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 for 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); 1–11. ©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 patency (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 for human VEGF, is an antiangiogenic drug that increases the survival of patients with metastatic colorectal cancer and non-small-cell lung cancer when given in combination with metastatic colorectal cancer and non-small-cell lung cancer when given in combination with conventional chemotherapy (4, 5). Small-molecule receptor tyrosine kinase inhibitors (RTKIs) that inhibit VEGF receptors (VEGFR) can also be used to induce antiangiogenesis. However, despite efficacy observed in clinical trials with several such RTKIs (6, 7), recent phase III trials showed very limited benefits when antiangiogenic RTKIs were combined with conventional chemotherapy (8–10). These observations raise the question as to how antiangiogenesis treatments affect tumor uptake of other chemotherapeutic agents and the overall anti-tumor activity of combination therapies.

Morphological normalization of surviving tumor blood vessels has been observed following treatment with a variety of antiangiogenic drugs (11). It has been proposed that antiangiogenesis may transiently normalize the tumor vasculature, leading to an increase in tumor drug uptake (12). Like the antiangiogenic antibodies bevacizumab and DC101, the VEGF receptor-selective RTKI axitinib (13) induces morphological normalization of the tumor vasculature and can increase the transport efficiency of individual blood vessels that survive antiangiogenesis treatment (14, 15). However, the total number of surviving blood vessels decrease and overall tumor blood perfusion is not improved by axitinib treatment, as indicated by the increase in tumor hypoxia and the decrease in tumor uptake of small molecules, including 4-OH-CPA, the active metabolite of the anticancer prodrug cyclophosphamide (CPA; refs. 14, 16). This lack of improved tumor vascular functionality (“normalization”) by axitinib and certain other antiangiogenic drugs (17–20) could be the result of doses that are optimized for maximal angiogenesis inhibition with acceptable host toxicity.

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

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doi: 10.1158/0008-5472.CAN-10-3242

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but are not optimal for vascular normalization. Supporting this possibility, increases in chemotherapeutic drug uptake are seen in some tumor 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 coinduction 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 antiangiogenesis 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 extracellular matrix, a decrease in tumor interstitial fluid pressure following antiangiogenesis treatment 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 antiangiogenesis 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 cytochrome 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 x 106) 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) x (L x W)2/3.

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 ± 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 (3–40 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 ± 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 ± 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 (3–40 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 ± SE, $n = 4$ to 6 tumors per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with controls.

Antiangiogenesis Increases Tumor Drug Retention

selective VEGFR-2 RTKI than axitinib (25). AG-028262 induced a progressive, dose-dependent decrease in intratumoral 4-OH-CPA over the full dose range tested (Fig. 1D). At 40 mg/kg, AG-028262 suppressed tumor 4-OH-CPA level by 48%, consistent with its strong antiangiogenic activity. Similar decreases in tumor uptake of CPA (Supplementary Fig. S1) and 4-OH-CPA (30) were seen in PC-3 tumors pretreated with axitinib at 25 mg/kg/day for times ranging from 0.5 to 6 days. Thus, VEGFR inhibition by either axitinib or AG-028262 is not associated with functional normalization of the tumor vasculature.

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 1,840 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., $K_m$ (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 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 $C_{max}$ 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, at the 5-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-OHCPA 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 $K_m$ 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 RTKs.

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 effected 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-hydroxylation 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-hydroxylation 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

Figure 2. Impact of axitinib on CPA and 4-OH-CPA pharmacokinetics following intratumoral CPA injection. Scid mice bearing s.c. 9L/2B11 tumor xenografts without (solid lines) or with axitinib pretreatment (dashed lines; 25 mg/kg, i.p., sid for 4 days) were given a single i.t. injection of CPA at 50 mg/kg at t = 0 minutes. CPA and 4-OH-CPA levels were determined in 9L/2B11 tumors (A, B) and liver (E, F) 5-240 minutes after CPA administration. CPA (G) and 4-OH-CPA (H) were also determined in 9L tumors grown s.c. in scid mice following i.t. CPA injection, without or with axitinib pretreatment, as in A and B. Mean ± SE, n = 3 mice (6 tumors) per time point. *, P < 0.05; **, P < 0.01; ***, P < 0.001 for axitinib pretreated compared with controls.
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 cytochrome P450 enzyme CYP2B11, neoadjuvant axitinib treatment, as is typical for this cytotoxic drug. Thus, the increases in therapeutic activity were achieved with two other antiangiogenic agents, indicating that this is the extended drug retention associated with neoadjuvant axitinib treatment. No additional toxicities were associated with the neoadjuvant 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.

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

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Note: Levels of CPA (A) or 4-OH-CPA (B) in 9L/C6 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. <sup>a</sup>, P < 0.05; <sup>b</sup>, P < 0.01, compared with untreated controls, with n = 3 mice and n = 6 tumors per group.

