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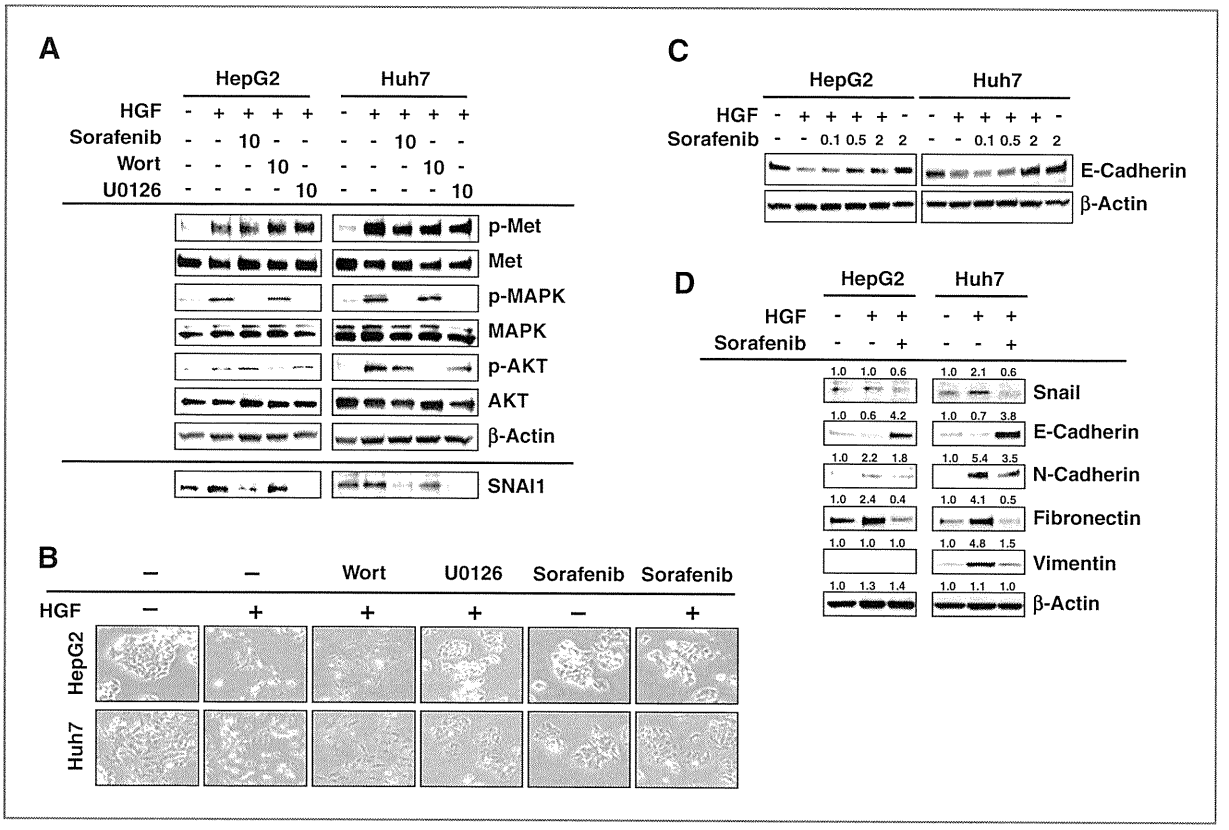

Figure 4. Sorafenib downregulates SNAI1 expression in HCC. A, as expected, sorafenib and the MEK inhibitor U0126 inhibited the HGF-mediated phosphorylation of MAPK, but the PI3K inhibitor wortmannin did not. Of note, SNAI1 expression was markedly downregulated by sorafenib and U0126. The HepG2 and Huh7 cells were exposed to 10 μmol/L of sorafenib or wortmannin or U0126 for 3 hours and were then stimulated with 10 ng/mL of HGF for 60 minutes. Wort, wortmannin. B, the HGF-mediated morphologic changes were canceled by sorafenib and U0126 but not by wortmannin in the HCC cells. The cells were exposed to sorafenib or wortmannin or U0126 for 48 hours with or without HGF (10 ng/mL) and then photographed. C, HGF-mediated downregulation of E-cadherin was canceled by sorafenib. The cells were stimulated with HGF (10 ng/mL) and treated with sorafenib at indicated concentration for 48 hours. D, HGF-mediated cadherin switching and upregulation of fibronectin and vimentin were canceled by sorafenib in the HCC cell lines. The cells were cultured with or without 2 μmol/L of sorafenib for 72 hours, with or without HGF (10 ng/mL), and then were analyzed using Western blot analysis. Densitometric data are shown above the Western blot.

expected, sorafenib and the MEK inhibitor U0126 (10 μmol/L) markedly inhibited the HGF-induced phosphorylation of MAPK, but the PI3K inhibitor wortmannin (10 μmol/L) did not. In contrast, only wortmannin inhibited the phosphorylation of AKT (Fig. 4A). Notably, SNAI1 expression was strongly downregulated by sorafenib and U0126 but not by wortmannin (Fig. 4A). These results showed that sorafenib downregulated SNAI1 expression via MAPK signaling. Meanwhile, we examined the HGF- and sorafenib-mediated expression changes of *SNAI2*, *ZEB1*, *ZEB2*, and *TWIST* using real-time RT-PCR and Western blot (Supplementary Fig. 3). Baseline and expression changes of SNAI2 and TWIST were very low compared with SNAI1, and the expression changes of ZEB1 and ZEB2 seemed not to be significant. Collectively, we considered that SNAI2, TWIST, ZEB1, and ZEB2 are not likely to be involved in the effect of HGF and sorafenib on EMT in this cell lines. Then, we examined the activity of sorafenib on HGF-mediated morphologic changes in HCC cells. HGF stimulation mediated the cell scattering and spindle-shaped changes, and these effects were clearly canceled by sorafenib and U0126, but not by wortmannin, in both HepG2 and Huh7 cells (Fig. 4B). These results were consistent with the results of Western blotting. To show whether sorafenib cancels the effect of HGF-mediated downregulation of E-cadherin, we examined the Western blot in dose–response analysis. Downregulation of E-cadherin was clearly canceled by sorafenib in a dose-dependent manner (Fig. 4C). Time-course analysis showed that HGF-mediated downregulation of E-cadherin was also canceled by sorafenib (Supplementary Fig. 4). HGF stimulation downregulated E-cadherin expression and upregulated N-cadherin, vimentin, and fibronectin in HCC cells; however, these effects were canceled by sorafenib in both HCC cell lines (Fig. 4D and Supplementary Fig. 2B). The mRNA data of N-cadherin in Huh7 cells were not correlated with protein level. These results show that sorafenib inhibits the

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RAF-MAPK pathway, thereby downregulating SNAI1 and inhibiting the EMT in HCC.

