The prevailing concept is that immediate mobilization of bone marrow–derived circulating endothelial progenitor cells (CEP) is a key mechanism mediating tumor resistance to vascular-disrupting agents (VDA). Here, we show that administration of VDA to tumor-bearing mice induces 2 distinct peaks in CEPs: an early, unspecific CEP efflux followed by a late yet more dramatic tumor-specific CEP burst that infiltrates tumors and is recruited to vessels. Combination with antiangiogenic drugs could not disrupt the early peak but completely abrogated the late VDA-induced CEP burst, blunted bone marrow–derived cell recruitment to tumors, and resulted in striking antitumor efficacy, indicating that the late CEP burst might be crucial to tumor recovery after VDA therapy. CEP and circulating endothelial cell kinetics in VDA-treated patients with cancer were remarkably consistent with our preclinical data. These findings expand the current understanding of vasculogenic “rebounds” that may be targeted to improve VDA-based strategies.

SIGNIFICANCE: Our findings suggest that resistance to VDA therapy may be strongly mediated by late, rather than early, tumor-specific recruitment of CEPs, the suppression of which resulted in increased VDA-mediated antitumor efficacy. VDA-based therapy might thus be significantly enhanced by combination strategies targeting late CEP mobilization.

Reversing Resistance to Vascular-Disrupting Agents by Blocking Late Mobilization of Circulating Endothelial Progenitor Cells

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ABSTRACT

The prevailing concept is that immediate mobilization of bone marrow–derived circulating endothelial progenitor cells (CEP) is a key mechanism mediating tumor resistance to vascular-disrupting agents (VDA). Here, we show that administration of VDA to tumor-bearing mice induces 2 distinct peaks in CEPs: an early, unspecific CEP efflux followed by a late yet more dramatic tumor-specific CEP burst that infiltrates tumors and is recruited to vessels. Combination with antiangiogenic drugs could not disrupt the early peak but completely abrogated the late VDA-induced CEP burst, blunted bone marrow–derived cell recruitment to tumors, and resulted in striking antitumor efficacy, indicating that the late CEP burst might be crucial to tumor recovery after VDA therapy. CEP and circulating endothelial cell kinetics in VDA-treated patients with cancer were remarkably consistent with our preclinical data. These findings expand the current understanding of vasculogenic “rebounds” that may be targeted to improve VDA-based strategies.

SIGNIFICANCE: Our findings suggest that resistance to VDA therapy may be strongly mediated by late, rather than early, tumor-specific recruitment of CEPs, the suppression of which resulted in increased VDA-mediated antitumor efficacy. VDA-based therapy might thus be significantly enhanced by combination strategies targeting late CEP mobilization.

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INTRODUCTION

Rapid tumor revascularization and repopulation compromise the expected clinical efficacy of vascular-disrupting agent (VDA)-based therapy (1–4). VDAs selectively target the already-established tumor vasculature and cause acute vascular shutdown (2, 3, 6), effectively resulting in severe tumor hypoxia, ischemia, and cell death (1, 7). Although VDAs are characterized by extensive necrosis of the tumor core, a viable peripheral rim is typically spared from which tumor cell repopulation and regrowth rapidly resumes (2, 3, 7). Tumor recovery at the periphery, representing treatment resistance, appears to be a consistent finding and may explain the minimal tumor shrinkage and lack of clinical benefit observed with VDAs when given as single agents (1, 2, 5).

For these reasons, combination strategies with cytotoxic agents or radiotherapy aiming to interfere with the tumor recovery that ensues after VDA therapy have been actively evaluated both preclinically (1, 8–10) and clinically (4, 11–13). The future success of VDAs will most likely rely on advances in deciphering the mechanisms underlying VDA-induced tumor resistance and determining optimal agents and schedules that will improve the antitumor activity of these drugs.

VDA-induced tumor repopulation is angiogenesis dependent, suggesting that combining VDAs with drugs targeting angiogenesis may be effective (2, 7). Initial studies in xenograft tumor models showed that the anti-VEGF antibody, bevacizumab (14), or a potent inhibitor of VEGF receptor-2 (VEGFR-2)-associated tyrosine kinase effectively enhanced the antitumor activity of VDAs (15). A mechanistic rationale for these findings was provided in a pioneering study by Shaked and colleagues (16) in which they showed that the treatment of tumor-bearing mice with VDAs led to an acute and immediate (within 4 hours) mobilization of circulating endothelial progenitor cells (CEP), which were reported to home to the viable peripheral rim and promote tumor regrowth. Blockade of this VDA-induced CEP mobilization with a monoclonal antibody to mouse VEGFR-2 (DC101) enhanced the antitumor efficacy of the VDA, thus highlighting the potential role of CEPs in tumor recovery and treatment escape after VDA therapy (16). On the basis of these findings, it seemed reasonable to postulate that combined complementary antiangiogenic strategies would readily translate into substantial tumor control and clinical benefit for patients (11, 17). However, in preclinical studies authors also have underscored the importance of schedule
and sequence to optimize the putative additive effects of combination approaches (18).

Accumulating evidence suggests that bone marrow-derived CEPs are pivotal mediators of tumor progression. Initial studies (19, 20) in preclinical models of acute vascular injury first showed how CEPs are mobilized and home to ischemic sites, incorporate into neovessels, and complement angiogenesis afforded by preexisting endothelium (21, 22). Since then, CEPs have attracted considerable interest and have been actively investigated for their role in angiogenesis-mediated tumor growth (21, 23, 24). CEP mobilization is regulated in part by proangiogenic cytokines released into the circulation by the tumor cell, or after ischemic lesions or vessel wall injury for vascular repair (23).

Although in some preclinical studies authors have shown the incorporation of CEPs in tumor neovessels (24–27), the contribution and general relevance of CEPs to tumor angiogenesis and progression have been controversial and the subject of intense scrutiny as the result of several studies in which investigators reported contradictory data (28–32). Alternatively, in other preclinical studies, authors have suggested that CEPs are not only key contributors to tumor neoangiogenesis and tumor growth (24–26) but are also critical regulators of the angiogenic switch promoting metastatic progression (33). Another subpopulation of endothelial cells that has been considerably studied in the context of vascular trauma and cancer is mature circulating endothelial cells [CEC (34, 35)]. CECs are shed from blood vessel walls into the circulation as the result of vascular injury. Although their role in tumor angiogenesis is less clear, the number of CECs in peripheral blood directly reflects the extent of endothelial insult, and CEC counts are currently considered as a useful biomarker of vascular damage in patients with vascular disorders (36).

