to activated CPA, as determined using cultured HUVEC cells (Fig. 5B).

**Axitinib enhances the antitumor activity of intratumoral CPA injection**

The therapeutic impact of axitinib-enhanced tumor drug retention was investigated in a tumor growth delay study. Four days of axitinib treatment resulted in a transient (~3 day) delay in 9L/2B11 tumor growth, whereas maximum
tolerated dose-scheduled CPA (2 intratumoral CPA administration spaced 24 hours apart) resulted in substantially longer tumor growth delay (Fig. 6A; Table 2). However, robust tumor growth eventually resumed, even when each cycle of CPA treatment was followed by axitinib treatment (CPA/axitinib schedule). In contrast, when axitinib was administered for 4 days prior to intratumoral CPA injection (axitinib/CPA schedule), i.e., conditions under which axitinib increases drug retention and intratumoral exposure to 4-OH-CPA, tumor growth stasis was sustained for at least 22 days after the last
cycle of drug treatment (Fig. 6A; \( P < 0.05 \), 1-way ANOVA, day 0 to day 49 for axitinib/CPA schedule vs. CPA/axitinib schedule). Similar body weight profiles were observed for the CPA monotherapy and for both axitinib-CPA schedules (Fig. 6B), indicating that no additional toxicities were associated with the axitinib/CPA schedule. A small body weight loss occurred after each CPA treatment, as is typical for this cytotoxic drug. Thus, the extended drug retention associated with neoadjuvant axitinib treatment significantly enhances CPA antitumor activity.

**Discussion**

In the present study, we investigated how VEGF receptor-targeted antiangiogenic agents modulate chemotherapeutic drug delivery and drug retention by the tumor. Antiangiogenesis was found to decrease tumor uptake of the anticancer prodrug CPA and its active metabolite, 4-OH-CPA, consistent with the established requirement for a functional tumor vasculature and blood flow for effective drug delivery. However, in mice bearing tumors that express the CPA-activating cytochrome P450 enzyme CYP2B11, neoadjuvant axitinib treatment significantly increased the AUC of intratumoral exposure following intratumoral CPA administration, leading to an increase in tumor cell apoptosis and a major increase in antitumor activity, as seen in tumor growth delay studies. Increased tumor drug retention was also seen with two other antiangiogenic agents, indicating that this effect is a general response to tumor antiangiogenesis. Importantly, the increases in therapeutic activity were achieved despite the transient nature of the tumor drug retention effect of neoadjuvant axitinib treatment, and they cannot be explained by an increase in tumor cell or endothelial cell chemosensitivity following axitinib treatment (14). Furthermore, no increase in antitumor activity was seen when CPA treatment preceded axitinib administration, consistent with the proposed mechanism for the improved therapeutic response, namely, antiangiogenesis-dependent drug retention leading to increased tumor cell exposure to 4-OH-CPA. Finally, when CPA was administered systemically and activated intra-tumorally, the decreases in tumor uptake of both CPA and 4-OH-CPA due to antiangiogenesis were counter-balanced, and fully compensated for, by antiangiogenesis-induced drug retention. Thus, when a tumor-activated prodrug is administered systemically, antiangiogenesis-induced drug retention can counteract the decrease in drug uptake while at the same time retaining the therapeutic benefits of antiangiogenesis-induced tumor cell starvation.

Antiangiogenesis induces morphological normalization of the tumor vasculature, which involves pruning of immature blood vessels, a decrease in blood vessel tortuosity and dilatation, and a closer association between pericytes and tumor endothelial cells (12). This can lead to functional improvements, as shown by the increases in tumor vascular patency and drug uptake and decreases in tumor hypoxia reported in preclinical studies with several antiangiogenic agents (31). However, these effects are short lived and they disappear with continued antiangiogenic drug treatment (32, 33). Moreover, for axitinib (15, 34) and certain other antiangiogenic drugs (17, 35, 36), although morphological normalization of the tumor vasculature and improved functionality of individual blood vessels, a decrease in blood vessel tortuosity and dilatation, and a closer association between pericytes and tumor endothelial cells (12). This can lead to functional improvements, as shown by the increases in tumor vascular patency and drug uptake and decreases in tumor hypoxia reported in preclinical studies with several antiangiogenic agents (31). However, these effects are short lived and they disappear with continued antiangiogenic drug treatment (32, 33).