Because sorafenib inhibits the HGF-mediated EMT in HCC cells, we next examined whether the inhibitory effect of sorafenib on the EMT leads to an inhibition of cellular migration in HCC cells. A scratch assay revealed that HGF stimulation increased cellular migration by about 2- fold in both HCC cell lines; however, sorafenib significantly inhibited this effect to the baseline levels (Fig. 5A). Similarly, a migration assay using the Boyden chamber method revealed that sorafenib canceled HGF-mediated cellular migration in both cell lines (Fig. 5B). These results suggest that sorafenib actually inhibits the cellular migrating phenotype of the EMT in HCC cells. The combination of migration data with siRNA and sorafenib (Fig. 3D and Fig. 5B) suggests that inhibitory effects of sorafenib on migration may be mediated by Snail downregulation in some tumors (e.g., Huh7) but not in others (e.g., HepG2). It is assumed that the inhibitory activity of sorafenib on the cellular migrating phenotype is due to its inhibitory effect of Raf-MAPK signaling pathway (Fig. 4A and B). Regarding HGF-dependent PI3K-AKT signaling pathway, wortmannin weakly inhibited the wound closure in Huh7 cells and to the same extent by sorafenib in HepG2 cells (Supplementary Fig. 5). In contrast, wortmannin has no effect on Snail levels or on HCC morphology changes (Fig. 4A and B). Collectively, we speculate that activation of HGF-dependent PI3K-AKT pathway may not be involved in SNAI1 induction or morphologic change but at least partially involved in cell migration independent of Raf-MAPK-SNAI1 signaling.

Taken together, these results indicate that sorafenib inhibits the HGF-mediated EMT, which is characterized by cadherin switching, morphologic changes, and an increase in the cellular migrating phenotype, by inhibiting Raf-MAPK signaling, resulting in the downregulation of SNAI1 in HCC cells (Fig. 6).

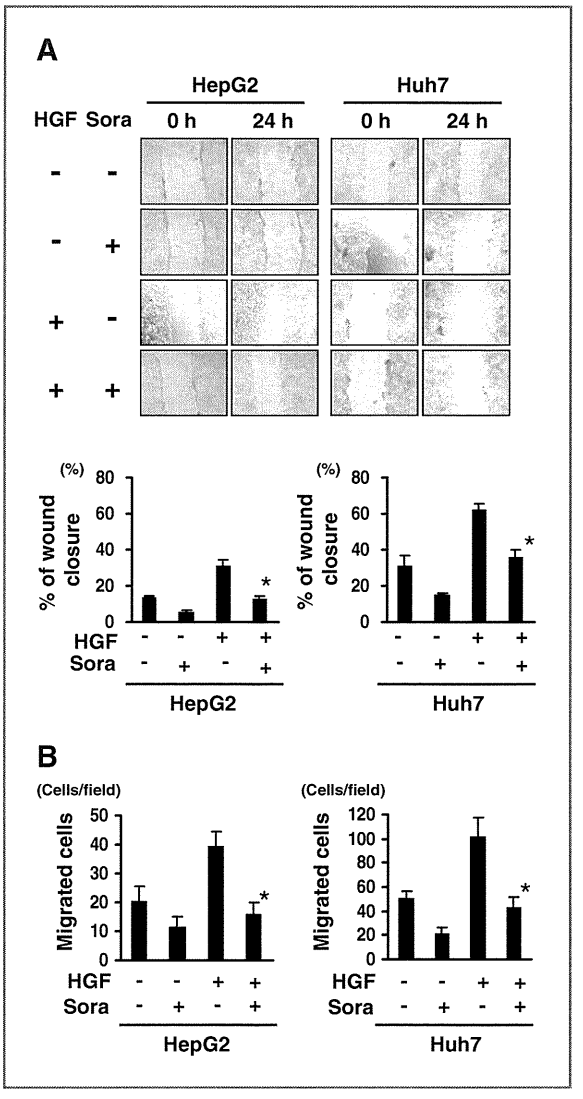

Figure 5. Sorafenib inhibits HGF-mediated cellular migration in HCC cells. A, a scratch assay revealed that HGF stimulation increased the cellular migration by about 2-fold, but sorafenib almost completely canceled the effect. The subconfluent HepG2 and Huh7 cells were scratched with a plastic pipette tip and incubated under the indicated conditions (control, 10 ng/mL of HGF; and HGF, 10 μmol/L of sorafenib). The scratch area was photographed and measured. The experiment was done in triplicate. *, sorafenib (–) versus (+), $P < 0.05$. B, migration assay using the Boyden chamber method revealed that sorafenib almost completely canceled the HGF-mediated cellular migration in both HCC cell lines. The cells were incubated under the indicated conditions: control, 10 ng/mL of HGF; and HGF, 10 μmol/L of sorafenib. *, sorafenib (–) versus (+), $P < 0.05$. Sora, sorafenib.