We hypothesized that CECs and CEPs might have the potential of being unique tools, possibly biomarkers, reflecting the pathophysiologic effects of VDAs. Previously, we observed the presence of CECs (13) and, more importantly, of VDA-induced CEP mobilization in a small series of cancer patients included in a phase I trial combining a VDA, ombrabulin (AVE8062; Sanofi-Aventis), with cisplatin and in whom CEP levels peaked 3 to 7 days after drug injection (37). CECs have also been documented in cancer patients in a phase I study evaluating another VDA, ZD6126 (38). These preliminary clinical findings supported the mechanistic hypothesis that CEC levels might directly reflect the antivascular and antitumor activity of the VDA, whereas CEP levels might express tumor resistance via vasulogenic rebounds and could therefore be crucial to identify possible targets as well as optimal schedules for combined strategies.

Herein, we show for the first time that VDA therapy induces not only an instantaneous CEP peak as previously described (16) but also a delayed yet more dramatic tumor-specific burst of CEPs that were recruited to VDA-disrupted tumor vessels. Blockade of this late CEP burst significantly increased the antitumor efficacy of the VDA. Taken together, these findings provide novel insight into host-reactive responses and vasulogenic “rebounds” mediating resistance to VDA therapy which might be more efficaciously targeted by optimally scheduled antiangiogenic strategies.

RESULTS

Treatment with CA-4-P Induces Early and Massive Necrosis with Hypoxia-Related Microvessel Destruction Followed by Revascularization

Preliminary experiments with tumors of different histologic types (see Supplementary Methods) showed that PC3 tumors displayed an important level of microvessel density with little to no spontaneous necrosis and were very sensitive to VDAs compared with other xenograft tumor types (data not shown). To investigate the precise cascade of events triggered by VDAs in tumors, PC3 xenograft tumors established in male nude mice were evaluated for necrosis, microvessel density, and upregulation of carbonic hydrase IX (CA-IX) expression (an indicator of hypoxia) after treatment with a potent VDA, combretastatin-A4-phosphate [CA-4-P (39)]. Once PC3 tumor volumes reached an average of 300 to 500 mm³, mice (n = 5) were randomized into groups and culled for tumor harvesting at different time points ranging from 2 to 120 hours after a single i.v. injection of 100 mg/kg CA-4-P.

Four hours after CA-4-P injection, PC3 tumors displayed significant and marked intratumoral necrosis compared with baseline values (73 ± 10% vs. 4.6 ± 1%, P < 0.0003; Fig. 1A). By 24 hours, necrosis was extensive (81% ± 7%, P < 0.0001 compared with baseline; Fig. 1A and D) and was associated with a significant reduction in the surface fraction of CD34⁺ vessels (microvessel density; 0.7% ± 0.3% vs. 3.7% ± 1% at baseline, P < 0.03; Fig. 1B and E), consistent with the effects of VDA described in different tumor models (2, 7, 39). Tumor expression of CA-IX peaked significantly 24 hours after CA-4-P (P < 0.005 compared with baseline values; Fig. 1C) and was concomitant to the lowest level of microvessel density. Microvessel density increased again by 48 hours and mirrored the downregulation of CA-IX expression, suggesting the existence of revascularization processes after immediate and extensive hypoxia-related tumor and vascular damage that ensued after CA-4-P treatment.

VDA-Induced Tumor Vascular Disruption Correlates with Peaks in CEC and CEP Levels

Next, we investigated whether VDA-induced tumor alterations and vascular injury were concomitantly reflected by changes in CEC and CEP levels, with special focus on the mobilization of CEPs that mediate vascular repair. We established flow cytometry assays to enumerate CECs and CEPs in mouse peripheral whole blood in accordance with the strategy we previously established in human samples [37, 40, 41 (Supplementary Data and Supplementary Figs. S1–S5)]. CECs were identified as
Two Distinct CEP Bursts Induced by VDA

Figure 1. CA-4-P-treated PC3 xenografts show rapid extensive necrosis and hypoxia-related vascular damage. Six- to 8-week-old nude mice bearing PC3-xenografts of 300 to 500 mm³ established subcutaneously 15 days previously were culled at indicated times ranging from baseline to 120 hours (n = 5 mice per group) after a single i.v. injection of 100 mg/kg CA-4-P, at which times tumors were harvested for analysis. A–C, summary graphs for the percentage of (A) intratumoral necrosis, (B) surface fraction of CD34⁺ vessels (microvessel density), and (C) area containing expression of CA-IX after CA-4-P treatment are presented. Data are expressed as mean ± SD. Significance was set as 0.05 (*) or 0.01 (**) or 0.001 (***) compared with baseline values. D, representative hematoxylin and eosin images of tumor sections show increased necrosis (N) over time (baseline to 96 hours) after CA-4-P treatment. Scale bars at 100 μm for lower images. E, representative images of immunohistochemical staining of the endothelial cell marker CD34 showing reduced and peripheral microvessel density 24 hours after CA-4-P (right) compared with baseline (left). Scale bars, 100 μm. Original magnification, ×25.

A rapid and significant peak in CEC levels was observed in PC3 tumor-bearing mice 4 hours after a single injection of 100 mg/kg CA-4-P (P < 0.008 compared with baseline values; Fig. 2A). In contrast, no changes in CEC levels were detected in control groups of non–tumor-bearing mice receiving CA-4-P (Fig. 2B) or tumor-bearing mice receiving vehicle (Fig. 2C). These data were consistent with our

viable CD45⁺Flk-1⁺CD31⁺MECA-32⁺ cells. CEPs were identified as viable CD45⁺Flk-1⁺CD31⁺MECA-32⁺ cells. Complementary flow cytometry experiments further characterized CEPs as expressing CD34⁺, as recently described by Yang and colleagues (42), and importantly, vascular endothelial (VE)-cadherin, which directly supports the endothelial phenotype (Supplementary Fig. S2).
histologic findings and suggested that the CEC peak observed in tumor-bearing mice was the direct consequence of the tumor vascular injury induced by CA-4-P.