**Table 1. Axitinib pretreatment increases tumor retention of CPA and 4-OH-CPA**

<table>
<thead>
<tr>
<th></th>
<th>( \text{AUC}_{0-\infty} ) nmol/g-min</th>
<th>( C_{\text{max}} ) nmol/g</th>
<th>( T_{\text{max}} ) min</th>
<th>( t_{1/2} ) min</th>
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<td><strong>A. CPA pharmacokinetics</strong></td>
<td></td>
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<tr>
<td>Tumor</td>
<td>Untreated</td>
<td>364 ± 46</td>
<td>5,040 ± 419</td>
<td>0.5 ± 0</td>
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<td></td>
<td>Axitinib</td>
<td>642 ± 79a</td>
<td>5,405 ± 449</td>
<td>0.5 ± 0</td>
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<tr>
<td>Plasma</td>
<td>Untreated</td>
<td>33 ± 4</td>
<td>123 ± 14</td>
<td>5 ± 0</td>
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<td></td>
<td>Axitinib</td>
<td>48 ± 9</td>
<td>143 ± 6</td>
<td>5 ± 0</td>
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<tr>
<td>Liver</td>
<td>Untreated</td>
<td>27 ± 3</td>
<td>113 ± 14</td>
<td>5 ± 0</td>
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<tr>
<td></td>
<td>Axitinib</td>
<td>45 ± 11</td>
<td>96 ± 7</td>
<td>5 ± 0</td>
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<td><strong>B. 4-OH-CPA pharmacokinetics</strong></td>
<td></td>
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<tr>
<td>Tumor</td>
<td>Untreated</td>
<td>48 ± 4</td>
<td>84 ± 6</td>
<td>12 ± 2</td>
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<tr>
<td></td>
<td>Axitinib</td>
<td>85 ± 6b</td>
<td>119 ± 9b</td>
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<tr>
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<tr>
<td></td>
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<tr>
<td></td>
<td>Axitinib</td>
<td>24 ± 3</td>
<td>22 ± 4</td>
<td>20 ± 5</td>
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Note: Levels of CPA (A) or 4-OH-CPA (B) in 9L/2B11 tumor, plasma, and liver were measured from 5 to 240 minutes following a single i.t. injection of CPA (50 mg/kg) based on Figure 2. Values are expressed as mean ± SE. a, \( P < 0.05 \); b, \( P < 0.01 \), compared with untreated controls, with \( n = 3 \) mice and \( n = 6 \) tumors per group.
vasculature over a more than 10-fold dose range of either axitinib or AG-028262. Moreover, our studies with AG-028262 rule out cross-inhibition with PDGFR-β as a reason for the absence of normalization, given the high selectivity of this RTKI for VEGFR inhibition (25).

The absence of tumor vessel normalization in these preclinical studies is consistent with the limited therapeutic benefit reported in several phase III clinical trials combining antiangiogenic agents with conventional chemotherapies (8–10). The poor performance of these combination therapies indicates a need to consider new approaches, such as the antiangiogenesis-induced tumor drug retention approach reported here. In particular, for cytotoxic agents that can be delivered or activated intratumorally, persistent angiogenesis inhibition may further increase drug retention and therapeutic activity. Other approaches may include optimization of the timing and sequencing of antiangiogenic agents given in combination with cytotoxic drugs, as discussed elsewhere (11). For antiangiogenic agents that show limited activity in a monotherapy setting and that transiently induce functional normalization of the tumor vasculature, intermittent neoadjuvant antiangiogenesis prior to each cycle of cytotoxic drug administration may increase tumor drug uptake. However, for combination therapies that include potent antiangiogenic drugs that do not induce tumor vascular normalization, a brief period of antiangiogenic drug treatment at a reduced dose, or temporary dilation of tumor blood vessels (37) prior to chemotherapy administration may be required to minimize the negative impact of angiogenesis inhibition on tumor drug uptake.

Tumor uptake of 4-OH-CPA, the active metabolite of CPA, was inhibited by axitinib and AG-028262, which reduce tumor

Figure 3. Impact of other antiangiogenic drugs on tumor drug retention following intratumoral CPA administration. Scid mice bearing s.c. 9L or 9L/2B11 tumors were pretreated for 4 days with AG-028262 (40 mg/kg, p.o., bid) or SU5416 (25 mg/kg, i.p., sid), followed by i.t. injection of CPA at 50 mg/kg on day 5. Tumor levels of CPA (A) and 4-OH-CPA (B) were determined 15 and 30 minutes later. Mean ± SE, n = 5 to 6 tumors per group. *, P < 0.05 compared with vehicle controls (Ctrl).

