Antitumor Activity of BIBF 1120, a Triple Angiokinase Inhibitor, and Use of VEGFR2\textsuperscript{\textasterrm{+}}pTyr\textsuperscript{+} Peripheral Blood Leukocytes as a Pharmacodynamic Biomarker \textit{In Vivo}

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Abstract

\textbf{Purpose:} BIBF 1120 is a potent, orally available triple angiokinase inhibitor that inhibits VEGF receptors (VEGFR) 1, 2, and 3, fibroblast growth factor receptors, and platelet-derived growth factor receptors. This study examined the antitumor effects of BIBF 1120 on hepatocellular carcinoma (HCC) and attempted to identify a pharmacodynamic biomarker for use in early clinical trials.

\textbf{Experimental Design:} We evaluated the antitumor and antiangiogenic effects of BIBF 1120 against HCC cell line both \textit{in vitro} and \textit{in vivo}. For the pharmacodynamic study, the phosphorylation levels of VEGFR2 in VEGF-stimulated peripheral blood leukocytes (PBL) were evaluated in mice inoculated with HCC cells and treated with BIBF 1120.

\textbf{Results:} BIBF 1120 (0.01 \textmu mol/L) clearly inhibited the VEGFR2 signaling \textit{in vitro}. The direct growth inhibitory effects of BIBF 1120 on four HCC cell lines were relatively mild \textit{in vitro} (IC\textsubscript{50} values: 2–5 \textmu mol/L); however, the oral administration of BIBF 1120 (50 or 100 mg/kg/d) significantly inhibited the tumor growth and angiogenesis in a HepG2 xenograft model. A flow cytometric analysis revealed that BIBF 1120 significantly decreased the phosphotyrosine (pTyr) levels of VEGFR2\textsuperscript{+}CD45\textsuperscript{dim} PBLs and the percentage of VEGFR2\textsuperscript{+}pTyr\textsuperscript{+} PBLs \textit{in vivo}; the latter parameter seemed to be a more feasible pharmacodynamic biomarker.

\textbf{Conclusions:} We found that BIBF 1120 exhibited potent antitumor and antiangiogenic activity against HCC and identified VEGFR2\textsuperscript{+}pTyr\textsuperscript{+} PBLs as a feasible and noninvasive pharmacodynamic biomarker \textit{in vivo}. \textit{Clin Cancer Res; 17(6); 1373–81. ©2010 AACR.}

Introduction

A number of antiangiogenic inhibitors have been studied in clinical settings, some of which have clearly exhibited a clinical benefit in oncology. Consequently, VEGFs and VEGF receptors (VEGFR) are now well-validated targets in cancer therapy (1). In hepatocellular carcinoma (HCC), 2 recent randomized controlled trials for HCC have reported a clinical benefit of single-agent sorafenib for extending the overall survival in both Western and Asian patients with advanced unresectable HCC (2, 3). On the basis of the clear results of these trials, sorafenib is presently regarded as the standard therapy for HCC.

Because antiangiogenic inhibitors may achieve therapeutic levels long before toxicities arise compared with conventional cytotoxic chemotherapies, identifying pharmacodynamic biomarkers that accurately reflect the effects of the drug on its known targets are needed (4, 5). Therefore, a wide variety of biomarkers of antiangiogenic inhibitors have been proposed and intensively investigated, including plasma proteins, angiogenesis-related signaling, immunohistochemistry of endothelial cell markers for evaluating microvessel density (MVD), circulating endothelial progenitor/cells, and functional imaging such as dynamic contrast-enhanced MRI and molecular imaging using positron emission tomography (6). These candidate biomarkers have been evaluated and characterized as prognostic, pharmacodynamic, or response-predictive markers. Although the utility of biomarkers for evaluating MVD was highly anticipated, these markers were not predictive for clinical response in patients treated with bevacizumab (7). Regarding growth factors and cytokines, the plasma VEGF level has been shown to be neither a pharmacodynamic nor a predictive biomarker of antiangiogenic drugs (7, 8), although the plasma VEGF level is a well-known prognostic biomarker (9–11). Plasma-soluble VEGFR2, on the other hand, may be a promising and specific biomarker of...
antiangiogenic drugs for evaluating their effects (12, 13). Indeed, we have shown that soluble VEGFR2 was certainly decreased by BIBF 1120 treatment in a phase I trial; however, this decrease was observed at a relatively late stage, 8 to 29 days after the start of treatment (14). These results suggest that soluble VEGFR2 is not a rapid-responding biomarker for monitoring effects of antiangiogenic drugs. As no other biomarkers have been validated for routine clinical use, a new pharmacodynamic biomarker is needed.

