

## BIBF 1120 (Nintedanib), a Triple Angiokinase Inhibitor, Induces Hypoxia but not EMT and Blocks Progression of Preclinical Models of Lung and Pancreatic Cancer

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### Abstract

Signaling from other angiokinasases may underlie resistance to VEGF-directed therapy. We evaluated the antitumor and biologic effects of BIBF 1120 (nintedanib), a tyrosine kinase inhibitor that targets VEGF receptor, platelet-derived growth factor receptor, and fibroblast growth factor receptor in preclinical models of lung and pancreatic cancer, including models resistant to VEGF-targeted treatments. *In vitro*, BIBF 1120 did not show antiproliferative effects, nor did it sensitize tumor cells to chemotherapy. However, *in vivo* BIBF 1120 inhibited primary tumor growth in all models as a single agent and in combination with standard chemotherapy. Analysis of tumor tissue posttreatment revealed that BIBF 1120 reduced proliferation (phospho-histone 3) and elevated apoptosis (cleaved caspase-3) to a greater extent than chemotherapy alone. Furthermore, BIBF 1120 showed potent antiangiogenic effects, including decreases in microvessel density (CD31), pericyte coverage (NG2), vessel permeability, and perfusion, while increasing hypoxia. Despite the induction of hypoxia, markers of epithelial-to-mesenchymal transition (EMT) were not elevated in BIBF 1120-treated tumors. In summary, BIBF 1120 showed potent antitumor and antiangiogenic activity in preclinical models of lung and pancreatic cancer where it induced hypoxia but not EMT. The absence of EMT induction, which has been implicated in resistance to antiangiogenic therapies, is noteworthy. Together, these results warrant further clinical studies of BIBF 1120. *Mol Cancer Ther*; 12(6); 992–1001. ©2013 AACR.

### Introduction

Despite initial promise and sound biologic rationale, antiangiogenic therapies targeting VEGF have shown only modest clinical effect and are prone to resistance in many disease contexts (1). In advanced non-small cell lung carcinoma (NSCLC), the anti-VEGF monoclonal antibody bevacizumab increases overall survival from approximately 10 to 12 months when added to carboplatin-paclitaxel chemotherapy (2) but conveys no survival benefit when added to cisplatin-gemcitabine chemotherapy (3). The addition of sorafenib, a VEGF receptor (VEGFR) tyrosine kinase inhibitor (TKI), to standard che-

motherapy does not improve clinical outcomes and is associated with inferior outcomes in tumors with squamous histology (4). In advanced pancreatic cancer, the addition of bevacizumab to gemcitabine does not improve overall survival (5).

In tumors such as lung and pancreatic cancers, proliferation and growth signaling through alternate angiogenic pathways, such as platelet-derived growth factor (PDGF; refs. 6, 7) and fibroblast growth factor (FGF; ref. 8), seems to participate in tumor escape from anti-VEGF therapy. The PDGF-PDGFR axis affects angiogenesis, fibroblast activation, and tumor interstitial pressure (9). In NSCLC, expression of FGF and PDGF are associated with poor prognosis (10). Similar associations have been observed in pancreatic cancer (11).

On the basis of these observations, it follows that targeting multiple angiokinasases would be a logical therapeutic approach (6, 12). However, most available drugs feature an imbalanced pharmacodynamic profile that may result in toxicities precluding optimal inhibition of key pathways (13), particularly the FGF-FGFR axis. Sorafenib, sunitinib, pazopanib, and cediranib have IC<sub>50</sub>s for FGFR that range 7 to 25 times greater than IC<sub>50</sub>s for VEGFR, 2 to 40 times greater than IC<sub>50</sub>s for PDGFR, and 2 to 300 times greater than IC<sub>50</sub>s for cKIT (inhibition of which can result in myelosuppression). Indeed, the FGFR IC<sub>50</sub>s achieved with sorafenib (580 nmol/L), sunitinib (2,900 nmol/L),

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and vandetanib (3,600 nmol/L) are likely greater than clinically sustainable drug concentrations. In contrast, the pharmacodynamic profile of the receptor TKI BIBF 1120 (nintedanib; Boehringer-Ingelheim) provides balanced inhibition of relevant therapeutic targets: VEGFRs 1, 2, and 3 (IC<sub>50</sub> 13–34 nmol/L); FGFRs 1, 2, and 3 (IC<sub>50</sub> 37–108 nmol/L); and PDGFR  $\alpha$  and  $\beta$  (IC<sub>50</sub> 59–65 nmol/L). Additional targets of BIBF 1120 include FLT-3 and members of the Src-family (Src, Lyn, and Lck), but there is no meaningful inhibition of cKIT (14).

Although BIBF 1120 has shown negligible *in vitro* anti-tumor activity, in animal models single-agent BIBF 1120 decreases growth of head and neck, kidney, ovarian, lung, colorectal, prostate, and liver cancer xenografts (14, 15), suggesting that *in vivo* efficacy is due to antistromal effects. To evaluate this hypothesis, we studied the effects of BIBF 1120 on tumor growth, metastatic potential, and stromal and vascular parameters in lung cancer and pancreatic cancer models, including selected tumors resistant to anti-VEGF therapies.

## Materials and Methods

### Cell lines

Human pancreatic cancer lines AsPC-1, HPAF-II, and MIA PaCa-2 and the lung cancer line A549 were obtained from the American Type Culture Collection (ATCC). The pancreatic cancer line Colo357 was a gift from Dr. Jason Fleming (Department of Surgical Oncology, MD Anderson Cancer Center, Houston, TX). Lung cancer lines Calu-3, Calu-6, H1703, and H1993 were kindly provided by Dr. John Minna (University of Texas Southwestern, Dallas, TX). All cell lines were grown in a humidified atmosphere with 5% CO<sub>2</sub>, at 37°C, DNA fingerprinted for provenance using the PowerPlex 1.2 kit (Promega), and confirmed to be the same as the DNA fingerprint library maintained by ATCC and the Minna/Gazdar laboratory. In addition, they were confirmed to be free of *Mycoplasma* by e-Myco kit (Boca Scientific) before injection into mice.