Similarly, a single injection of 100 mg/kg CA-4-P caused an acute increase in CEP levels in tumor-bearing mice at 2 and 4 hours after treatment ($P < 0.0001$ and $P < 0.0004$, respectively, compared with baseline values; Fig. 2D), which then returned to basal levels by 24 hours. CEP levels also rapidly increased in non–tumor-bearing mice at 2 and 4 hours after CA-4-P ($P < 0.002$ compared with baseline values; Fig 2E), as previously described (16). Interestingly, the CEP levels observed at 2 and 4 hours after CA-4-P did not significantly differ between tumor-bearing and non–tumor-bearing mice ($P = 0.66$ and $P = 0.86$, respectively; Fig. 2D and E). Also, in tumor-bearing mice, the increase in CEPs occurred before the peak in CEC levels. These results suggested that the early peak in CEP levels—observed immediately after CA-4-P in both tumor-bearing and non–tumor-bearing mice but before the peak in CEC levels—did not actually reflect a VDA-induced tumor vascular-repair process but was most likely a general host response to the injection of CA-4-P. Alternatively, the significant peaks in CECs observed exclusively in tumor-bearing animals 4 hours after CA-4-P treatment were highly suggestive of tumor-specific VDA-induced vascular lesions. We therefore hypothesized that the mobilization of CEPs recruited for tumor-specific vascular repair might occur at later time points after CA-4-P administration.

Figure 2. CA-4-P–induced tumor vascular damage is associated with peaks in CEC and CEP levels and a characteristic delayed tumor-specific burst in CEPs. CEC and CEP levels were evaluated using 4-color flow cytometry in mice bearing PC3-xenografts of 300 to 500 mm$^3$ at sequential time points after treatment with 100 mg/kg CA-4-P. A–C, CEC levels were evaluated in mice ($n = 6–8$ per time point in each group) at times ranging from baseline to 24 hours after CA-4-P (A) in tumor-bearing mice and (B) in non–tumor-bearing mice, or (C) in tumor-bearing mice receiving vehicle. Data are expressed as mean ± SD. Significance was set as $0.05 > P > 0.01 (**), 0.01 > P > 0.001 (***), or $P < 0.001 (****) compared with baseline values. D–F, in a separate experiment, CEP levels were measured in mice ($n = 8–10$ per time point in each group) at sequential time points ranging from baseline to 144 hours in (D) tumor-bearing and in (E) non–tumor-bearing mice after CA-4-P treatment and in (F) a control group of tumor-bearing mice receiving vehicle. Data are expressed as mean ± SD. Significance was set as $0.05 > P > 0.01 (**), 0.01 > P > 0.001 (***), or $P < 0.001 (****) compared with baseline values unless expressed otherwise.
Figure 3. Circulating G-CSF, MMP-9, SDF-1 serum levels, and bone marrow SDF-1 content differ in CA-4-P-treated tumor-bearing and non-tumor-bearing mice. A-D, blood samples collected from PC3-tumor-bearing and non-tumor-bearing mice at baseline, 4 hours, 24 hours, 48 hours, 72 hours, and 96 hours after administration of 100 mg/kg CA-4-P were evaluated with the use of ELISA for serum levels of (A) G-CSF, (B) MMP-9, and (C) SDF-1, as well as levels for (D) bone marrow SDF-1 content (n = 4–6 mice per time point). Results obtained were plotted as a fold increase from the baseline value. Data are expressed as mean ± SD. Significance was set as 0.05 compared with baseline values.

To test this hypothesis, previous experiments were repeated in separate cohorts of tumor-bearing and non–tumor-bearing mice to extend CEP measurements from 4 to 144 hours after CA-4-P administration. Tumor-bearing mice displayed a dramatic burst in CEP levels 96 hours after CA-4-P treatment (P < 0.0001 compared with baseline values) that was 2-fold greater than the previous CEP peak at 4 hours (Fig. 2D). In contrast, control groups showed no changes in CEP levels after 24 hours (Fig. 2E and F) suggesting that the CA-4-P-induced burst in CEPs at 96 hours was specific to tumor-bearing animals.

Comparable experiments in a different xenograft-tumor model (H69) yielded similar results with a rapid increase in CEC levels (within 6 hours) and 2 distinct CEP peaks at 4 and 72 hours after treatment of H69-tumor-bearing mice with 100 mg/kg CA-4-P (Supplementary Fig. S6). Administration of different doses of CA-4-P (50 mg/kg or 100 mg/kg) to H69 tumor-bearing animals showed that CEC and CEP levels varied in a drug-dose dependent manner (Supplementary Fig. S6). These results showed that VDA-induced tumor vascular lesions were reflected by changes in CEC and CEP levels that were both tumor-specific and drug-dose dependent.

CEP Kinetics Correlate with Serum Matrix Metalloproteinase-9, Granulocyte Colony-Stimulating Factor, and Bone Marrow Stromal-Derived Factor-1 Levels

To investigate which growth factors may be responsible for the mobilization of CEPs after VDA treatment, we measured circulating levels of murine VEGF, granulocyte colony-stimulating factor (G-CSF), matrix metalloproteinase-9 (MMP-9), and stromal-derived factor-1 (SDF-1) in serum samples obtained from tumor-bearing and non–tumor-bearing mice at various time points after the administration of CA-4-P (Fig. 3). The results in Figure 3A and B show that the kinetics of G-CSF and MMP-9 levels paralleled those observed for CEPs with an initial unspecified rise at 4 hours in both non–tumor-bearing and tumor-bearing CA-4-P-treated mice followed by a second increase in G-CSF and MMP-9 levels at 72 to 96 hours exclusively in tumor-bearing mice. VEGF levels at baseline were similar among tumor-bearing and non–tumor-bearing mice and were not correlated to changes in CEP levels in tumor-bearing animals (data not shown) as reported in a previous study describing CEP mobilization after VDA-like chemotherapeutic agents (43).

Systemic induction of SDF-1 accounts for the rapid mobilization of CEPs induced by VDAs and by paclitaxel in non–tumor-bearing mice (43). Here, we found that serum SDF-1 levels did not change after treatment with CA-4-P and did not differ between tumor-bearing and non–tumor-bearing mice (Fig. 3C). SDF-1 levels measured directly within bone marrow samples showed, however, a rapid increase at 24 hours after the administration of CA-4-P in tumor-bearing and non–tumor-bearing animals as well as a significant increase at 96 hours exclusively in tumor-bearing mice (P < 0.03 compared with baseline values; Fig. 3D), suggesting that the second burst of CEPs was associated with changes in bone marrow and not serum SDF-1 content.
Antiangiogenic Treatment Blocks the Delayed CEP Burst and Is Associated with Enhanced Antitumor Activity

We next evaluated the capacity of an antiangiogenic agent to inhibit the 2 CEP bursts induced by CA-4-P treatment. Because preclinical studies have investigated therapies that combine VDAs with an antiangiogenic drug targeting VEGFR-2 (15), and previous reports have shown that antiangiogenic drugs can suppress the mobilization of CEPs (16, 43), we postulated that sunitinib, a potent receptor tyrosine-kinase inhibitor including VEGFR and PDGFR, might target the VEGFR-2 receptor expressed on CEPs.