BIBF 1120 is a potent triple angiokinase inhibitor that inhibits VEGFR1, 2, and 3, fibroblast growth factor receptors (FGFR), and platelet-derived growth factor receptors (PDGFR). In vitro studies have shown that VEGFR2 tyrosine kinase activity was potently inhibited by BIBF 1120 (IC₅₀ = 21 nmol/L) and was also active against VEGFR1 and 3 (IC₅₀ = 34 and 13 nmol/L, respectively; ref. 15). BIBF 1120 dose dependently inhibited the growth of various human tumor xenografts and tumor angiogenesis in vivo studies, consistent with the potent inhibition of VEGF signaling (15). BIBF 1120 also exhibited a relatively strong direct growth inhibitory effect on cancer cell lines, influencing 9 of 14 acute myeloid leukemia cell lines in a colony formation assay with an IC₅₀ value of less than 1 μmol/L (16).

We previously reported the antitumor activity of VEGFR2 tyrosine kinase inhibitors (TKI) against non–small cell lung cancer and gastric cancer, identifying a biomarker and the mode of action (17–19). In the present study, we focused on the antitumor activity of BIBF 1120 against HCC, which is hypervascular in nature. In addition, to identify a pharmacodynamic biomarker, we examined the phosphorylation levels of VEGFR-positive peripheral blood leukocytes (PBL) as a surrogate tissue in an in vivo model.

Materials and Methods

Compounds

BIBF 1120 was provided by Boehringer Ingelheim Pharma GmbH & KG, 5-Fluorouracil (5FU, Sigma-Aldrich) and an epidermal growth factor receptor (EGFR) TKI, AG1478 (Biomol International), were purchased from the indicated companies.

Cell lines and cultures

HepG2, HLF, HLE, and Huh7 (human hepatoblastoma and HCC cell lines, respectively) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS (Gibco BRL). HUVECs (human umbilical vein endothelial cells) were purchased from Kurabo and were maintained in Humedia-EG2 (Kurabo) medium with 2% FBS, 2 ng/mL of VEGF-A (R&D Systems), 10 ng/mL of EGF, 5 ng/mL of FGF, 10 μg/mL of heparin, and 1 μg/mL of cortisol. These cells were cultured in an atmosphere of 5% CO₂ at 37°C.

In vitro growth inhibition assay

The growth inhibitory effects of BIBF 1120 on the HepG2, HLF, HLE, and Huh7 cell lines were examined using an MTT assay as previously described (17, 18). The optical density was measured at 570 nm. Three independent experiments were conducted.

Western blot analysis

The antibodies used for the Western blot analysis were anti-KDR (IBL), anti–phospho (p)-VEGFR2 (Tyr1175), anti-VEGFR1, anti-p44/42 MAPK (mitogen-activated protein kinase), anti–p-p44/42 MAPK, anti–c-Kit, anti–PDGFRβ, anti–FGFR1, 2, and 3, horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology), and anti–β-actin (Santa Cruz Biotechnology). The methods have been previously described (18). Two independent immunoblotting experiments were conducted.