### *In vitro* cytotoxicity and drug response assay

Cell proliferation assays were conducted in 96-well format as described previously (16). For gemcitabine (Eli Lilly and Company), gemcitabine–BIBF 1120, or gemcitabine–cisplatin–BIBF 1120 the highest dose of gemcitabine administered was 2,000 nmol/L. For cisplatin (APP Pharmaceuticals; codiluted with gemcitabine) or gemcitabine–cisplatin–BIBF 1120 (Fig. 1A) the highest dose of cisplatin administered was 140 nmol/L. For BIBF 1120 alone, the highest dose was 25.6  $\mu$ mol/L. For combination studies, a fixed concentration of BIBF 1120 (225 nmol/L) was added to serial dilutions of gemcitabine or gemcitabine plus cisplatin. Relative cell number was calculated on day 5 by adding the MTS reagent (Promega; final concentration: 333  $\mu$ g/mL), incubating for 1 to 3 hours at 37°C, and reading absorbance in a 490-nm plate reader (Spectra Max 190; Molecular Devices). Drug sensitivity curves and IC<sub>50</sub> values were calculated using in-house software.

### Animal studies

All animals were housed in a pathogen-free facility with continuous access to food and water. Experiments were approved by and carried out in accordance with the Institutional Animal Care and Use Committee at the University of Texas Southwestern. Mice were purchased from the core breeding facility at University of Texas Southwestern. Six- to 8-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were injected with  $2.5 \times 10^6$  lung (A459, Calu-6, and H1993) or  $1 \times 10^6$  pancreatic (HPAF-II, MIA PaCa-2, and AsPC-1) cancer cells. Lung cancer cells were injected subcutaneously. Pancreatic cancer cells were injected orthotopically, as described previously (17). Subcutaneous lung tumor volumes were followed by twice weekly measurements with Vernier calipers. Pancreas tumors were followed by palpation and, if necessary, by ultrasound. Animals were randomized and treatment was initiated as indicated.

BIBF 1120 was suspended in 0.5% hydroxy-ethylcellulose (HEC) as described previously (14) and administered at a dose of 50 mg/kg 5 days a week via oral gavage. In lung cancer models, gemcitabine was administered twice weekly at a dose of 25 mg/kg [intraperitoneally (i.p.)] and cisplatin was administered once weekly at a dose of 1 mg/kg (i.p.). For the pancreas model, gemcitabine was administered at a dose of 12.5 mg/kg (i.p.) 3 times per week. Animals were sacrificed when the average volume of control-treated tumors reached 1,500 mm<sup>3</sup> or when animals became moribund.

### Perfusion and hypoxia studies

**Perfusion studies with labeled dextrans.** Three mice per group were injected intravenously with a 1:1 mixture of fluorescein isothiocyanate (FITC)–conjugated dextran (25 mg/mL,  $2 \times 10^6$  kDa; Molecular Probes/Invitrogen) and rhodamine B–conjugated dextran (12.5 mg/mL,  $1 \times 10^4$  kDa; Molecular Probes/Invitrogen) in 0.9% saline in a volume of 200  $\mu$ L. The probes were allowed to circulate for 10 minutes. Afterward, animals were sacrificed, tissues were removed, snap-frozen, embedded in OCT, and 8- $\mu$ m sections were cut and evaluated as described previously (18).

**Hypoxia studies with pimonidazole.** Three mice per group were injected intravenously with 60 mg/kg of pimonidazole (30 mg/mL in 0.9% saline, Hypoxyprobe Plus; HPI Inc.) that was allowed to circulate for 90 minutes before sacrificing animals. Frozen tissue sections were interrogated with FITC-conjugated anti-pimonidazole primary antibody (Chemicon) and endothelial cell markers (CD31, Dianova; Meca-32, DSHB; or endomucin, Santa Cruz Biotechnology) as described previously (18). Eight images per tissue area were obtained and analyzed using NIS Elements.

**Drug delivery studies with doxorubicin.** Three mice per group from the acute and chronic AsPC-1 endpoint study were injected intravenously with 20 mg/kg doxorubicin (Johnson & Johnson Pharmaceuticals).

Doxorubicin was allowed to circulate for 5 minutes before sacrificing animals. Frozen tissue sections were stained with endothelial cell markers and visualized under fluorescent microscopy and analyzed as above.

### Histology

Tissues were fixed in 4% formalin, embedded in paraffin, sectioned, and stained with routine hematoxylin and eosin (H&E) or used for immunohistochemistry. After routine deparaffinization, tissue sections were incubated in primary antibody overnight at 4°C. Primary antibodies were used at 5 to 10 µg/mL (see Supplementary Table S1 for complete list of antibodies). Detection with appropriate secondary antibodies and imaging was done as described previously (18).

### Statistical analysis

Quantification of immunohistochemistry was conducted using NIS Elements 3.2 software (Nikon Instruments). All data were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc.). Datasets were analyzed by Student *t* test or ANOVA followed by Dunn posttest and results were considered as significant at  $P < 0.05$ . Results are shown as mean  $\pm$  SEM. Notation on graphs is as following: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; and \*\*\*\*,  $P < 0.0001$ .

### Results

#### **BIBF 1120 does not have antiproliferative effects and does not sensitize tumors cells to chemotherapy *in vitro***

We conducted cellular proliferation assays in selected lung and pancreatic cancer cell lines using BIBF 1120 as a single agent and in combination with standard of care chemotherapy. As monotherapy, BIBF 1120 did not show cytotoxic effects nor did it sensitize the majority of cell lines to chemotherapy (Supplementary Table S2). Single-agent BIBF 1120 had  $IC_{50}$  values more than 20 µmol/L, which are above the pharmacologically achievable concentration in mammals (200–450 nmol/L; ref. 14). In combination studies, a fixed concentration (225 nmol/L) of BIBF 1120 did not alter the cytotoxicity of chemotherapy in A549, Calu-3, Calu-6, H1993, AsPC-1, Colo357, or MIA PaCa-2 cells. However, BIBF 1120 induced a significant shift in the  $IC_{50}$  of chemotherapy in H1703 and HPAF-II cells (Supplementary Table S2).