Discussion

Recent accumulating evidence has shown that the EMT is involved in drug sensitivity to several anticancer agents (25). Within this topic, the most intensively investigated drugs have been endothelial growth factor receptor (EGFR)–targeting drugs for the treatment of lung cancer. A clinical trial has revealed that lung cancer cells with strong E-cadherin expression exhibit a significantly longer time to progression after EGFR-TKI (tyrosine kinase inhibitor) treatment (26). Other studies on EGFR-targeting drugs have shown that mesenchymal type lung cancer cells exhibit an EMT-dependent acquisition of PDGFR, FGF receptor, and TGF-β receptor signaling pathways (27), and integrin-linked kinase is a novel target for overcoming HCC resistance to EGFR inhibition (28). Regarding sensitivity to gemcitabine, mesenchymal type cancer cells are reportedly associated with gemcitabine resistance in pancreatic cancer cells (29). The mechanism of resistance to gemcitabine has been shown to involve the activation of Notch signaling, which is mechanistically linked with the mesenchymal chemoresistance phenotype of pancreatic cancer cells (30). Thus, baseline cellular characteristics based on the EMT phenotype might be useful not only as prognostic biomarkers for a malignant phenotype but also as predictive markers

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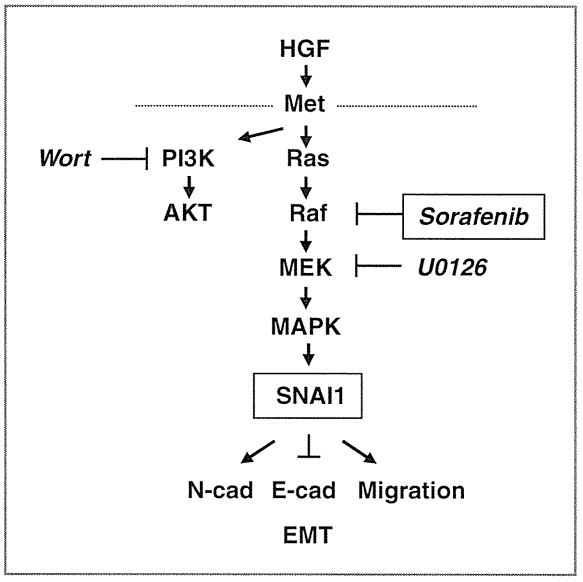

Figure 6. Diagram of the proposed mechanism by which sorafenib inhibits the EMT. Sorafenib inhibits the HGF-mediated EMT, which is characterized by morphologic changes, cadherin switching, and an increase in the cellular migrating phenotype. The anti-EMT effect of sorafenib occurs through the downregulation of SNAI1 by the inhibition of MAPK phosphorylation in HCC cells. Wort, wortmannin; N-cad, N-cadherin; E-cad, E-cadherin.

of sensitivity to anticancer agents. In this study, we focused on the signaling pathway responsible for inducing the EMT and showed that the multitarget TKI sorafenib downregulates SNAI1 by inhibiting Raf-MAPK signaling, thereby inhibiting the HGF-mediated EMT in HCC cells. Our findings may provide a novel insight into the actions of TKIs and their anti-EMT effects.

The mechanisms underlying the SNAI1-induced metastatic and aggressive phenotypes of cancer cells have recently been intensively investigated in both basic and clinical research studies. A novel aspect of the activity of SNAI1 is its involvement in immunosuppression. The SNAI1-induced EMT mediates regulatory T cells and impairs dendritic cells, accelerating cancer metastasis not only by enhancing invasion but also by inducing immunosuppression (31). A complex of histone deacetylase (HDAC) and SNAI1 plays an essential role in silencing E-cadherin (32), suggesting that the use of HDAC inhibitors to inhibit SNAI1 function might represent a promising therapeutic approach. On the other hand, large-scale clinical data on SNAI1 expression and the prognosis of patients with HCC were recently reported (33) and the overexpression of SNAI2 and/or TWIST was correlated with a worse prognosis. In contrast, no such significant differences were observed in samples that overexpressed SNAI2. The coexpression of Snail and TWIST was correlated with the worst prognosis for HCC (33). This evidence suggests that SNAI1 might be a useful therapeutic target for oncology. Our findings showed that sorafenib completely canceled the HGF-mediated SNAI1 induction in HepG2 and Huh7 cells. This activity of sorafenib, in addition to sorafenib's antiangiogenic effects, might contribute to a clinical benefit against metastatic and aggressive phenotypes in patients with HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Clinical Cancer Research

Cancer Therapy: Preclinical

Antitumor Activity of BIBF 1120, a Triple Angiokinase Inhibitor, and Use of VEGFR2$^+$pTyr$^+$ Peripheral Blood Leukocytes as a Pharmacodynamic Biomarker *In Vivo*

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Abstract

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.

Experimental Design: We evaluated the antitumor and antiangiogenic effects of BIBF 1120 against HCC cell line both *in vitro* and *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.

Results: BIBF 1120 (0.01 μmol/L) clearly inhibited the VEGFR2 signaling *in vitro*. The direct growth inhibitory effects of BIBF 1120 on four HCC cell lines were relatively mild *in vitro* (IC_{50} values: 2–5 μmol/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$^+$CD45^{dim} PBLs and the percentage of VEGFR2$^+$pTyr$^+$ PBLs *in vivo*; the latter parameter seemed to be a more feasible pharmacodynamic biomarker.

Conclusions: We found that BIBF 1120 exhibited potent antitumor and antiangiogenic activity against HCC and identified VEGFR2$^+$pTyr$^+$ PBLs as a feasible and noninvasive pharmacodynamic biomarker *in vivo*. *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.

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

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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

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Translational Relevance

A wide variety of biomarkers of antiangiogenic inhibitors have been proposed and intensively investigated; however, no biomarkers have been validated for routine clinical use and a new pharmacodynamic biomarker is needed. We have shown in this study that (i) BIBF 1120, a VEGF receptor 2 (VEGFR2) inhibitor, exhibited potent antitumor and antiangiogenic activity against hepatocellular carcinoma *in vivo* and (ii) $VEGFR2^{+}pTyr^{+}$ peripheral blood leukocytes (PBL) were useful pharmacodynamic biomarker *in vivo*. Our findings indicate the clinical utility of $VEGFR2^{+}pTyr^{+}$ PBLs as a feasible, noninvasive, and VEGF signal-specific biomarker of VEGFR2 tyrosine kinase inhibitors for use in early clinical trials.