Tube formation assay

HUVECs were cultured without VEGF-A for 24 hours. A total of 40 μL of Matrigel (BD Bioscience) and 20 μL of PBS were mixed and incubated in 96-well plates. After the gel had solidified, a 100-μL volume of HUVECs (2 × 10⁴ cells/well) was seeded onto the plates with 20 ng/mL of VEGF-A and the indicated concentration of BIBF 1120. The 96-well plates were then incubated for 4 hours. Capillary morphogenesis was evaluated under a microscope (Olympus). This assay was carried out in 3 independent experiments.

Real-time reverse transcriptase PCR

The method has been previously described (17). The primers used for real-time reverse transcriptase PCR (RT-PCR) are shown in Supplementary Table 1. GAPD was used to normalize the expression levels in the subsequent quantitative analyses.

Flow cytometric analysis for HUVECs

HUVECs were seeded on 6-well plates without VEGF-A for 24 hours. After exposure to BIBF 1120, AG1478, or 5FU for 3 hours, the cells were stimulated with 20 ng/mL of VEGF-A for 30 minutes. The flow cytometric procedure was carried out according to the manufacturer’s protocols.
using the Fixation/Permeabilization Kit (BD Biosciences); the data were obtained using a FACSCalibur flow cytometer (BD Biosciences). Anti-phosphotyrosine (pTyr) antibody (P-Tyr-100; Cell Signaling) was used to detect the phosphorylation levels.

Flow cytometric analysis for PBLs in the in vivo model
In the in vivo model, about 0.5 to 1 mL of peripheral blood was obtained from treated mice and 20 ng/mL of VEGF was added to the whole blood samples for 20 minutes. The red cells were then lysed using a lysis buffer (155 mmol/L NH₄Cl, 10 mmol/L NaHCO₃, and 1 mmol/L EDTA2Na, pH 7.3) for 10 minutes, and leukocytes were fixed and permeabilized using a Fixation/Permeabilization Kit for analysis. The following antibodies were used: anti-mouse CD45-PerCP, anti-mouse Flk-1-PE (BD Biosciences), anti-pTyr (P-Tyr-100; Cell Signaling), and Alexa Fluor Mouse IgG1 Isotype Control (BD Parningen). The analysis was carried out using the WinMDI software (20).

HCC xenograft model
Nude mice (BALB/c nu/nu; 6-wk-old females; CLEA Japan Inc.) were used for the in vivo studies and were cared for in accordance with the recommendations for the handling of laboratory animals for biomedical research, compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University. The ethical procedures followed and met the requirements of the United Kingdom Coordinating Committee on Cancer Research Guidelines.

Mice were subcutaneously inoculated with a total of 6 × 10⁶ HepG2 cells. Two weeks after inoculation, the mice were randomized according to tumor size into 3 groups to equalize the mean pretreatment tumor size among the 3 groups (n = 6 in each group). The mice were then treated with BIBF 1120 (50 mg/kg/d, p.o.), BIBF 1120 (100 mg/kg/d, p.o.), or the vehicle control (saline, p.o.) for 14 days (Fig. 3A–C). On day 14, the mice were euthanized, blood samples were collected by cardiac puncture, and tumor specimens were collected for immunohistochemistry. The tumor volume was calculated as the length × width² × 0.5 and was assessed every 2 to 3 days.

Immunohistochemical analysis
A mouse anti-CD31 monoclonal antibody (1:100; BD Biosciences) was used to detect the endothelial cells. The paraffin-embedded samples were cut into 4-μm sections, deparaffinized, and placed in a preheated antigen retrieval solution (Dako) in a steamer for 10 minutes. All the samples were then blocked in 3% H₂O₂ in methanol for 15 minutes and rinsed with PBS. The slides were then placed in a Sequenza slide staining system (Thermo Fisher Scientific) and blocked in 1% normal goat serum for 20 minutes. The slides were incubated overnight at 4°C with the CD31 antibody. A standard avidin–biotin peroxidase complex assay was then carried out using the ABC Elite Kit (Vector Laboratories). The slides were developed with 3,3'-diaminobenzidine (DAB; Zymed Laboratories) and counterstained with 10% hematoxylin. Microvessel density (MVD) was quantified by measuring the number of CD31-positive endothelial cells in the tumors. Ten random fields per tumor sample at 200× magnification were captured and saved for computer-assisted image analysis using the ImageJ software package (21). An algorithm for color deconvolution was used to segregate the brown DAB-positive CD31 endothelial cells and the blue tumor cells. Thresholds were adjusted to remove background and non-specific signals. MVD was reported as the average ratio of CD31-positive cells to tumor cells.