#### **BIBF 1120 inhibits growth of subcutaneous lung xenografts**

The *in vivo* efficacy of BIBF 1120, as a single agent and in combination with chemotherapy, was evaluated in SCID mice bearing subcutaneous human lung xenografts (A549, Calu-6, and H1993). Cell lines were selected on the basis of prior knowledge of sensitivity to chemotherapy and antiangiogenic therapy. For example, we have found A549 to be poorly responsive to bevacizumab (19) and resistant to gemcitabine and cisplatin *in vivo* (20).

Calu-6 also responds poorly to bevacizumab but is sensitive to chemotherapy. H1993 is sensitive to bevacizumab and chemotherapy (20).

We initiated single-agent therapy studies with A549 xenografts when primary tumors were less than 200 mm<sup>3</sup> or larger than 250 mm<sup>3</sup>. BIBF 1120 effectively reduced primary tumor size in each setting (Supplementary Fig. S1A). To extend these observations, we pursued combination therapy studies in A549, H1993, and Calu-6 xenografts. Therapy with vehicle (control), BIBF 1120, chemotherapy, or BIBF 1120 plus chemotherapy commenced when tumors were established. A decrease in tumor growth rate was observed across all models, particularly in the combination groups, where the growth curve gradually became linear (Fig. 1B).

End tumor volumes and weights were lower in BIBF 1120 and the combination groups compared with controls, across all models ( $P < 0.0001$ , ANOVA; Fig. 1B). In A549 and H1993 xenografts, combination was more effective than single-agent therapy ( $P < 0.001$  and  $P < 0.01$  respectively, Dunn posttest, Fig. 1C); however, in Calu-6 xenografts combination therapy was not different from BIBF 1120 single-agent therapy.

We next investigated whether the reduction in primary tumor size could be attributed to changes in tumor cell viability *in vivo*. To assess apoptosis and proliferation in these models, tumors were evaluated immunohistochemically for cleaved caspase-3 and phospho-histone H3. This analysis showed a decrease in proliferation (phospho-histone H3) and an increase in apoptosis (cleaved caspase-3) in 2 lung cancer models (Supplementary Fig. S2A–S2D, data not shown for Calu-6).

#### **BIBF 1120 inhibits growth of orthotopic pancreatic xenografts**

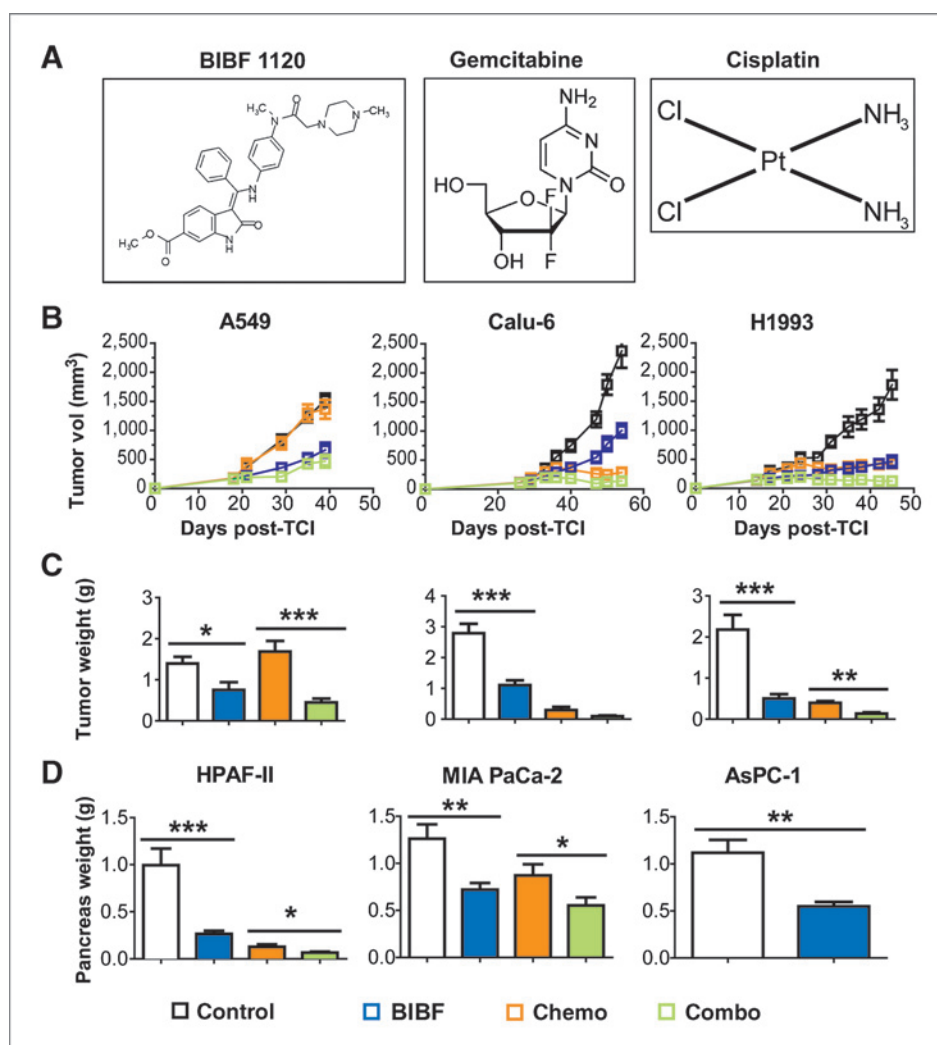
Pancreatic cancer cells (HPAF-II, MIA PaCa-2, and AsPC-1) were injected orthotopically into SCID mice. The efficacy of BIBF 1120 as a single agent or in combination with chemotherapy was assessed. *In vitro* the cell lines selected showed differential response to gemcitabine but were not affected by BIBF 1120 (Supplementary Table S2). However, *in vivo* BIBF 1120 significantly reduced tumor size as a single agent in each model and enhanced the activity of gemcitabine in HPAF-II and MIA PaCa-2 xenografts (Fig. 1D).

As observed in A549 xenografts, BIBF 1120 alone or in combination with chemotherapy induced a striking increase in apoptosis (cleaved caspase-3) and decrease in proliferation (phosphorylated histone H3) in Mia-PaCa-2 (Supplementary Fig. S2E) and HPAF-II xenografts (data not shown). Gemcitabine treatment alone had little effect on either measure.