PC3-tumor-bearing mice were treated with vehicle alone or with CA-4-P (100 mg/kg) alone or in combination with sunitinib. We reasoned that the antiangiogenic agent had to be given sufficiently in advance to be able to suppress the VDA-induced CEP peaks. To selectively target either of the CEP peaks and do so with an equivalent treatment period, sunitinib was given to tumor-bearing mice orally once daily for 4 days either before or after the CA-4-P injection. As shown in Figure 4A, treatment with sunitinib before CA-4-P did not block the early CEP peak. Alternatively, sunitinib given after CA-4-P completely abrogated the second CEP peak at 96 hours ($P < 0.008$ compared with CA-4-P alone). In mice receiving sunitinib before the injection of CA-4-P, a moderate inhibition of CEP levels at 96 hours was observed and was probably attributable to a sustained effect of sunitinib despite its discontinuation. CEP levels were unchanged in mice treated with sunitinib alone. These results suggested that only the second tumor-specific CA-4-P-induced CEP burst could be blocked by the combination of an antiangiogenic agent.

To assess whether inhibition of VDA-induced CEP peaks with an antiangiogenic agent was associated with therapeutic antitumor activity, we evaluated tumor growth in mice receiving combined therapy. PC3-xenograft tumors were allowed to reach approximately 100 mm$^3$, at which point treatment with vehicle, sunitinib, CA-4-P alone, or combined with sunitinib was initiated according to the same drug combination and schedule described previously. The results in Figure 4B show that the most significant tumor growth inhibition was observed in tumor-bearing mice treated with CA-4-P followed by 4 days of sunitinib as compared with those receiving vehicle...
(P < 0.0001), CA-4-P alone (P < 0.002) or sunitinib before CA-4-P (P < 0.02). Tumors treated with sunitinib alone displayed moderate growth delay at day 16 compared with untreated tumors (P < 0.02), which suggested a mild local antiangiogenic effect of sunitinib despite its known lack of antitumor activity in the PC3-xenograft model (44). Overall, the best tumor growth suppression was observed with a sequence that combined sunitinib after CA-4-P administration and that was concurrent with marked abrogation of the second burst of CEPs.

Next, the long-term antitumor efficacy of this combination strategy was evaluated. When tumor volumes reached 100 mm³, PC3-xenograft tumors received a single dose of CA-4-P followed by sunitinib given once a day, 5 days a week, until tumor progression. As shown in Figure 4C, this combination sequence yielded substantial antitumor effect manifested by prolonged control of tumor progression at day 18 compared with tumor-bearing mice treated with vehicle (P < 0.0001), CA-4-P, or sunitinib alone (P < 0.001). Tumor control was maintained at day 25 compared with controls receiving vehicle for which the tumor end point was reached by day 18 (P < 0.005; Fig. 4C). Sunitinib alone showed only a delay in tumor growth by a few days. CA-4-P alone resulted in potent tumor regrowth with volumes that were comparable with untreated tumors, most likely attributable to VDA-induced tumor repopulation (Fig. 4B and C). It is of note that the combination treatment with sunitinib given before CA-4-P injection was not evaluated here because this strategy did not prove relevant in previous experiments.

Taken together, these results indicated that in our PC3-xenograft model, selective blocking of the late CA-4-P-induced CEP peak was significantly associated with marked tumor growth suppression and the optimal therapeutic combination scheme, allowing for this enhanced antitumor efficacy relied on administering sunitinib after CA-4-P.

The Late VDA-Induced CEP Peak Correlates with Bone Marrow–Derived Cell Tumor Infiltration That Can Be Blocked by Antiangiogenic Drugs

To further characterize host-derived vascular repair mechanisms caused by VDAs and determine the functional role of bone marrow–derived CEPs, we focused subsequent experiments on a syngeneic C57Bl/6 mouse model transplanted with GFP⁺ bone marrow (GFP⁺ bone marrow). First, Lewis lung carcinoma (LLC) tumors established in immunocompetent C57Bl/6 mice were allowed to reach 300 to 500 mm³, at which point CA-4-P treatment was initiated. As observed in nude mice bearing PC3 xenografts, CEP levels peaked in LLC-tumor-bearing C57Bl/6 mice at 2 distinct times after CA-4-P, with an early CEP peak at 4 hours (P < 0.002 compared with baseline) followed by a delayed but significantly greater (by 7-fold) tumor-specific CEP burst at 72 hours (P < 0.0001 compared with untreated controls; Fig. 5A). The lower CEP values and slightly earlier occurrence of the second CEP burst in C57Bl/6 mice compared with nude mice were likely the result of inherent differences in immunogenic background. Initial studies by Shaked and colleagues (16) showed that DC101 administered 24 hours before VDA treatment could block the early-occurring CEP surge and resulted in increased antitumor efficacy.

We evaluated the capacity of a single dose of DC101 or sunitinib, administered 24 hours before, to suppress the successive CEP mobilizations in CA-4-P-treated LLC-tumor-bearing mice. As shown in Figure 5A, neither DC101 nor sunitinib blocked the early CEP peak. A single dose of DC101 or sunitinib given 24 hours before the second CEP peak, however, completely blunted this late CEP mobilization (P < 0.006 compared with CA-4-P alone; Fig. 5A). These data extended our results observed with combined treatment in the PC3-xenograft model and, taken together, implied that (i) only the second delayed CA-4-P-induced tumor-specific CEP burst could in fact be blocked by antiangiogenic agents in combined strategies, and (ii) the mechanisms underlying the mobilization of the two distinct CEP bursts were different. These experiments were repeated to explore the capacity of CA-4-P-mobilized CEPs to home to tumors and incorporate to the tumor vasculature of LLC tumors established in GFP⁺ bone marrow–transplanted C57Bl/6 mice (Fig. 5B–D). After CA-4-P treatment, tumors were removed 3 days after each CEP peak (at day 3 or day 6). Immunohistochemical analyses of tumors from untreated control mice showed moderate incorporation of GFP⁺ bone marrow–derived cells (Fig. 5B and D). Mice treated with sunitinib or DC101 alone also showed mild incorporation of GFP⁺ bone marrow–derived cells in tumors, similar to that observed in untreated tumors (data not shown). CA-4-P-treated tumors presented a 2-fold increase in total GFP⁺ bone marrow–derived cells at day 3 (P < 0.009 compared with untreated controls), which increased further by 8-fold with significant tumor infiltration at day 6 (P < 0.0003 compared with untreated controls; Fig. 5B and D). These results suggested a correlation between the CEP peaks detected in peripheral blood and the level of GFP⁺ bone marrow–derived cells observed in tumors and supported the finding that GFP⁺ bone marrow–derived cells infiltrating VDA-treated tumors could, at least in part, derive from the VE-cadherin⁺-CEP bursts.