Statistical analysis
The statistical analyses were carried out using Microsoft Excel (Microsoft) to calculate the SD and to test for statistically significant differences between the samples using a Student’s t test. A value of P < 0.05 was considered statistically significant.

Results

BIBF 1120 potently inhibits VEGFR2 signaling in HUVECs
We evaluated the inhibitory effect of BIBF 1120 at various concentrations (0.0001–10 μmol/L) on VEGFR2 signaling, using HUVECs stimulated with 20 ng/mL of VEGF. BIBF 1120 at a concentration of 0.01 μmol/L completely inhibited the phosphorylation of VEGFR2 and MAPK in HUVECs (Fig. 1A). BIBF 1120 at a concentration of 0.01 μmol/L partially inhibited tube formation in HUVECs stimulated with VEGF, whereas BIBF 1120 at a concentration of 1 μmol/L completely inhibited tube formation (Fig. 1B). These data indicate that BIBF 1120 potently inhibits VEGFR2 signaling in endothelial cells.

Flow cytometry detects BIBF 1120–induced inhibition of pTyr levels
To detect the BIBF 1120–induced inhibition of pTyr levels by flow cytometry, the VEGF-induced pTyr levels of proteins in HUVECs were evaluated after exposure to BIBF 1120, the EGFR TKI AG1478 as a TKI control, or 5FU as a cytotoxic drug control. The controls agents were used to show that another target of TKI did not induce (AG1478) or to exclude the possibility that nonspecific effects such as cytotoxic cellular responses were not induced (5FU). Flow cytometry revealed that the VEGF-induced pTyr levels in HUVECs were significantly inhibited by BIBF 1120 at concentration of 1 and 5 μmol/L but not by AG1478 or by 5FU (Fig. 1C and D). This flow cytometric method is considered a feasible means of detecting the inhibition of VEGF-induced pTyr levels induced by VEGFR2 TKIs.

Growth inhibitory effects and expression status of targeted receptors in HCC cell lines in vitro
To evaluate the expression status of the putative targeted receptors of BIBF 1120 in the 4 HCC cell lines and HUVECs as a control, we examined the protein expression levels of VEGFR1, VEGFR2, FGFR1, FGFR2, FGFR3, PDGFRβ, and...
c-Kit (the kinase activities of which are reportedly inhibited by BIBF 1120 (15) and p-VEGFR2, MAPK, and p-MAPK by Western blotting. The protein expression of these receptors were not highly upregulated in any of the HCC cell lines, except for PDGFRβ in HLE and HLF cells (Fig. 2A). A comparable expression level of MAPK was observed among the cell lines, and an increase in p-MAPK expression was observed in HLE cells. The mRNA expression levels of the target receptors VEGFR1, VEGFR2, VEGFR3, PDGFRα, PDGFRβ, FGFR1, FGFR2, FGFR3, and FGFR4 were determined using real-time RT-PCR in the HUVEC line and the HCC cell line. Higher receptor expression levels were observed for VEGFR2 in HUVECs, PDGFRβ in HLE and HLF, FGFR1 in HUVECs and HLE, FGFR3 in HepG2, and FGFR4 in Huh7 (Fig. 2B). The expression levels were consistent with the Western blotting results.