#### **BIBF 1120 decreases metastatic burden in pancreatic cancer models**

The orthotopic pancreatic cancer models also provided means to assess the effect of BIBF 1120 on metastatic burden. Gross metastatic events counted at sacrifice

**Figure 1.** BIBF 1120 inhibits primary tumor growth in subcutaneous lung and orthotopic pancreatic cancer xenografts. **A**, Chemical structures of BIBF 1120 (left panel) and chemotherapeutics gemcitabine and cisplatin (middle and right panels). **B**, The indicated NSCLC cells were injected subcutaneously into mice. Treatment with vehicle (control), BIBF 1120 (BIBF, 50 mg/kg, daily), Chemo [gemcitabine (25 mg/kg 2×/wk) + cisplatin (1 mg/kg 1×/wk)] was initiated when average tumor volume reached 100 to 150 mm<sup>3</sup> (*n* = 10/group). Tumor growth curves displaying mean tumor volume ± SEM and mean final tumor weights ± SEM (**C**) are shown. **D**, The indicated pancreatic cancer cells were injected orthotopically into SCID mice. Treatment was initiated 7 to 10 days after tumor cell injection (TCI). Animals were treated as above with the exception of chemotherapy; gemcitabine was given at 12.5 mg/kg 3×/wk. Mean final pancreas weight ± SEM is shown (*n* = 10/group). \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001 by Dunn posttest.



(which included liver, spleen, peritoneum, lymph node, diaphragmatic, and gastrointestinal metastatic foci) were decreased in BIBF 1120-treated animals (Fig. 2). In the chemotherapy combination studies (HPAF-II, MIA PaCa-2), gross metastatic burden was decreased significantly in the BIBF 1120-treated groups, compared with control. In the HPAF-II model, there were an average of 7 metastatic events in the control animals compared with 0, 1, and 0 in the BIBF 1120, gemcitabine, and combination groups, respectively (*P* < 0.001, ANOVA; Fig. 2A). In the MIA PaCa-2 model, there were an average of 7 metastatic events in the control animals compared with 2, 4, and 2 in the BIBF 1120, gemcitabine, and combination groups, respectively (*P* < 0.0001, ANOVA). There was an additional decrease in metastatic events in the combination group in the MIA PaCa-2 study, compared with gemcitabine only (*P* < 0.0001, Dunn posttest; Fig. 2A).

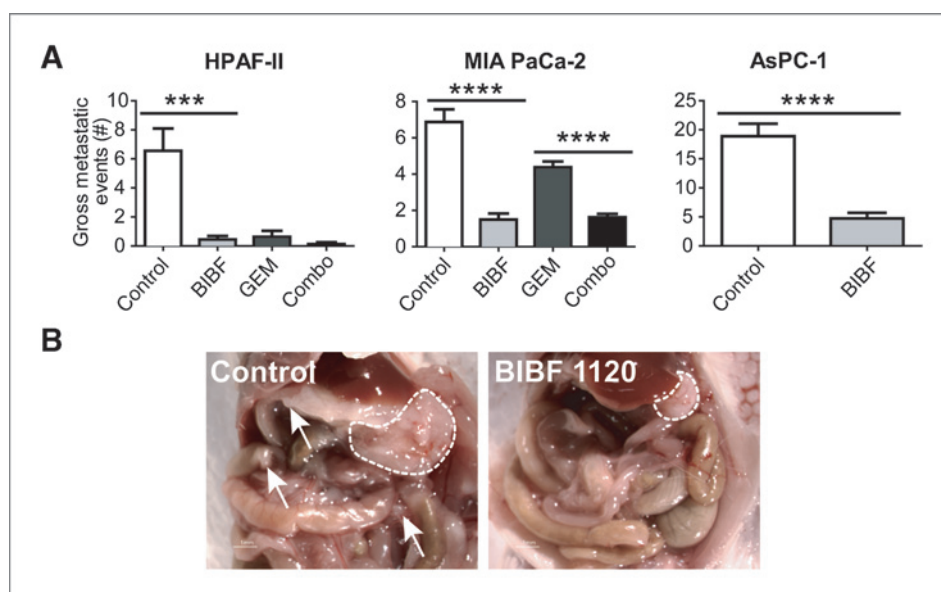
In the AsPC-1 study, animals treated chronically with single-agent BIBF 1120 also had a significantly reduced number of gross metastases compared with the control group (*P* < 0.0001, Student *t* test; Fig. 2A and B).

### BIBF 1120 is a potent antiangiogenic agent

BIBF 1120 inhibits the activity of multiple angiokines (14); therefore, tumor sections from lung and pancreatic xenografts were assessed for microvessel density (MVD) and vascular function. MVD was determined using CD31 (PECAM-1). Sections were also stained for Meca-32 (a pan-endothelial cell marker) and endomucin. Because these results closely resembled the CD31 data, the Meca-32 and endomucin data are not shown.

Tumor sections were also assessed for pericyte coverage, which has been implicated in resistance to anti-VEGF therapy (21, 22). Pericyte coverage was determined by colocalizing the pericyte marker NG2 with CD31. Similar results were confirmed with the colocalization of the pericyte marker [ $\alpha$ -smooth muscle actin (SMA)] with endomucin (data not shown). In lung xenografts, there was a substantial decrease in MVD and pericyte coverage (Fig. 3A, D, and E) in BIBF 1120-treated animals. Chemotherapy alone had little effect on MVD or pericyte coverage.

We hypothesized that this decrease in MVD would negatively affect vascular perfusion, resulting in hypoxia



**Figure 2.** BIBF 1120 decreases gross metastatic burden in orthotopic pancreatic xenografts. **A**, at sacrifice, the number of gross metastatic events was recorded. Gross metastatic burden was decreased significantly in BIBF 1120-treated animals compared with control groups in each model. **B**, representative image of a HPAF-II tumor presentation at the time of sacrifice. Dashed lines, primary tumor borders; arrows, metastatic foci; Gem, gemcitabine. Bargraphs indicate mean + SEM. \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  by Student *t* test/Dunn posttest.

and decreased blood flow into tumor tissue. We therefore conducted functional staining with labeled dextrans, pimonidazole, and doxorubicin to assess these parameters. Permeability and perfusion studies conducted in A549-bearing SCID mice with labeled dextrans showed a decrease in permeability of high molecular weight (FITC) dextran and perfusion of low molecular weight (rhodamine) dextran (Fig. 3B and F).