In contrast to previously reported data (16), the number of GFP⁺ bone marrow–derived cells recruited to CD31⁺ tumor vessels did not differ between untreated tumors and CA-4-P-treated tumors removed at day 3 (Fig. 5C). Tumors treated with sunitinib or DC101 alone showed levels of GFP⁺ bone marrow–derived cells incorporated into tumor vessels that were comparable with untreated tumors (data not shown). In contrast, a substantial increase in GFP⁺ bone marrow–derived cells per tumor vessel was observed at day 6 after CA-4-P (P < 0.0004 compared with untreated controls; Fig. 5C). Because the early CEP peak could not be suppressed, it was not possible to determine whether the greater number of GFP⁺ bone marrow–derived cells detected at day 6 in CA-4-P-treated tumors resulted from the accumulation of both CEP bursts, from the intratumoral proliferation of GFP⁺ bone marrow–derived cells already present at day 3, or were exclusively derived from the second CEP burst. The finding that GFP⁺ bone marrow–derived cell numbers significantly increased in tumor vessels 3 days after the delayed CEP burst suggested, however, that these cells were recruited to injured tumor vessels and were most likely, in part, direct descendents of the second CEP burst.

To investigate this hypothesis, we asked whether the administration of antiangiogenic agents could block the recruitment of GFP⁺ bone marrow–derived cells to the tumor and tumor vasculature after CA-4-P to a similar extent as was observed with the CEP peaks. Treatment with DC101...
Figure 5. Homing and incorporation of GFP+ bone marrow-derived cells in tumors and tumor vessels parallel levels of CEPs in blood of tumor-bearing mice after CA-4-P and can be blocked by antiangiogenic treatment. A, B–D, 8- to 10-week-old C57Bl/6 mice bearing 300 mm3 LLC tumors were monitored for CEP levels at 4 and 72 hours after treatment with CA-4-P (100 mg/kg i.v.), sunitinib (SU; 40 mg/kg), DC101 (800 μg i.p.), or a combination of the drugs. Combination treatment consisted in CA-4-P administered with sunitinib (n = 5 mice per time point) or with DC101 (n = 13 mice per time point) given 24 hours before the 4-hour or the 72-hour CEP peak. Control groups received vehicle (n = 5 mice), CA-4-P alone (n = 8 mice per time point), or sunitinib or DC101 alone given 24 hours before blood sampling (n = 5 mice per time point). CEPs were measured in all groups at 4 and 72 hours after CA-4-P or at 4 and 72 hours after start of sunitinib or DC101 alone. Summary graphs of CEP levels are presented. Data are expressed as mean ± SD. Significance was set as 0.05 > P > 0.01 (*), 0.01 > P > 0.001 (**) or P < 0.001 (***) compared with baseline unless expressed otherwise. B–D, LLC cells (0.5 × 106) were injected subcutaneously to GFP+ bone marrow–transplanted C57Bl/6 mice. When tumor reached 300 to 500 mm3, mice were treated with CA-4-P, DC101, sunitinib, or combinations of the drugs at the same doses and schedule presented in A. Three days after the 4- or 72-hour CEP peak, tumors were removed (at day 3 and day 6, respectively), and cryosections were prepared for assessment of GFP–cell colonization (green), CD31+ endothelial cells (red) representing blood vessels, and colocalization of CD31+ and GFP+ cells. DAPI-staining (blue) for nuclei. Summary graphs of the quantifications of B–D GFP+ bone marrow–derived events in LLC tumors and (C) of GFP+ bone marrow–derived events per tumor vessel are presented (n = 5 mice per group). Data are expressed as mean ± SD. Significance was set as 0.05 > P > 0.01 (*), 0.01 > P > 0.001 (**) or P < 0.001 (***) compared with baseline unless expressed otherwise. D, representative immunofluorescent images of LLC tumors after treatment with CA-4-P or CA-4-P combined with sunitinib (SU) as described in B and C are presented; original magnification, ×20. Scale bar, 50 μm.
Two Distinct CEP Bursts Induced by VDA

or sunitinib 24 hours before the early CEP peak had no effect on the total number of GFP bone marrow–derived cells per tumor or tumor vessel detected at day 3 (Fig. 5B and C) whereas DC101 or sunitinib administered 24 hours before the second CEP burst markedly reduced the amount of GFP bone marrow–derived cells infiltrating tumors and incorporating tumor vessels at day 6 (Fig. 5B–D). Previous treatment with DC101 or sunitinib had no effect on the number of other circulating GFP hematopoietic cell populations in CA-4-P-treated mice (Supplementary Fig. S7). These data further suggested that the delayed CA-4-P-induced VE-cadherin−CEP burst was an important source of proangiogenic bone marrow–derived cells that were mobilized in response to tumor vascular injury and were recruited to tumor neovessels to promote tumor regrowth and recovery via angiogenesis mechanisms.