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 (IC_{50} = 21 nmol/L) and was also active against VEGFR1 and 3 (IC_{50} = 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 IC_{50} 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% CO_2 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 (2×10^4 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,

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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 NH_4Cl, 10 mmol/L $NaHCO_3$, 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 Parmingen). 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 6×10^6 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 $\times$ width$^2 \times 0.5$ 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% H_2O_2 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 nonspecific 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

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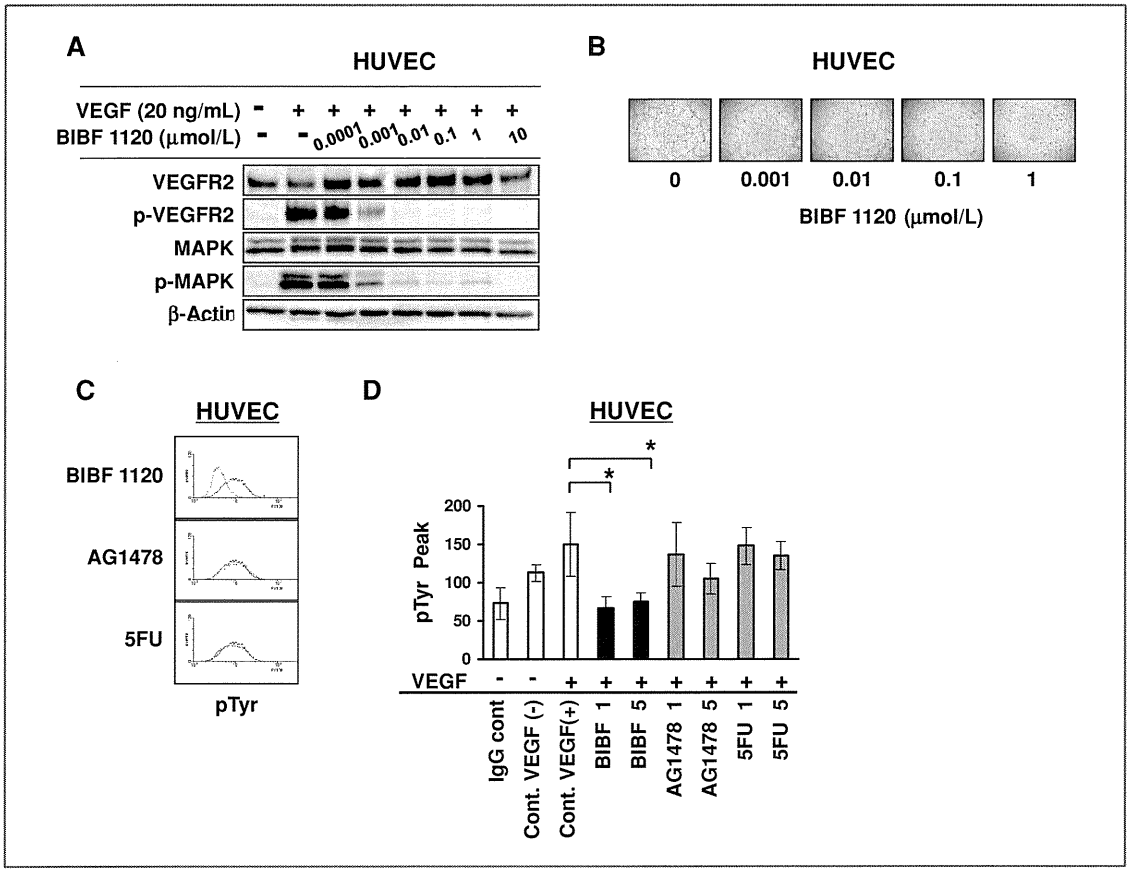

Figure 1. Inhibition of VEGFR2 signaling by BIBF 1120 and detection of the inhibition of pTyr by flow cytometry in HUVECs. A, the inhibition of VEGFR2 and MAPK phosphorylation by BIBF 1120 was determined using a Western blot analysis. HUVECs cultured in a medium containing 2% FBS were exposed to BIBF 1120 (0.0001–10 μmol/L) for 3 hours, stimulated with 20 ng/mL of VEGF for 15 minutes, and lysed for analysis. B, effect of BIBF 1120 on the inhibition of tube formation. HUVECs were seeded with 20 ng/mL of VEGF-A and exposed to BIBF 1120 (0.001–1 μmol/L) on Matrigel-layered 96-well plates for 4 hours. Capillary morphogenesis was evaluated under a microscope. This assay was conducted in 3 independent experiments. C and D, 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 inhibition of pTyr level was detected by flow cytometry with an anti-pTyr antibody. Note that only BIBF 1120 significantly inhibited the VEGF-induced phosphorylation levels of tyrosine. This assay was conducted in 3 independent experiments; bars, SD. *, $P < 0.05$.

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*, *PDGFRA*, *PDGFRB*, *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, *PDGFRB* 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

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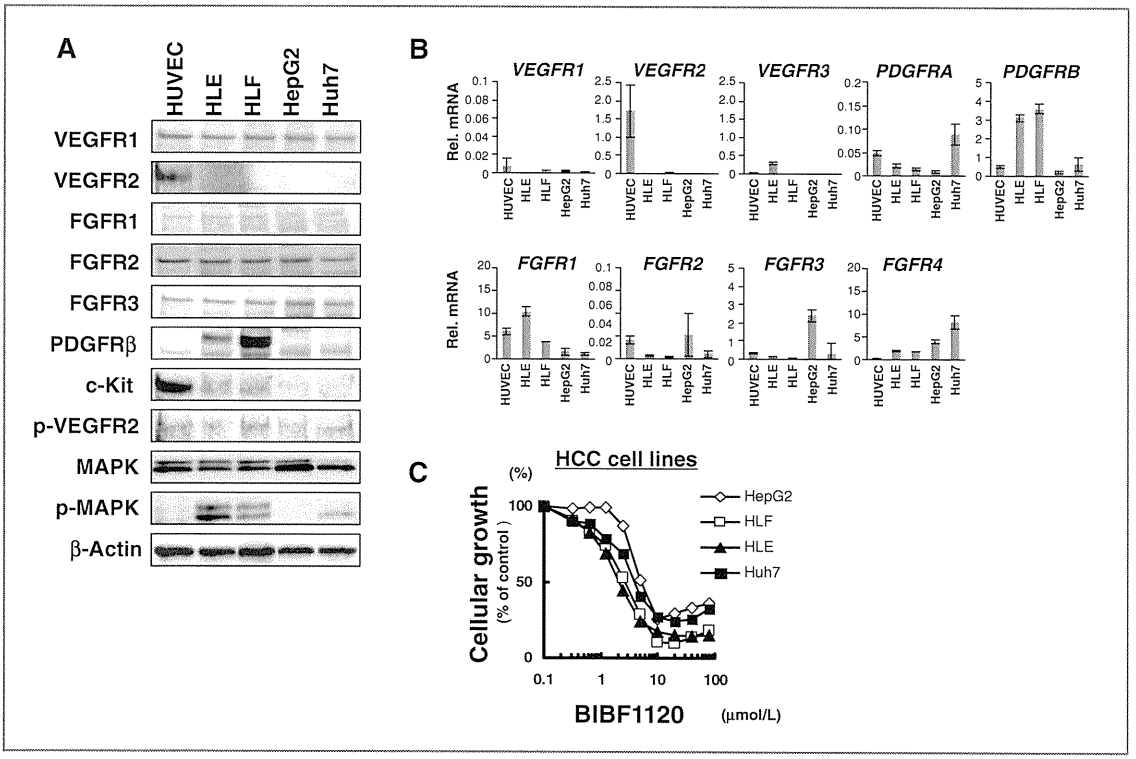