Pimonidazole is a 2-nitroimidazole that is reductively activated in hypoxic cells and forms stable adducts with thiol groups in proteins, peptides, and amino acids. Pimonidazole in these adducts was detected by immunohistochemistry (18, 23). Pimonidazole staining in H1993 (Fig. 3C and F) showed a significant increase in hypoxic areas after BIBF 1120 treatment concurrent with the decrease in MVD.

The MVD findings were similar in the pancreatic cancer xenografts. In MIA PaCa-2 xenografts, MVD was significantly decreased in BIBF 1120-treated groups compared with the control or chemotherapy groups ( $P < 0.0001$ ; Fig. 4A and D). These findings were also observed in the HPAF-II model (Fig. 4D). Because of the strong sensitivity of HPAF-II to gemcitabine tissue from gemcitabine-treated mice could not be analyzed. MVD analysis in AsPC-1 xenografts showed that BIBF 1120 reduces MVD within 5 days of treatment initiation ( $P < 0.001$ ; Fig. 4D) but was more pronounced after chronic treatment ( $P < 0.001$ ; Fig. 4D). Pericyte coverage in MIA PaCa-2 xenografts was similarly decreased by BIBF 1120 therapy (Fig. 4A and E). In addition, in MIA PaCa-2 xenografts BIBF 1120 alone or in combination with gemcitabine dramatically elevated hypoxia (Fig. 4B and F).

To investigate the effect of BIBF 1120 on drug delivery, AsPC-1-bearing mice treated acutely (5 days) or chronically with BIBF 1120 were perfused intravenously with the naturally fluorescing chemotherapeutic agent doxorubicin (Fig. 4C and G; ref. 24). In BIBF 1120-treated mice, the

perfusion of tumor tissue with doxorubicin was decreased significantly compared with the control group in the acute ( $P < 0.01$ ) and chronic ( $P < 0.05$ ) treatment groups, with a more pronounced effect after chronic treatment (Fig. 4G).

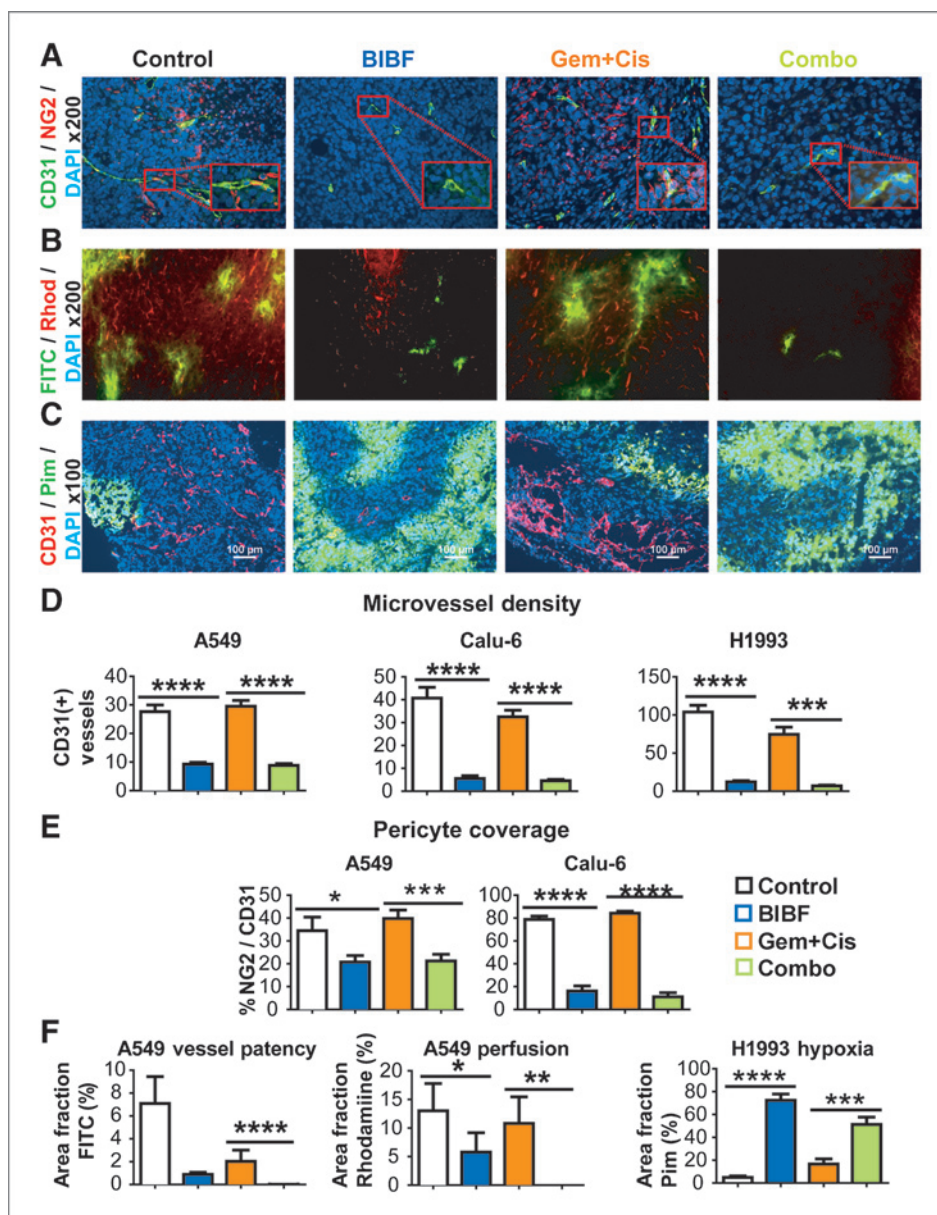
#### BIBF 1120 does not promote an invasive phenotype

It has been reported that antiangiogenic therapy may promote a more invasive phenotype (25–27). The mechanism underlying this phenotypic change is not fully elucidated but is thought to involve hypoxia-induced epithelial-to-mesenchymal transition (EMT; refs. 28–31). To investigate whether the BIBF 1120-mediated hypoxia promoted a more invasive phenotype in our models, tissues from the lung and pancreatic cancer models were stained with canonical markers of EMT. A549 xenografts from each treatment group were evaluated for the expression of E-cadherin and vimentin. The level of E-cadherin, a marker of epithelial cells, was not affected by chemotherapy but was elevated by BIBF 1120 (Fig. 5A and C). Although the level of vimentin, a mesenchymal marker, was unchanged compared with control in animals receiving single-agent BIBF 1120 therapy but was decreased by combination therapy in A549 xenografts (Fig. 5B and C). We also evaluated fibroblast recruitment, which is a component of EMT and invasion. Mature myofibroblasts were determined by  $\alpha$ -SMA (Fig. 5B) and S100A4 staining (data not shown).  $\alpha$ -SMA levels were significantly decreased in BIBF 1120-treated animals bearing A549 xenografts (Fig. 5C; data not shown for Calu-6 and H1993).