Moreover, 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 intratumorally, 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 dilation, 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 may occur, overall tumor vascular patency and capacity for drug uptake actually decrease. Presently, we investigated the hypothesis that tumor blood vessel normalization leading to increased drug uptake can be achieved by reducing the dose of the antiangiogenic RTKI; however, we obtained no evidence for such functional normalization of tumor.
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/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.

Figure 5. Effects of axitinib on CPA-induced tumor cell apoptosis and endothelial cell chemosensitivity. A, Scid mice bearing s.c. 9L/2B11 tumor xenografts pretreated for 4 days with vehicle or axitinib (25 mg/kg, i.p., sid) were injected with phosphate-buffered-saline (control) or CPA (50 mg/kg, i.t., 2 injections 24 hours apart). Tumor cell apoptosis was determined 24 hours after the last drug treatment by measuring caspase activity in the absence or in the presence of the caspase inhibitor caspatin. Mean ± SE, n = 6 tumors per treatment. *, P < 0.05; **, P < 0.01 for the comparisons indicated. B, cultured HUVEC cells were treated with chemically activated CPA, 4-HC, alone or in combination with axitinib. The effect of drug treatment on final cell number (cell proliferation) was determined by crystal violet staining. Mean ± SE, n = 3 per data point.

Figure 6. Impact of neoadjuvant axitinib treatment on antitumor activity of intratumoral CPA treatment. 9L/2B11 tumors were implanted s.c. in scid mice. Arrows along the x-axis indicate the days on which CPA was administered (150 mg/kg, 2 i.t. injections 24 hours apart, drug treatment cycle repeated beginning on day 21) and the horizontal lines below the x-axis indicate the time period of axitinib treatment (25 mg/kg, i.p., sid). Drug treatment was initiated on day 0, when the average tumor size of each group reached 800 to 900 mm⁳. A, antitumor activity of intratumoral CPA injection was substantially prolonged in mice given 4 daily axitinib injections prior to CPA treatment (Axitinib/CPA schedule) compared with CPA treatment alone. Also see Table 2. B, impact of axitinib and CPA treatment schedule on body weight for the same mice shown in panel A. No additional body weight loss was induced by the combination drug treatments compared with CPA treated tumor-bearing mice. Mean ± SE, n = 10 to 16 tumors per group.
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 chemotherapeutic drug is present at low concentrations. Most important, the same antiangiogenic mechanisms that increase tumor drug retention also decrease chemotherapeutic drug uptake by the tumor. Thus, to take advantage of the increase in tumor drug retention and translate it into a meaningful therapeutic benefit, it needs to be utilized in a way that overcomes or circumvents the associated decrease in drug uptake from systemic circulation. This can be achieved by combining neoadjuvant antiangiogenic treatment with direct delivery of a chemotherapeutic drug into target tissues. Although intratumoral drug delivery is not suitable for all solid tumors and may impose certain practical limitations, it has been used in the clinic for treatment of head and neck cancer, lung cancer, and breast cancer (38–40) and can also be used in cases where tumors are not amenable to resection or as an adjuvant following tumor resection (41, 42). The benefits of antiangiogenesis induced tumor drug retention can also be realized in combination therapies involving systemic administration of a tumor-activated produg. This approach was exemplified for the P450 produg CPA in mice bearing tumors that express CYP2B11, where the axitinib-dependent decrease in tumor uptake of CPA and 4-OH-CPA from systemic circulation was fully compensated by the increase in tumor retention of 4-OH-CPA generated intratumorally. This drug retention effect helps explain our earlier finding that maximal antitumor activity is achieved in mice bearing 9L/2B11 tumors when systemic CPA administration is preceded by axitinib treatment (30). Finally, the potential limitations imposed by the subcutaneous tumor xenografts model used here should be noted. The tumor microenvironment can have a significant impact on angiogenesis (43), and malignant gliomas grown orthotopically may be more hypoxic and less highly vascularized than the subcutaneous 9L tumors used in our studies (14, 44). These differences in tumor microenvironment may impact drug uptake as well as the extent to which antiangiogenesis increases overall drug exposure via the drug retention effect described here.

The principle of antiangiogenesis-induced tumor drug retention presented here may be applied to systemic treatments based on other therapeutic agents that can be activated intratumorally, including other P450 prodrugs (29), produgs activated by other enzymes (45), and bioreductive drugs, which are activated within hypoxic tumor regions (46). Antiangiogenesis-induced tumor drug retention may also be extended to increase the retention of tumor cell replicating, oncolytic viral vectors (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 paucity 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 disruption agent 5,6-dimethylxanthenone-4-acetic acid (50), 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.
Disclosure of Potential Conflicts of Interests

No potential conflicts of interests were disclosed.

Acknowledgements

We thank Pfizer Global Research and Development for providing axitinib and AG-028262, and Dr. Dana Hu-Lowe for useful discussions.

References


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Cancer Res  Published OnlineFirst March 29, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-3242

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