To further characterize GFP bone marrow–derived cells recruited to tumor vessels, we sought to identify the luminal incorporation of these cells into CA-4-P-treated tumor vessels. Thick cryosections of LLC tumors established in GFP bone marrow–transplanted C57BL/6 mice were harvested at day 6 after CA-4-P and were analyzed by the use of high-resolution confocal/Z-stack microscopy. Analyses of multiple Z-stacks showed the presence of GFP bone marrow–derived cells that were incorporated into the vessels of CA-4-P-treated tumors (Fig. 6) and colocalized with the CD31 endothelial marker (Fig. 6A). GFP platelets may also express CD31 and can be closely associated to CD31 blood vessels or vessel debris, especially after VDA treatment (Supplementary Fig. S8 and Supplementary Movie SM2). In some instances, the nuclei of GFP bone marrow–derived cells coexpressing CD31 could not be clearly identified within the endothelial layer (Fig. 6A). Nuclei were observed in the immediate perivascular environment of these cells. This limitation could be attributable to technical aspects (nucleus situated above or below the cryosection). The presence of such GFP CD31 signals could, however, also result from aggregated platelets which, in these cases, could prevent the quantification of CEP incorporation in blood vessels. GFP bone marrow–derived cells luminally incorporated into vessels did not coexpress the macrophage marker F4/80 or myeloid marker CD11b (Fig. 6B–D and Supplementary Movie SM1). GFP F4/80 and GFP CD11b cells consistently occupied a perivascular location.

The amount of GFP bone marrow–derived cells detected in CA-4-P-treated tumors was not correlated with the extent of necrosis and was therefore not caused by unspecific GFP monoocyte and macrophage infiltrates (Supplementary Table 1). CD41 or CD31 platelets were observed within the lumen of damaged CD31 blood vessels in close proximity to GFP platelets (Supplementary Fig. S8 and Movie SM2). GFP bone marrow–derived cells incorporated into blood vessels and identified as CEPs presented a nucleus and did not colocalize with the CD41 platelet–specific marker (Fig. 6E and F, Supplementary Movies SM3 and SM4). On the basis of the proximity of CD31 and CD41 platelet signals, it is impossible to exclude, however, that GFP CD31 signals could, in some instances, originate from platelets and lead to artifactual assessment of CEPs.

**Cancer Patients Treated with VDAs Display Acute Peaks in CEC Levels and Delayed CEP Bursts**

To evaluate whether our preclinical findings were extended to the clinical setting, we measured CEC and CEP levels in patients with advanced solid tumors enrolled at our institute in phase I clinical studies evaluating a novel VDA, ombrafulin (Fig. 7). Sequential measurements of CEC levels in patients treated with ombrafulin alone (n = 19) showed an early and significant increase in CEC levels occurring between 6 and 10 hours after drug injection (P < 0.03), which returned to baseline values at 24 hours (Fig. 7A and B) and were very similar to the CEC kinetics observed in mice after CA-4-P. We have previously reported preliminary data on the changes in CEP levels in 5 patients receiving treatment with ombrafulin combined with CDDP (37).

CEP monitoring in this ongoing study has now been extended to 9 patients, in whom CEP levels dramatically increased by 4-fold at days 2 to 4 and remained increased by 3-fold at day 7 (Fig. 7C). Finally, close monitoring of CEP kinetics was performed in 2 patients included in an ongoing dose-escalating study evaluating ombrafulin combined with bevacizumab and in whom CEP levels increased significantly in a delayed fashion between day 4 and day 8 after VDA, similar to our preclinical observations (Fig. 7D). Of note, CEP levels in these 2 patients did not peak at 4 hours after ombrafulin (low doses of 8.5 and 11.5 mg/m²), administration, in contrast to previously published data; this was not attributable to the effect of combined bevacizumab therapy, which was administered 24 hours after ombrafulin. Also, we did not observe an inhibition of the delayed CEP burst in these 2 patients receiving combined angiogenic therapy, but this may be the result of low initial doses of bevacizumab at 5 mg/kg or the fact that CEP inhibition could be effective only after several cycles of this combined therapy. These clinical findings were, nonetheless, remarkably consistent with our preclinical data and strongly support our mechanistic hypothesis favoring the existence of a late (rather than early) tumor-dependent CEP mobilization recruited specifically after VDA-induced tumor vascular injury.

**DISCUSSION**

Results from clinical trials evaluating VDAs in combination strategies have highlighted the urgent need to identify VDA-induced resistance mechanisms that lead to treatment escape (4, 17, 37). Our findings expand the current understanding of the mechanisms that underscore tumor vascular injury and revascularization, as well as subsequent host-reactive resistance responses triggered by VDA therapy. By studying the cascade of histologic and functional events that ensue VDA treatment in a highly VDA-sensitive PC3-xenograft tumor model, we show that VDA-induced vascular shutdown correlated with an acute and tumor-specific peak in CEC levels, thereby rendering these cells a potential pharmacodynamic biomarker of VDA activity.

Importantly, we describe for the first time 2 timely yet very distinct VDA-induced CEP bursts. The initial CEP peak, present as early as 2 to 4 hours after VDA administration and previously described (16), was apparently an unspecific, general, and transient chemokine–mediated response to the VDA—a finding emphasized by the fact that this initial peak was similarly detected in both tumor and non–tumor-bearing animals and occurred before the increase in CEC levels resulting from tumor vascular injury in tumor-bearing animals. Remarkably, a second delayed yet
more important burst in CEP levels was unexpectedly observed 72 to 96 hours after drug administration exclusively in tumor-bearing animals and subsequently verified in different mouse strains and tumor models, suggesting that this delayed but tumor-specific CEP mobilization was the actual VDA-induced tumor-dependent response for vascular repair and regrowth.

Functional analyses in the syngeneic C57Bl/6 mouse model transplanted with GFP+-tagged bone marrow showed a significant correlation between the late CEP burst detected in peripheral blood and tumor recruitment and infiltration of GFP+ bone marrow–derived cells, with a fraction of these cells incorporating into the lumen of tumor neovessels. Inhibition of the second VDA-induced CEP burst via the use of combined treatment with antiangiogenic agents (sunitinib or DC101) was associated with markedly reduced GFP+ bone marrow–derived cell tumor colonization and recruitment to vessels and with significantly enhanced antitumor activity and efficacy of CA-4-P treatment, thereby suggesting a distinct but relevant role of the late VE-cadherin+-CEP burst in tumor recovery after VDA treatment.