We also investigated EMT in MIA PaCa-2 xenografts. We found that the expression of zeb1, a transcription factor that can induce EMT (32), did not differ across the 4 treatment groups nor did levels of vimentin or E-cadherin (Fig. 6A, C, and D). In addition,  $\beta$ -catenin, a marker that shows membranous staining in epithelial cells and is transported to the nucleus in mesenchymal cells (33) did not differ significantly across groups (data not shown) in

**Figure 3.** BIBF 1120 shows potent antiangiogenic effects, decreases perfusion, and induces hypoxia in lung cancer xenografts.

Vascular parameters of NSCLC tumors were evaluated by immunohistochemistry and perfusion studies. **A**, representative images of MVD (CD31) and pericyte coverage (NG2) in Calu-6 xenografts at  $\times 200$  magnification (inset,  $\times 400$ ), with 4',6-diamidino-2-phenylindole (DAPI)-labeling nuclei. **B**, representative images of perfusion of high-molecular weight (FITC; MW, 2 million daltons) and low-molecular weight [rhodamine (Rhod); MW, 10,000 daltons] dextrans in A549 xenografts. **C**, representative images of hypoxia as assessed by pimonidazole staining in H1975 xenografts. Scale bar, 100  $\mu\text{m}$ . Quantification of MVD (**D**), pericyte coverage (**E**), and dextran perfusion data and hypoxia (**F**). Vessel patency reflects signal from high-molecular weight dextran and perfusion reflects signal from low-molecular weight dextran. Bar graphs indicate mean  $\pm$  SEM. A minimum of 5 images were acquired per group. Results were given as mean percentage of threshold area or absolute vessel counts per  $\times 200$  field. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  by Dunn posttest. Gem, gemcitabine; Cis, cisplatin.



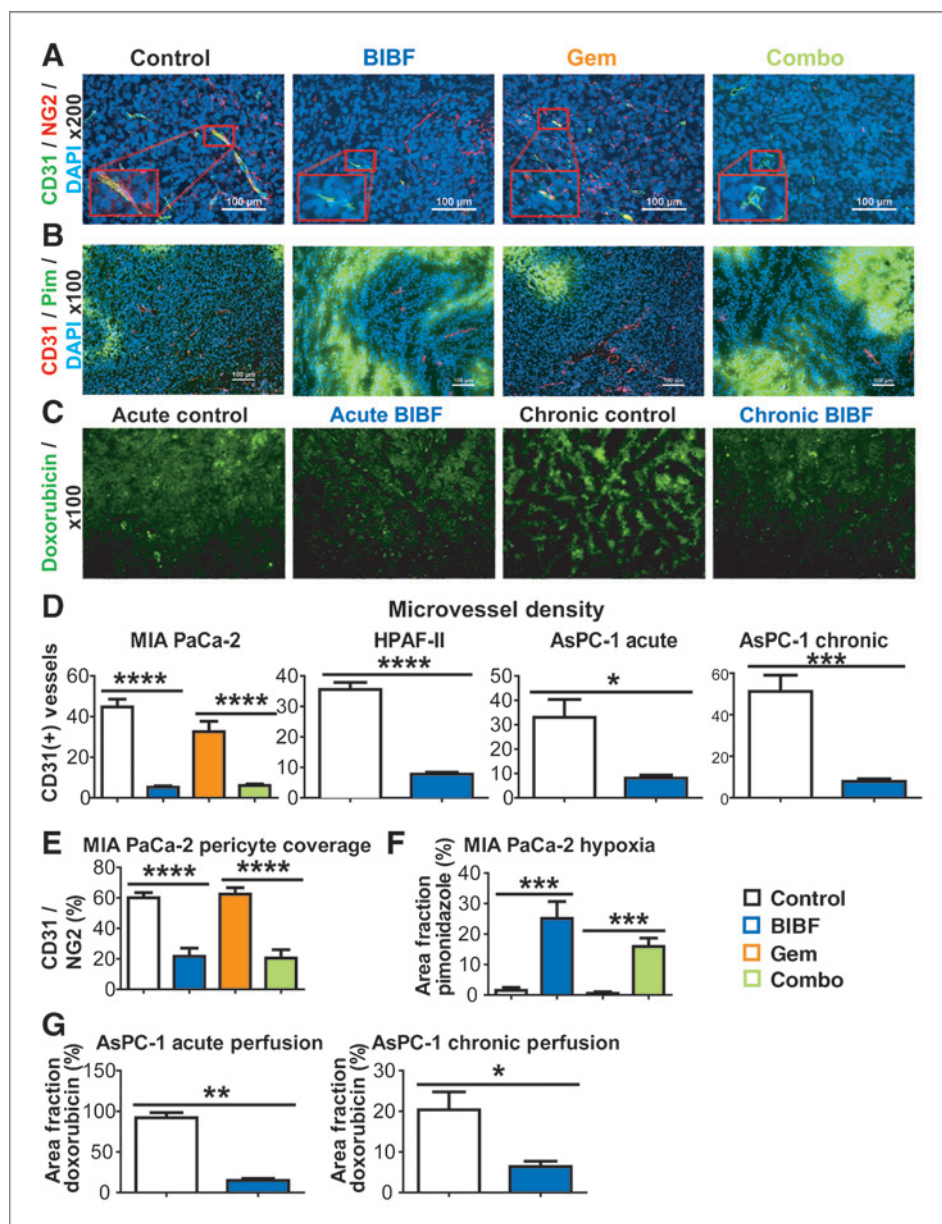
MIA PaCa-2 xenografts. Furthermore, BIBF 1120 reduced the expression level of  $\alpha$ -SMA in MIA PaCA-2 xenografts similar to the results in A549 tumors (Fig. 6B and D).