Figure 2. Expression levels of target receptors and sensitivity to BIBF 1120 in HCC cell lines. A, Western blot analysis of the expression levels of VEGFR1, VEGFR2, FGFR1, FGFR2, FGFR3, PDGFRβ, c-Kit, p-VEGFR2, MAPK, p-MAPK, and β-actin in HCC cell lines and HUVECs as a control. B, the mRNA expression levels of *VEGFR1*, *VEGFR2*, *VEGFR3*, *PDGFRA*, *PDGFRB*, *FGFR1*, *FGFR2*, *FGFR3*, and *FGFR4* were determined using real-time RT-PCR. Rel mRNA, mRNA expression levels normalized using *GAPD* (target gene/GAPD × 10^3). C, *in vitro* growth inhibitory effect of BIBF 1120 in 4 HCC cell lines by an MTT assay; bars, SD of 3 independent experiments. This assay was conducted in 3 independent experiments.

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 ± 634 mm^3; 50 mg/kg/d, 488 ± 489 mm^3; and 100 mg/kg/d, 572 ± 556 mm^3. 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$^+$pTyr$^+$ PBLs are a pharmacodynamic biomarker *in vivo*

VEGFR2$^+$CD45^{dim} PBLs are generally regarded as circulating endothelial cells (22); therefore, we hypothesized that VEGFR2$^+$CD45^{dim} PBLs might be useful as a biological biomarker of VEGFR2 TKIs. The effects of BIBF 1120 on the pTyr levels of VEGFR2$^+$CD45^{dim} PBLs and the percentage of VEGFR2$^+$pTyr$^+$ 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$^+$CD45^{dim} 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$^+$pTyr$^+$ 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$^+$CD45^{dim} 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$^+$pTyr$^+$ 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

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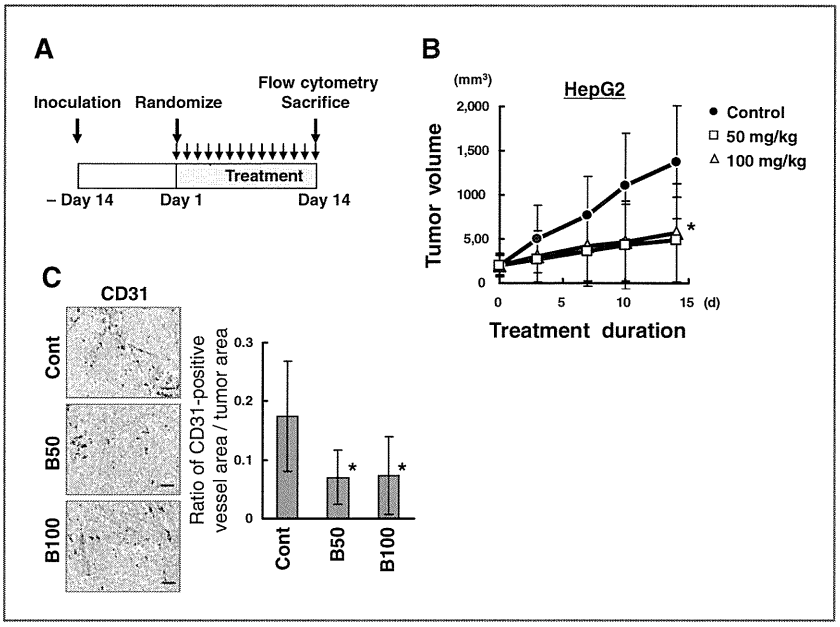

Figure 3. BIBF 1120 exhibited the antitumor and antiangiogenic effects against HCC *in vivo*. A, schema of the BIBF 1120 treatment schedules. Mice were inoculated with HepG2 cells for 14 days. The mice were then randomized into 3 groups ($n = 6$ in each group) and treated with BIBF 1120 (50 mg/kg/d, p.o.), BIBF 1120 (100 mg/kg/d, p.o.), or the vehicle control (p.o.) for 14 days. On day14, the mice were euthanized; blood was collected for the following biomarker study, and tumor specimens were collected for immunohistochemistry. B, inhibition of tumor growth by BIBF 1120 treatment. The tumor volume was assessed every 2 to 3 days ($n = 6$ in each group). Bars, SD. *, $P < 0.05$. C, inhibition of tumor angiogenesis by BIBF 1120 treatment was evaluated using the CD31 staining of tumor samples. Representative data are shown. MVD was quantified by measuring the number of CD31-positive endothelial cells in the tumors. Ten random fields per tumor sample at a magnification of ×200 were captured and saved for computer-assisted image analysis using the ImageJ software package. The *y*-axis represents the ratio of the CD31-positive vessel area/tumor area. Scale bar, 100 μm. Cont, tumor sample treated with vehicle control. B50 and B100, tumor sample treated with BIBF 1120 (50 mg/kg/d, 100 mg/kg/d, p.o.); *, $P < 0.05$.