Despite the consensus on the important role of bone marrow–derived cells in solid tumors, controversy has shifted to the critical role played by CEPs and their contribution to tumor neovessels as illustrated by studies evidencing variable extent to which CEPs participate in the generation of tumor endothelium (24, 25, 28, 30). It is of note that many of these studies supporting such a role of CEPs have focused on tumor models analyzed in untreated steady states or in spontaneous conditions (27, 29, 45), which may be very different from the biologic processes triggered by anticancer therapies. Although CEP levels were indeed typically low in untreated PC3, H69, and LLC tumors, we found that these levels could increase dramatically
Two Distinct CEP Bursts Induced by VDA

Confocal microscopy experiments showed that GFP+ bone marrow–derived cells were incorporated into tumor vessels, colocalized with the CD31-endothelial marker, and did not express CD11b-macrophoid, F4/80-macrophage, or CD41-platelet markers. Platelets from the bone marrow of GFP+-bone marrow–transplanted mouse models can aggregate to damaged blood vessels, especially in the context of VDA. The presence of GFP+ platelets is an important limitation that can interfere with the assessment of CEP incorporation in tumors and tumor blood vessels, as described herein and elsewhere (16, 43). Our findings supported, however, that bone marrow–derived cells including CEPs are recruited to tumor neovessels and may therefore have a catalytic role in tumor recovery following VDA therapy.

This is, to our knowledge, the first study investigating delayed biologic and cellular effects (vasculogenic rebounds) after VDA therapy. Vascular repair processes after VDA treatment have been previously analyzed in preclinical models but within a limited time-frame almost always restricted to the first 24 hours after drug administration (16, 46, 47). We confirmed the existence of an early (within 2–4 hours) VDA-induced CEP peak in both non–tumor-bearing and tumor-bearing animals. In our hands, however, disruption of this immediate VDA-induced CEP peak using DC101 or another antiangiogenic agent (sunitinib) was not observed. The variations in CEP levels reported herein were synchronized with changes in levels of G-CSF, MMP-9, and SDF-1, 3 key proteins known to orchestrate CEP mobilization (21, 31, 43, 48, 49). Interestingly, although antiangiogenic strategies had no effect on the initial CEP peak, their administration after CA-4-P completely abrogated the late CEP burst and showed the most potent antitumor activity with concomitant disruption of GFP+ bone marrow–derived cell tumor infiltration.

One hypothesis for the differential effects observed when aiming to block these VDA-induced CEP peaks with antiangiogenic agents may be that these 2 distinct CEP bursts rely on different molecular pathways for mobilization. VDA therapy may have a direct and an indirect effect on CEP and

Figure 6 continued. D, maximum intensity projection of a full z-stack of confocal images from an LLC cryosection stained with anti-CD31 (red), anti-CD11b (blue), DAPI (white), and analyzed for GFP (green) expression. White arrow indicates an incorporated GFP+ CD31+ bone marrow–derived cell, and corresponding nucleus, which does not express CD11b. CD11b+ myeloid cells were located in the perivascular surrounding. Note that several CD11b+ myeloid cells did not express GFP and none incorporated the CD31+ blood vessel. Scale bar, 10 μm. E-F, LLC cryosections stained with anti-CD31 (red), anti-CD41 (blue), DAPI (white), and analyzed for GFP (green) expression. Maximum intensity projections of full z-stacks of confocal images are shown. CD41+ platelets were observed within the vessel lumen and were closely associated to damaged CD31+ blood vessels but did not co-stain for CD31. E, the GFP+ bone marrow–derived cells (yellow arrows) replacing the damaged endothelial wall presented a nucleus and did not express CD41 platelet-specific marker. Note that a minority of CD41+ platelet aggregates expressed GFP. Scale bar, 50 μm. F, yellow arrows show a GFP+ bone marrow–derived cell that does not express CD41 and is incorporated into a damaged CD31+ blood vessel. Scale bar, 10 μm. See Supplementary Movies SM3 and SM4.
bone marrow–derived cells: first, the VDA may trigger an instantaneous and violent, but unspecific vascular effect on bone marrow vessels resulting in a passive “efflux” of CEPs already present within the bone marrow compartment; second, the intense hypoxia resulting from the tumor vascular injury might drive a VEGFR-dependent chemokine cascade powered to stimulate CEP proliferation, differentiation, and maturation for mobilization and subsequent recruitment for tumor-specific regrowth. This proposed mechanism would explain why the early CEP peak (or efflux) could not be blocked.

Taken together, our data suggest the existence of powerful regenerative mechanisms involving a complex chemokine cascade via G-CSF (46), MMP-9 (48, 49) and bone marrow SDF-1 after VDA therapy, instigated to restore tumor endothelium integrity and culminating into a relevant burst of mobilized CEPs 4 days after VDA-induced vascular injury. Importantly, combined antiangiogenic strategies scheduled after VDA and associated with complete blunting of the late VE-cadherin– CEP mobilization resulted in enhanced antitumor activity of the VDA-based strategy.

Several studies support the emerging concept that bone marrow–derived cells recruited to sites of tumor neoangiogenesis are composed of various proangiogenic subsets that all help to promote tumor growth and neovascularization, even when
present in small numbers (50). These different bone marrow–derived cell subsets include not only CEPs but also myeloid progenitors, especially Gr1+CD11b+ (51), and tumor-associated macrophages (52), including a particular subset expressing Tie2+ (TEM), which contribute indirectly to neoangiogenesis by incorporating perivascularly or by delivering cytokines essential to angiogenesis (29). Recently, Welford and colleagues (47) examined the role played by TEMs in the early stages after CA-4-P treatment. The authors reported that TEMs were detected within 4 to 24 hours after drug injection and were critical for perivascular support during tumor recovery.

Our results do not exclude the potential contribution of other bone marrow–derived cells to tumor revascularization and recovery after CA-4-P treatment. In fact, a complementary role between various bone marrow–derived subsets that indirectly respond to VEGFR-2-mediated processes is most probable. We observed that antiangiogenic agents, DC101 and sunitinib, blocked the late burst of CA-4-P-induced CEPs in peripheral blood and were associated with a significant reduction of GFP+ bone marrow–derived cell infiltrates in tumors and tumor vessels. The precise mechanism by which DC101 treatment—which is supposed to specifically target the VEGFR2+CEP population—broadly inhibited the recruitment of GFP+ bone marrow–derived cells (other than CEPs) to tumors remains unclear, although this phenomenon has been previously observed after VDA treatment (16) and chemotherapy (45).