## Discussion

Antiangiogenic therapies—including anti-VEGF monoclonal antibodies and VEGFR TKIs—are currently U.S. Food and Drug Administration (FDA) approved for lung, colorectal, kidney, thyroid, and brain cancer, as well as sarcomas. However, in many instances, use of these drugs has been fraught with toxicities, lack of predictive biomarkers, resistance, and only modest clinical benefit. The addition of bevacizumab to chemotherapy does not improve overall survival in advanced pancreatic cancer (5, 34, 35). In advanced nonsquamous NSCLC, bevacizu-

mal contributes a modest survival benefit when combined with carboplatin-paclitaxel, but not when combined with such other regimens as cisplatin-gemcitabine or erlotinib (2, 3, 36, 37). Numerous phase III trials have shown that adding VEGFR TKIs (sunitinib, sorafenib, vandetanib, and cediranib) to chemotherapy does not extend survival (4, 38, 39), despite promising results in preclinical, phase I, and phase II studies.

These disappointing results may be due to intrinsic or evasive resistance. Evasive resistance may arise from phenotypic changes due to EMT driven by hypoxia, a consequence of effective antiangiogenic therapy. In the present study, we show that BIBF 1120, a triple angiokinase inhibitor of VEGFR, PDGFR, and FGFR, blunts primary tumor growth and metastasis, reduces MVD and



**Figure 4.** BIBF 1120 shows potent antiangiogenic effects, induces hypoxia, and decreases drug delivery in pancreatic cancer xenografts. Vascular parameters of pancreatic tumors were evaluated by immunohistochemistry and perfusion studies. **A**, representative images of MVD (CD31) and pericyte coverage (NG2) in Mia PaCa-2 xenografts at  $\times 200$  magnification (inset,  $\times 400$ ) with 4',6-diamidino-2-phenylindole (DAPI)-labeling nuclei. Scale bar, 100  $\mu\text{m}$ . **B**, representative images of CD31 and pimonidazole reactivity in Mia PaCa-2 xenografts. Scale bar, 100  $\mu\text{m}$ . **C**, representative images of doxorubicin perfusion in AsPC-1 xenografts after acute or chronic exposure to BIBF 1120. Quantification of MVD (**D**), pericyte coverage (**E**), hypoxia (**F**), and doxorubicin (perfusion) fluorescence (**G**) is shown. Bar graphs indicate means  $\pm$  SEM. A minimum of 5 images were acquired per group. Results are mean percentage of thresholded area or absolute vessel counts per field. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  by Dunn posttest. Gem, gemcitabine.

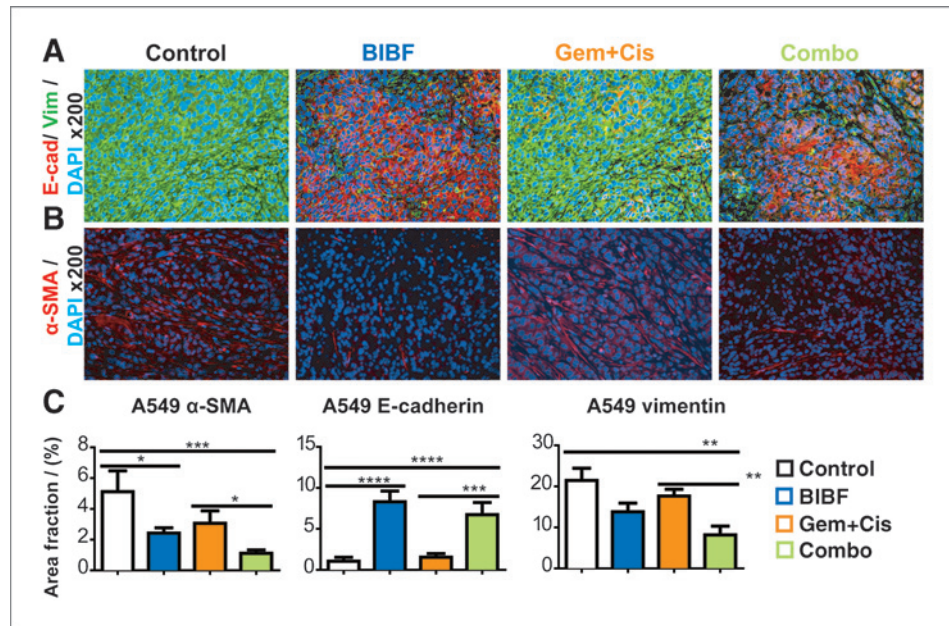
fibroblast activation, induces hypoxia, but does not promote EMT in multiple preclinical models of lung and pancreatic cancer.

EMT predicts poor prognosis, promotes metastasis, and is associated with resistance to therapy (40, 41). Because hypoxia is a known driver of EMT (42) and we observed extensive hypoxia in BIBF 1120-treated tumors, we anticipated EMT induction as a possible limitation of the drug. However, we observed no evidence of EMT after evaluating of the expression of accepted markers of EMT. Instead, in A549 tumors, we observed reversal of EMT and promotion of an epithelial phenotype after BIBF 1120 treatment.