We next evaluated the direct growth inhibitory activity of BIBF 1120 in 4 HCC cell lines in vitro. The IC_{50} value of BIBF 1120 for the HLE, HLF, HepG2, and Huh7 cell lines were 2.7 ± 1.7, 2.7 ± 0.5, 5.3 ± 0.6, and 4.3 ± 0.9 μmol/L, respectively (Fig. 2C). These results indicate that the direct growth inhibitory activity of BIBF 1120 against HCC cells was relatively mild (IC_{50}: 2–5 μmol/L).

**BIBF 1120 potently inhibits tumor growth and angiogenesis of HCC xenografts in vivo**

Next, we examined the antitumor and antiangiogenic effects of BIBF 1120 in vivo. Mice inoculated with HepG2...
cells were orally given a low (50 mg/kg/d) or high (100 mg/kg/d) dose of BIBF 1120, or vehicle alone, for 2 weeks (Fig. 3A). The mean tumor volumes on day 14, for each group of mice, were as follows: vehicle alone, 1,367 ± 364 mm³; 50 mg/kg/d, 488 ± 489 mm³; and 100 mg/kg/d, 572 ± 556 mm³. Both doses of BIBF 1120 significantly inhibited tumor growth (T/C = 0.36 and 0.42, respectively), indicating that BIBF 1120 has a potent antitumor activity against HCC in vivo (Fig. 3B). Body weight loss was not observed after the administration of BIBF 1120 at either dose (Supplementary Fig. S1). The CD31 staining of tumor tissues showed that BIBF 1120 administration also significantly inhibited tumor angiogenesis (Fig. 3C). Combined with the observation of the direct growth inhibitory activity against HCC in vitro, these findings suggest that the antitumor activity of BIBF 1120 in vivo mainly result from the drug's antiangiogenic activity, which blocks VEGF signaling.

VEGFR2<sup>pTyr</sup>+ PBLs are a pharmacodynamic biomarker in vivo

VEGFR2<sup>CD45<sup>dim</sup></sup> PBLs are generally regarded as circulating endothelial cells (22); therefore, we hypothesized that VEGFR2<sup>CD45<sup>dim</sup></sup> PBLs might be useful as a biological biomarker of VEGFR2 TKIs. The effects of BIBF 1120 on the pTyr levels of VEGFR2<sup>CD45<sup>dim</sup></sup> PBLs and the percentage of VEGFR2<sup>pTyr</sup>+ PBLs was examined in vivo (Fig. 4A). Murine blood samples were obtained from tumor-bearing, BIBF 1120–treated mice, as described previously. The pTyr levels of the VEGFR2<sup>CD45<sup>dim</sup></sup> PBLs were significantly inhibited by BIBF 1120 treatment, but the difference was relatively small (Fig. 4B and C). On the other hand, the percentage of VEGFR2<sup>pTyr</sup>+ PBLs was markedly decreased by BIBF 1120 administration (Cont: 1.8% ± 1.1%, B50: 0.34% ± 0.21%, B100: 0.37% ± 0.29%; Fig. 5A and B). These findings raise the possibility that evaluating the VEGFR2<sup>CD45<sup>dim</sup></sup> PBLs by flow cytometry as a surrogate tissue may contribute to the proof of concept of VEGFR2-targeting drugs or the monitoring of drug effects in vivo. Thus, VEGFR2<sup>pTyr</sup>+ PBLs might be a useful pharmacodynamic biomarker of VEGFR2 TKIs in early clinical trials.

Discussion

HCC is one of the most hypervascular tumors, and vascular embolization has been used as a therapeutic strategy. A recent study showed that sorafenib exhibits
clinical benefits in patients with advanced HCC (2, 3). This encouraging result suggests that molecular targeting drugs might be active against HCC, especially those that block VEGFR signaling. Our data showed that BIBF 1120 inhibited tumor growth and angiogenesis in HCCs in vivo, suggesting that BIBF 1120 may be an active and promising drug against HCC.