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

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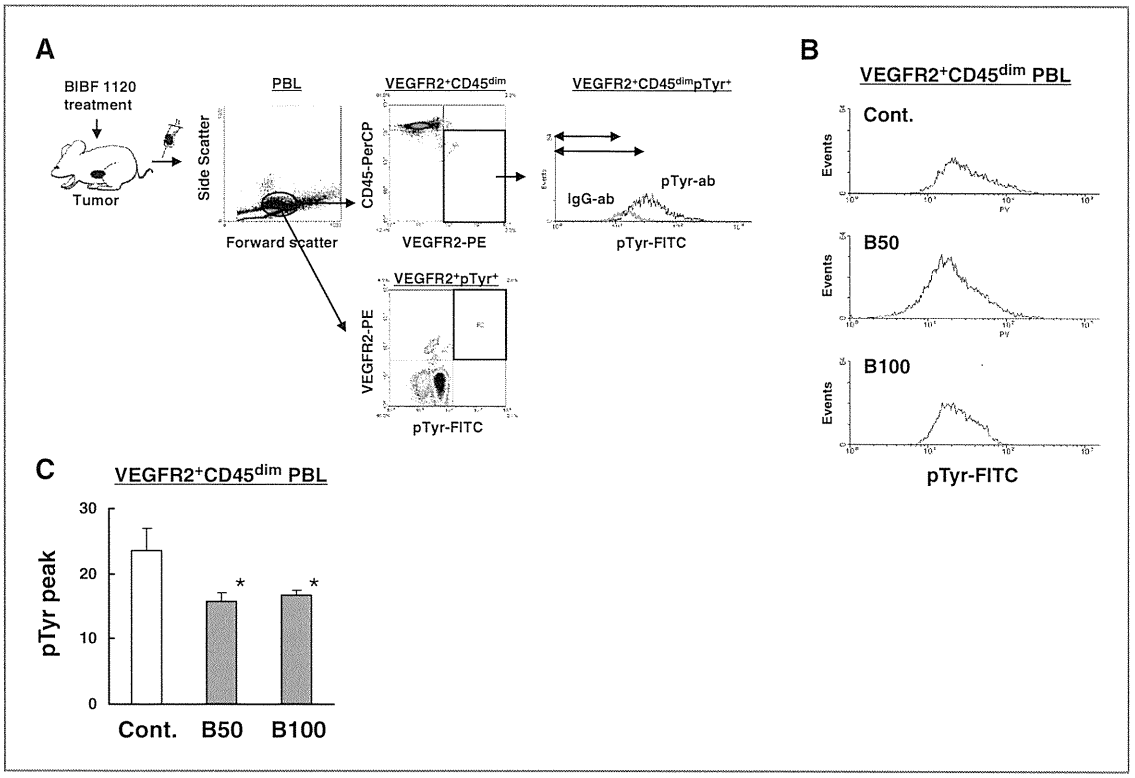

Figure 4. Evaluation of VEGFR2$^+$CD45^{dim} PBLs as a biomarker *in vivo*. A, schema of treatment schedules of BIBF 1120 and detection methods. Peripheral blood samples obtained from BIBF 1120–treated mice were stimulated with 20 ng/mL of VEGF for 30 minutes. The cells were fixed, permeabilized, and reacted with the following antibodies: anti-mouse CD45-PerCP, anti-mouse Flk-1-PE, and anti-pTyr-FITC (fluorescein isothiocyanate). Two methods, the tyrosine phosphorylation levels of VEGFR2$^+$CD45^{dim} PBLs and the percentage of VEGFR2$^+$pTyr$^+$ PBLs, were examined. B and C, BIBF 1120 significantly inhibited the pTyr levels of VEGFR2$^+$CD45^{dim} PBLs *in vivo*. Cont, blood sample from vehicle control. B50 and B100, blood samples from BIBF 1120 (50 mg/kg/d, 100 mg/kg/d; p.o.) treatment groups; bars, SD. *, $P < 0.05$.

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

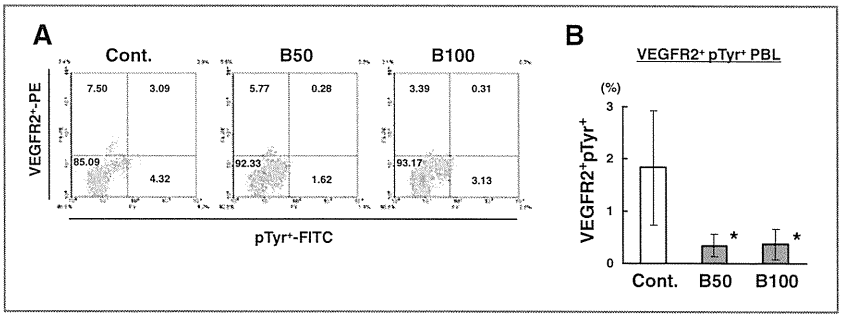

Figure 5. VEGFR2$^+$pTyr$^+$ PBLs can be used as a pharmacodynamic biomarker *in vivo*. A, the percentage of VEGFR2$^+$pTyr$^+$ PBLs obtained from BIBF 1120–treated mice. The numeral data indicate the percentage (%) in each quadrant. Representative data are shown. B, BIBF 1120 significantly inhibited the percentage of VEGFR2$^+$pTyr$^+$ PBLs. Cont, blood samples from vehicle control group ($n = 6$, not treated with drug). B50 and B100, blood samples from BIBF 1120 treatment groups ($n = 6$, 50 mg/kg/d; $n = 6$, 100 mg/kg/d; p.o.); bars, SD. *, $P < 0.05$.

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(LUME-Lung 1: 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 may 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 (IC_{50} values: 2–5 μmol/L) *in vitro*. BIBF 1120 exhibited potent antitumor and antiangiogenic 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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Molecular Targeted Therapy for Hepatocellular Carcinoma: Bench to Bedside

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Key Words
Hepatocellular carcinoma · Molecular targeted therapy · Signal transduction · Sorafenib

Abstract
According to the International Agency for Research on Cancer, approximately 670,000 new cases of hepatocellular carcinoma (HCC) developed in 2005, making it the fifth most common cancer and third most common cause of cancer-related death worldwide. HCC is a complex and heterogeneous tumor with several genomic alterations. There is evidence of aberrant activation of several signaling cascades such as EGFR, Ras/Raf/MEK, PI3K/mTOR, HGF/MET, Wnt, Hedgehog and apoptotic signaling pathway. Recently a multikinase inhibitor, sorafenib, has shown survival benefits in patients with advanced HCC. It has been proposed that signaling pathway disruption in cancer can be grouped in six function capabilities, some of which need to be altered for cancer development: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tumor invasion and metastases. The aim is to integrate these concepts into the molecular pathogenesis of HCC. It has also been proposed that there are common disturbances universal to all liver cancers on top of the more specific mechanisms. Based on this basic research, a molecular targeted agent has recently been developed. There have been no effective chemotherapeutic agents for advanced HCC. Sorafenib, an oral multikinase inhibitor, has set a milestone in the management of HCC in that it is the first agent to significantly improve the overall survival in patients with advanced HCC in a double-blind, placebo-controlled, phase III study. Clinical trials testing new agents for first- and second-line agents, as well as in combination with existing treatment options such as transarterial chemoembolization or arterial infusion chemotherapy, are ongoing. The results of these trials are therefore eagerly awaited.