These effects may be due to the multitargeted nature of BIBF 1120, which inhibits FGFR and PDGFR as well as

VEGFR. Therapeutic strategies targeting the VEGF-VEGFR axis exclusively have been implicated in EMT induction in other malignancies (43). The absence of EMT in this study might be attributed to BIBF 1120 inhibition of fibroblast function, which has been implicated in regulating tumor cell phenotype (Ostapoff and colleagues; submitted for publication). Consistent with this hypothesis, we observed a decrease in the level of  $\alpha$ -SMA<sup>+</sup> and S100A4<sup>+</sup> fibroblasts in tumors from BIBF 1120-treated animals. Alternatively, FGF pathway activation may provide an escape mechanism from VEGF-targeted strategies. For example, FGF signaling is activated in response to anti-VEGF therapy in glioblastoma multiforme (8, 44) and renal cell carcinoma (45). Furthermore, dual VEGF/FGF inhibition with the TKI brivanib shows activity

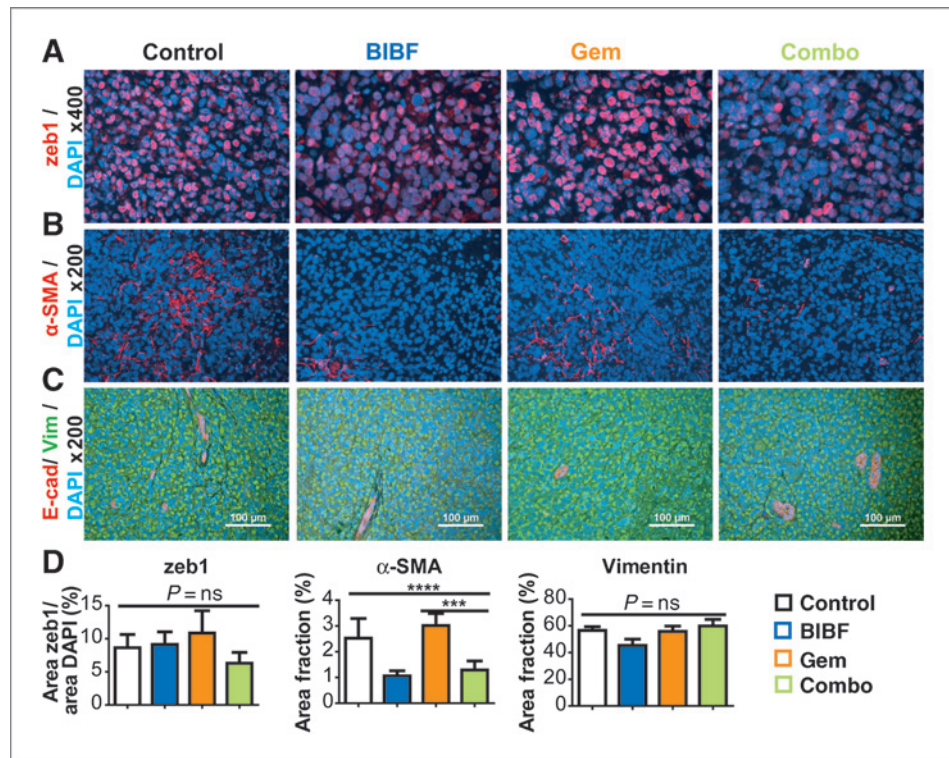
**Figure 5.** BIBF 1120 does not drive an invasive phenotype in lung cancer. EMT markers from lung tumors were evaluated by immunohistochemistry. A, representative images of mature myofibroblasts ( $\alpha$ -SMA) at  $\times 200$  magnification with 4',6-diamidino-2-phenylindole (DAPI)-labeling nuclei. B, representative images of E-cadherin and vimentin at  $\times 200$  magnification. C, quantification of fibroblasts, E-cadherin, and vimentin. Bar graphs indicate mean  $\pm$  SEM. A minimum of 5 images were acquired per group. Results were given as mean percentage of thresholded area per field. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  by Dunn posttest. Gem, gemcitabine; Cis, cisplatin.



against pancreatic neuroendocrine tumors that develop evasive resistance to anti-VEGF therapy (12). In addition, stromal and tumor cell PDGFR targeting inhibits tumor growth and enhances the effect of chemotherapy in preclinical lung cancer models (9). Furthermore, targeted inhibition of VEGFR expressed on endothelial cells and PDGFR on pericytes provided enhanced therapeutic activity compared with the usage of either

inhibitor as a single agent in pancreatic islet cell carcinoma (6). Similarly, we found that BIBF 1120, which inhibits pathways associated with endothelial and pericyte function, decreased pericyte coverage. This is in contrast to the concept of antiangiogenic induction of vascular normalization. Consistent with a decrease in vascular function, we observed decreased delivery of doxorubicin in AsPC-1 tumors after acute or chronic

**Figure 6.** BIBF 1120 does not drive an invasive phenotype in pancreatic cancer. EMT markers from pancreatic tumors were evaluated by immunohistochemistry. A, representative images of zeb1 at  $\times 200$  magnification with 4',6-diamidino-2-phenylindole (DAPI)-labeling nuclei. B, representative images of mature myofibroblasts ( $\alpha$ -SMA) at  $\times 200$  magnification with DAPI-labeling nuclei. C, representative images of E-cadherin and vimentin at  $\times 200$  magnification. Scale bar, 100  $\mu$ m. D, quantification of zeb1, fibroblasts, E-cadherin, and vimentin. Bar graphs indicate mean  $\pm$  SEM. A minimum of 5 images were acquired per group. Results were given as mean percentage of thresholded area per field. \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  by Dunn posttest. Gem, gemcitabine; ns, not significant.





therapy with BIBF 1120. These results are consistent with recent clinical data that show a rapid decrease in the delivery of docetaxel after treatment with bevacizumab (46, 47).

To date, a number of BIBF 1120 clinical studies have been reported, including combination studies with standard lung cancer chemotherapy regimens (carboplatin-paclitaxel and single-agent pemetrexed; refs. 48, 49). On the basis of encouraging safety and efficacy data, 2 phase III trials [LUME-Lung 1 (NCT00805194) and LUME-Lung 2 (NCT00806819)] evaluating BIBF 1120 in combination with docetaxel and pemetrexed, respectively, have been conducted.

Other strategies targeting VEGF/PDGF/FGF have shown efficacy in preclinical cancer models (50). Our study supports this strategy and, for the first time, examines its efficacy and biologic effects in combination with standard chemotherapy. In addition, to our knowledge, this is the first study to examine the impact of multitargeted angiokinase inhibition on EMT. In conjunction with encouraging clinical safety and efficacy data, these findings warrant further clinical investigation of this agent, including ongoing trials in lung cancer and further development in pancreatic cancer.

#### Disclosure of Potential Conflicts of Interest

D.E. Gerber has commercial research grant from Boehringer-Ingelheim and is a consultant/advisory board member of the same. R.A. Brekken has commercial research grant from Boehringer-Ingelheim and is a consul-

tant/advisory board member of Peregrine Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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