Figure 4. Impact of axitinib on tumor drug retention following systemic CPA administration. Scid mice bearing s.c. 9L or 9L/2B11 tumors were pretreated for 4 days with vehicle or axitinib (25 mg/kg/day, i.p., sid), followed by i.p. injection of CPA at 50 mg/kg on day 5. Tumor levels of CPA (A) and 4-OH-CPA (B) were determined 15 and 30 minutes later. Mean ± SE, n = 6 to 12 tumors per group. ***, P < 0.001 compared with vehicle controls.
blood perfusion and total vascular volume and decrease the number of patent tumor blood vessels (16, 34). These responses not only decrease chemotherapeutic drug uptake, but as shown here, they also decrease the rate at which drug molecules exit from the tumor, resulting in prolonged drug retention and increased tumor drug exposure. In the case of 9L/2B11 tumors given a single intratumoral injection of CPA, axitinib increased tumor cell exposure to CPA, and to 4-OH-CPA almost 2-fold, as judged by AUC values. As axitinib also decreases tumor uptake of liver-derived 4-OH-CPA, with no change in the intrinsic CPA 4-hydroxylase activity of 9L/2B11 tumors, these increases in tumor drug exposure likely underestimate the extent to which axitinib inhibits tumor efflux of 4-OH-CPA per se. Indeed, axitinib decreased the initial rate of tumor efflux of CPA by approximately 3-fold, as judged from the 3.2-fold higher residual intratumoral CPA concentration determined 5 minutes after drug injection (1,840 vs. 580 μmol/L). P450 2B11 has a K_m (CPA) of 70 μmol/L in 9L/2B11 cells (28), indicating that tumor cell capacity for CPA 4-hydroxylation (drug activation) is saturated during the initial 15 to 30 minutes period after intratumoral CPA injection. Thus, the increase in tumor cell apoptosis and therapeutic activity seen in our experiments may very well underestimate the drug retention effect of antiangiogenesis. A larger increase in therapeutic activity can therefore be anticipated for other, direct-acting drugs, or in the case of CPA, for tumors that express a prodrug activation enzyme less efficient than CYP2B11.

There are several important limitations of the antiangiogenesis-induced tumor drug retention effect reported here.
First, antiangiogenesis slows down but does not completely block tumor drug efflux. Moreover, drug retention becomes less prominent when baseline intratumoral drug concentrations are very low. Presumably, the residual efflux capability of the tumor vasculature is sufficient for export when the concentrations are very low. Antiangiogenic therapies involving systemic administration of a tumor-specific delivery of nanoparticles (47) as well as tumor-targeted nanoparticles (48). As tumor-specific delivery of nanoparticles in part depends on the enhanced permeability and leakiness of the tumor vasculature (48), the net impact of antiangiogenesis on tumor vascular permeability and drug retention is uncertain and will require further study. Increased tumor drug retention can also be expected for agents that transiently normalize tumor vasculature, once they ultimately decrease tumor vascular patency with continued use, and for vascular disrupting agents, which induce an acute interruption of tumor blood perfusion (49). The latter possibility is supported by the increased tumor exposure to the alkylating agent melphalan following pretreatment with the vascular disrupting agent 5,6-dimethylxanthene-4-acetic acid (30), and by the increase in activity when doxorubicin was combined with the vascular disruption agent ICT2588, where maximal antitumor activity was achieved when doxorubicin was administered after the collapse of the tumor vasculature induced by ICT2588 (51).

In conclusion, antiangiogenesis-induced tumor drug retention is an intrinsic action of antiangiogenic drugs and can be applied to a variety of antiangiogenesis treatments. This drug retention effect was employed to significantly increase the therapeutic activity of CPA treatment in a 9L xenograft model, and similar benefits can be expected with other tumor types. An even more pronounced drug retention effect can be anticipated for chronic antiangiogenesis treatment, when the decrease in functional tumor vasculature becomes more substantial, and perhaps for vascular disrupting agents as well. These findings provide a novel perspective and insight into the complex pharmacokinetic and pharmacodynamic effects and interactions between antiangiogenic drugs and chemotherapeutic agents and may stimulate further research to take advantage of these findings in a way that may circumvent the decrease in drug uptake that is also intrinsic to antiangiogenic therapies. Finally, certain normal tissues are also sensitive to VEGF/VEGFR inhibition (25, 52), indicating that the vasculature in these tissues may also be targeted for antiangiogenesis-induced drug retention.

Table 2. Effect of drug schedule on tumor doubling time.

<table>
<thead>
<tr>
<th>Tumor doubling time (days)</th>
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<tr>
<td>Vehicle (10)</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td>Axitinib (10)</td>
<td>8.0 ± 0.4</td>
</tr>
<tr>
<td>CPA (10)</td>
<td>28.4 ± 3.6</td>
</tr>
<tr>
<td>CPA/Axitinib (16)</td>
<td>21.3 ± 2.16</td>
</tr>
<tr>
<td>Axitinib/CPA (14)</td>
<td>&gt; 49$^a$</td>
</tr>
</tbody>
</table>

NOTE: Data shown are based on data presented in Fig. 6. Tumor sizes were measured every 3–4 days using digital calipers and volumes calculated as $(3.14/6) \times (L \times W)^{3/2}$. Values are expressed as mean ± SE. The number of individual tumors included in each treatment group is shown in parentheses. Statistical significance of differences was assessed by comparing each drug treatment group with vehicle control using 2-tailed Student’s t test.

$^a$Tumor growth was blocked after initiation of drug treatment (see Fig. 6) and 10 of 14 tumors never doubled in volume.

**Antiangiogenesis Increases Tumor Drug Retention**
Disclosure of Potential Conflicts of Interests

No potential conflicts of interests were disclosed.

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References


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