Introduction

The 7th Japan-Korea Liver Symposium, the main theme of which was 'Molecular Targeted Therapy for Hepatocellular Carcinoma: Bench to Bedside', was held in Kyoto, Japan, on July 17–18, 2010, to focus on and discuss current topics of basic science and clinical application of

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molecular targeted agents. The symposium was full of eye-opening lectures by world-leading scientists, followed by extensive discussion. This special issue of *Digestive Diseases* selects the most important articles presented at this congress.

Molecular Pathogenesis

Kim and Lee's group [1] described the possibility of TGFBR3 polymorphisms and its haplotypes might be associated with HBV clearance and age of HCC occurrence.

Molecular alterations such as the p53 mutation, p16 gene silencing, and AKT signaling activation are found in the late stage of HCC progression. The overexpression of some marker molecules is observed at the early stage. Transforming growth factor-β (TGF-β), a potent inhibitor of cell proliferation, is frequently overexpressed in HCC, although the role of TGF-β signaling during HCC development remains controversial [2]. Authors previously reported that HCC cells show TGF-β receptor-dependent growth inhibition in response to TGF-β. Also, reduced TFG-β receptor II in HCC correlates with intrahepatic metastasis and shorter time to recurrence, suggesting a role of TGF-β signaling in tumor suppression. In contrast, TGF-β overexpression in HCC is known to correlate with malignant potential, suggesting a role in tumor promotion. Enhanced formation of stroma is a feature of advanced HCC, and TGF-β also promotes the proliferation of stromal fibroblasts. The microenvironment produced via tumor-stromal interactions may be the key to the modulation of the dual roles of TGF-β signaling in HCC progression [2].

Signaling Pathways and HCC

As in other cancers, the molecular mechanisms involved in the development and progression of HCC are complex. It has been shown that, after HBV/HCV infection and alcohol or aflatoxin B_1 exposure, genetic and epigenetic changes occur, including oncogene activation and tumor-suppressor gene inactivation due to inflammation-induced increase in hepatocyte turnover and oxidative stress-induced DNA damage. Through apoptosis and cell proliferation, these changes lead to the multistep development and progression of a hyperplastic to dysplastic nodule, early HCC, and advanced HCC. A number of studies have reported changes in gene expression, chromosomal amplification, mutations, deletions and copy number alterations (gain/loss), somatic mutations, CpG hypermethylation, and DNA hypomethylation, as well as molecular abnormalities, which can constitute therapeutic targets [3–7].

The binding of growth factors to their receptor proteins activates protein-phosphorylating enzymes, thus activating a cascade of proliferative signaling pathways to transmit proliferative signals into the nucleus. Growth factors, such as epidermal growth factor (EGF), TGF-α/-β, insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF), also function in liver regeneration after injury, while fibroblast growth factor and the platelet-derived growth factor (PDGF) family are involved in liver fibrosis and HCC growth [6, 7]. The receptors for these growth factors are broadly classified into G-protein-coupled receptors and protein kinases. On ligand binding, these receptors activate their downstream intracellular molecules in a cascade fashion. Many of the growth factor receptors and oncogenes have tyrosine kinase activity, and the tyrosine kinases are classified into transmembrane receptor tyrosine kinases such as the EGFR and VEGFR, and cytoplasmic non-receptor tyrosine kinases such as Abl and Src. On the other hand, Raf, MAP kinase/ERK kinase (MEK) and mammalian target of rapamycin (mTOR) are serine/threonine kinases.

In general, the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt/mTOR, c-MET, IGF, Wnt-β-catenin and Hedgehog signaling pathways, and the VEGFR and PDGFR signaling cascades show altered activity in HCC, and agents targeting these pathways are under development. Many molecular targeted agents are now under development and the target signaling pathways and growth factors are outlined below.

Sorafenib for HCC as a Stepping Stone

Disease stabilization with sorafenib lasts a few months, possibly due to the development of resistance, and thus the survival advantage was modest, even in patients with preserved liver function. Furthermore, there is currently no biomarker for monitoring the response or resistance to sorafenib. Currently, various kinds of molecularly targeted agents have been developed and are being evaluated in clinical trials. There are several steps required to improve the outcome from sorafenib therapy. First, a reliable predictive and prognostic biomarker is urgently needed. A compelling indication of sorafenib treatment

for HCC needs more clinical studies and consensus. The actual benefits of sorafenib to patients with advanced liver dysfunction should be clarified and a more effective strategy for targeted therapy needs to be developed, for example, using a combination of targeted agents acting on different pathways or different levels of a key pathway. Finally, sorafenib could be used with other treatment modalities, such as local ablation or transarterial chemoembolization (TACE), to synergize efficacy. Based on the successful introduction of sorafenib, future studies should focus on plans to further improve the outcome of HCC patients by overcoming resistance and maximizing the efficacy of molecularly targeted therapy.

Des-γ-Carboxy Prothrombin as a Promising Biomarker of Sorafenib to HCC

Murata et al. [8–10] reported that hypoxia induces des-γ-carboxyprothrombin (DCP). They explained this phenomenon as follows. The fine filamentous actin network, which plays a crucial role in clathrin-mediated endocytosis of vitamin K, is disrupted in DCP-producing cells because of hypoxia. It is considered that this offers one explanation for the elevated serum DCP level in patients with HCC, for which sorafenib is effective. In this issue, Ueshima et al. [11] reported that TTP in patients with a rapid increase in DCP within 2 weeks after starting sorafenib was significantly longer than that in patients with no increase in DCP. The CT findings for HCC with rapid DCP elevation tended to include reduced vascularity or presence of necrosis. This indicates that hypoxia was responsible for the change in DCP production. Accordingly, DCP may offer a surrogate marker for hypoxia.