Treatment with DC101 and sunitinib after CA-4-P did not affect the levels of other circulating GFP+ hematopoietic cell populations. Therefore, these antiangiogenic drugs may exert a broader effect on bone marrow–derived cells and may block their intratumoral recruitment, possibly through the inhibition of local VEGFR-2-angiogenic pathways. The improved tumor control observed with combined antiangiogenic agents most likely involves additional mechanisms including the inhibition of endothelial cell proliferation from pre-existing blood vessels, which might also be more sensitive to antiangiogenic drugs after the administration of VDA. Our data showed, nevertheless, that combined antiangiogenic agents scheduled after VDA therapy and associated with blockade of the delayed CEP burst could critically impact the systemic recruitment of bone marrow–derived cells (including CEPs) to tumors to an extent that was sufficient to achieve significant anticaner efficacy with VDA therapy.

Our preclinical findings were extended by data obtained from cancer patients enrolled in dose-escalating clinical trials with a VDA, ombrabulin, where CEC peaks were observed in patients 4 hours after drug injection. Importantly, we also observed delayed and dramatic bursts in CEP levels in patients monitored over time after VDA administration. The delayed CEP bursts we report in cancer patients here and elsewhere (37) is strongly supported by other clinical studies documenting the kinetics of CEP mobilization in response to vascular injury (53, 54). Research evaluating CECs or CEPs has focused on their role during antiangiogenic treatment and despite the need to identify biomarkers of drug activity and resistance mechanisms, studies validating these cells as surrogate biomarkers in cancer patients are few (13, 23, 37, 55). Although VDAs are a particular model of vascular injury and repair, we observed in our study that the acute shedding of CECs directly reflected the vascular lesion and could be a pharmacodynamic biomarker of VDA activity, as shown by the exclusive peak in VDA-treated tumor-bearing animals and by CEC kinetics in cancer patients. Importantly, the late CEP burst could be considered as a remarkable host response and biomarker of resistance to VDA treatments.

The results presented here provide complementary insights to the intrinsic host responses and vasculogenic rebounds instigated after VDA treatment and call for a reconsideration of the current paradigm offered by initial studies supporting the role of very early (within hours) CEP responses after VDA therapy. Our data strongly suggest that the delayed VDA-induced tumor-specific burst in VE-cadherin+CEP levels is a crucial mediator that needs to be targeted for enhanced anticaner efficacy with VDAs. Because the future of VDA-based treatments in clinical oncology may rely exclusively on combination strategies, optimal scheduling is critical. Our findings provide a rationale for improving the sequence of administration of combined antiangiogenic agents in order to target the second CEP burst, which may prove to significantly enhance the clinical efficacy of VDA treatment.

METHODS

Blood Samples Obtained from Cancer Patients

Blood samples were collected from patients with advanced solid tumors who had given consent to participate in phase 1 studies evaluating escalating doses of a novel VDA, ombrabulin (Sanofi-Aventis), at the Service des Innovations Thérapeutiques Preocces (SITEP), Institut Gustave Roussy, France. Patients were enrolled in 1 of 3 trials evaluating ombrabulin alone (n = 19 patients) or combined with CDDP (n = 9 patients) or with bevacizumab (n = 2 patients). These studies were approved by our institutional ethical review board, who also granted approval for CEC and CEP analyses. Written informed consent was obtained from all patients. Blood samples were collected from patients at baseline and at indicated times after ombrabulin treatment. CECs and CEPs were quantified using 4-color flow cytometry according to previously published methods (37, 40, 41).

Tumor and Animal Models

PC3-xenografts established in male Foxn1-immunodeficient athymic Swiss nude nu/nu mice (Gustave Roussy Institute breeding) and LLC tumors established in immunocompetent C57BL/6 mice or C57BL/6 mice previously transplanted with GFP+tagged bone marrow cells were done according to procedures described in the Supplementary Methods.

Drug Administration and Scheduling

Treatment was given as CA-4-P (100 mg/kg via tail injection), SU11248/sunitinib malate (40 mg/kg orally), or DC101 (800 μg/mouse administered intraperitoneally) administered alone or in combination according to schedules described in the Supplementary Methods.

Evaluation of CEC and CEP Levels by Flow Cytometry

Viable CEC and CEP levels were measured each in 250 μL of whole blood using flow cytometry. In summary, immunofluorescent staining for CEC measurement was performed with the following rat anti-mouse monoclonal antibodies: MEGA-32 biotin, CD31-FTTC, CD45-PerCP, Flk-1-PE, and streptavidin-APC (all from BD Pharmingen). For CEP measurement, the following rat anti-mouse monoclonal antibodies were used: CD45-PerCP, CD117-APC, Sca-1-FTTC, and Flk-1-PE (all from BD Pharmingen; refs. 16, 23, 43). The detailed staining procedures, acquisition, and analyses of CECs and CEPs as well as complementary characterization of CEPs are described in the Supplementary Methods and Supplementary Figures S1 to S4.
Determination of Proangiogenic Factor Levels in Serum and Bone Marrow Samples

Murine serum levels of VEGF, MMP-9, SDF-1, G-CSF, and levels of bone marrow SDF-1 were determined using commercially available mouse ELISA kits (R&D Systems) as described in the Supplementary Methods.

Tissue Processing

Tumors were fixed and paraffin-embedded for analysis of tumor necrosis, microvessel density, and hypoxia or immediately frozen (LLC tumors) for GFP visualization using confocal microscopy as described in the Supplementary Methods.

Image Acquisition and Analysis

The measurement of necrosis, microvessel density (CD31-stained endothelial cells), and CAIX-stained hypoxic tumor cell surfaces from digitized whole histological tumor sections was done as described in the Supplementary Methods. A protocol to measure GFP bone marrow–derived cell numbers in LLC tumors and tumor blood vessels using a Leica SPE confocal microscope was established as detailed in the Supplementary Methods. To summarize, full Z-stacks of consecutive optical sections (0.8–1 μm step size) of thick (40–50 μm thickness) tumor cryosections were captured at a magnification of ×20 or ×63 using the Leica confocal software. Maximum intensity projections of full Z-stacks of confocal images were done using ImageJ software and are presented.

Statistical Analysis

Data are expressed as mean ± SD. The Wilcoxon–Mann–Whitney test was used to compare the distribution of marker levels between tumor and control groups or to compare levels at baseline and at a specific time. All tests were 2-sided and P values below 0.05 were considered to denote statistical significance. Data were analyzed by the use of SAS software (V 9.1, SAS institute). Significance was set as 0.05 > P > 0.01 (*), 0.01 ≥ P > 0.001 (**), or P ≤ 0.001 (***).

Disclosure of Potential Conflicts of Interest

J-C. Soria is a consultant for Sanofi-Aventis. No potential conflicts of interest were disclosed by the other authors.

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