BIBF 1120 has a potent inhibitory effect on VEGFRs, similar to that of sorafenib and sunitinib, and it also has activities against FGFRs and Src (refs. 15, 23, 24; Supplementary Table S2). Recent evidence has shown that Src expression is elevated and active in HCC and that Src may play a key role in supporting HCC progression (25); furthermore, HBx increased the activation of the androgen receptor through c-Src kinase, which acts as a major switch in the activation of HCC (26). We conducted a Western blot analysis to detect the inhibitory effect of BIBF 1120 on Src activity, using HUVECs and HepG2, Huh7, HLE, and HLF cells (Supplementary Fig. S2). The inhibitory effect of BIBF 1120 on p-Src was observed in HUVECs and HLE and HepG2 cells, suggesting that BIBF 1120 actually has an inhibitory effect on Src. This effect may benefit HCC therapy in a manner independent of its antiangiogenic effect, although this topic needs to be further investigated. Similarly, we showed an inhibitory effect of BIBF 1120 on p-FGFR2 by using FGFR2-amplified gastric cancer cell lines (Supplementary Fig. S3). Brivanib (BMS-540215), a dual inhibitor of VEGFR and FGFR, is currently in development for the treatment of HCC and colon carcinoma, and preclinical studies have shown that FGFR signaling in HCC cells seems to be a promising therapeutic target (27, 28). These results suggest that the effect of BIBF 1120 on FGFR may contribute the antitumor effect, although further investigation is needed.

Numerous candidate biomarkers of angiogenesis have been identified, but the use of these markers for diagnosis, prognosis, and treatment monitoring remains investigational and of uncertain utility (4). Among them, biomarkers for detecting the blockade of VEGFR signaling have received particular attention because of the intimate involvement of this mechanism in drug activity of VEGFR TKIs. We have shown that VEGF-induced VEGFR2 pTyr+ PBLs in peripheral blood samples were markedly decreased by BIBF 1120 treatment in vivo. This analysis was done using only peripheral blood collection, VEGF stimulation, and analysis of 2-color flow cytometry; thus, this method is feasible...
and specific to VEGF signaling. Our method may contribute to the proof of concept for VEGFR2 TKIs and may help to determine the biological optimal dose, especially in phase I clinical trials.

Phase II studies of BIBF 1120 against lung cancer and ovarian cancer have been completed and phase I/II study of BIBF 1120 is currently evaluated in HCC (NCT 01004003). Two large phase III clinical trials against lung cancer...
growth inhibitory effects on 4 HCC cell lines (IC50 values: 2–5 μmol/L) in vitro. BIBF 1120 exhibited potent antitumor and antimigratory activities against HCC in vivo, and the antitumor effect did not fail or show signs of weakening during the long-term administration period.

In addition, VEGFR2 pTyr+ PBLs were found to be a noninvasive pharmacodynamic biomarker in a murine model.

References

14. Kudo et al. LUME-Lung 2: docetaxel ± BIBF 1120; LUME-Lung 2: pemetrexed ± BIBF 1120) and 1 against ovarian cancer (LUME-Ovar 1: carboplatin/paclitaxel ± BIBF 1120) are now underway. We have shown that BIBF 1120 exhibited antiangiogenic and antitumor activity against HCC in vivo. These results provide the scientific rationale for introducing BIBF 1120 as a treatment of HCC in the future. In addition, our approach of evaluating VEGFR2 pTyr+ PBLs in VEGFR TKI might be applicable to future phase I trials. We plan to use this method in clinical settings.

In conclusion, BIBF 1120 clearly inhibited VEGFR2 signaling in endothelial cells and exhibited relatively mild growth inhibitory effects on 4 HCC cell lines (IC50 values: 2–5 μmol/L) in vitro. BIBF 1120 exhibited potent antitumor and antimigratory activities against HCC in vivo, and the antitumor effect did not fail or show signs of weakening during the long-term administration period. In addition, VEGFR2 pTyr+ PBLs were found to be a noninvasive pharmacodynamic biomarker in a murine model.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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