Sorafenib induces hypoxia in HCC by inhibiting angiogenesis. TACE exposes the HCC to hypoxia, as does sorafenib, but this change is very rapid and most of the tumor cells become necrotic. It is thought that not enough DCP is produced after TACE. On the other hand, sorafenib induces tissue hypoxia relatively slowly and many viable HCC cells are exposed to hypoxia. During sustained hypoxia, the tumor cells gradually die and the serum level of DCP subsequently decreases.

During molecular targeted HCC therapy using sorafenib, Ueshima et al. [11] found that the rapid increase in DCP after starting sorafenib does not indicate tumor progression, but rather indicates HCC tissue hypoxia. Therefore, DCP may be a useful predictive marker for the duration of tumor suppression.

Branched-Chain Amino Acid Granules and HCC Incidence

Protein/energy malnutrition is commonly observed in patients with cirrhosis, and is represented by a decreased serum albumin level and skeletal muscle volume, and a decline in the non-protein respiratory quotient [12]. A decreased blood concentration of branched-chain amino acid (BCAA), caused by enhanced uptake and consumption of BCAA by skeletal muscle for ammonia metabolism and energy generation, is another manifestation of protein/energy malnutrition in patients with cirrhosis and is associated with disorders of protein synthesis and liver regeneration, and hyperammonemia [13]. Therefore, BCAA supplementation is a rational treatment for patients with cirrhosis.

Two large randomized controlled trials recently demonstrated that oral BCAA supplementation decreased the frequency of complications of cirrhosis and improved event-free survival in patients with decompensated cirrhosis [14, 15]. Based on these findings, oral BCAA supplementation is now recommended in Japanese guidelines as part of the treatment of HCV-related cirrhosis [16]. Similar to the reports mentioned above, Hayaishi et al. [17] showed that the event-free survival of patients with Child-Pugh A cirrhosis was better among patients given oral BCAA supplementation than in those without, although the difference did not reach statistical significance. BCAA supplementation has also been reported to be useful as an adjuvant nutritional therapy following hepatectomy and TACE, showing reduced risk of complications and better maintenance of liver function [18–20]. According to the earlier reports, BCAA seems to reduce the incidence of complications of cirrhosis by enhancing ammonia detoxification, upregulating protein synthesis and downregulating proteolysis, enhancing liver regeneration, and improving immune function [21–24].

Consensus-Based Clinical Practice Manual for HCC Proposed by the Japan Society for HCC of Hepatology

Following the publication by the European Society of Study of the Liver (EASL) in 2001 [25], the American Association for the Study of Liver Disease (AASLD) published the Clinical Practice Guidelines of hepatocellular carcinoma (HCC) in *Hepatology* in November 2005 [26] and updated in 2010 [27].

In Japan, the original Evidence-Based Clinical Practice Guidelines of HCC were published in 2005 [28] and updated in 2009 [29], disclosed on the website of the Japan Society of Hepatology (JSH) (www.jsh.or.jp/), and then widely used for liver cancer treatment in Japan. An excerpted version has also been published in an English journal by Makuuchi and Kokudo's group [29–31]. These guidelines were prepared after critical evaluations based on about 100 reports with a high evidence level in each field selected from 7,118 reports on HCC published between 1966 and 2002. In the 2009 revised version, 2,950 articles were reviewed and 532 were incorporated into the new version. Since the guidelines were prepared based as much as possible on highly evidenced data, some points may slightly deviate from actual practices related to HCC routinely performed based on the experience and consensus of HCC experts in Japan.

Considering this situation, the JSH summarized HCC treatment as performed in Japan with the consensus opinions of many experts, even though clear evidence was not available, and published a simple manual in 2007 [32, 33] and updated in 2010 [34, 35]. This was an experience- or consensus-based manual based on evidence-based guideline in respect to the evidence level, and summarized the consensus of expert opinions, widely reflecting the actual state of HCC treatment in Japan.

The manual was prepared in accordance with the Evidence-Based Clinical Practice Guidelines reported by Makuuchi and Kokudo [30], Kokudo et al. [31] and Arii et al. [36], and thus contains no conflict with those guidelines. Points that slightly differ are a more detailed explanation of liver cancer treatments based on expert opinions, and a summary of the consensus by the expert panel. Although it may seem unusual that two different guidelines are available and followed in Japan, they both have different roles and are not contradictory.

The report in this issue introduces the revised version of Consensus-Based Clinical Practice Manual of HCC published by the JSH in 2010, and focuses on prevention, surveillance, pathology, diagnosis, staging, and treatment. This constitutes a 'practice manual' summarized by the expert panel of the JSH, and is different from the Clinical Practice Guidelines. The contents of this report may be considered as the current state of the most advanced HCC treatment practices in Japan.

Disclosure Statement

The author has no conflict of interest to declare.

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Signaling Pathway and Molecular-Targeted Therapy for Hepatocellular Carcinoma

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Key Words
Hepatocellular carcinoma · Molecular-targeted agent · Sorafenib · Sunitinib · Brivanib · Complete remission

Abstract
In recent years, molecular-targeted agents have been used clinically to treat various malignant tumors. In May 2009, sorafenib (Nexavar®) was approved in Japan for 'unresectable hepatocellular carcinoma (HCC)', and was the first molecular-targeted agent for use in HCC. To date, sorafenib is the only molecular-targeted agent whose survival benefit has been demonstrated in two global phase III randomized controlled trials, and has now been approved worldwide. Phase III clinical trials of other molecular-targeted agents comparing them with sorafenib as first-line treatment agents are now ongoing. Those agents target the vascular endothelial growth factor, platelet-derived growth factor receptors, as well as target the epidermal growth factor receptor, insulin-like growth factor receptor and mammalian target of rapamycin, in addition to other molecules targeting other components of the signal transduction pathways. This review outlines the main pathways involved in the development and progression of HCC and the agents that target these pathways. Finally, current status and future perspective will also be discussed.

Introduction

The advances in molecular cell biology over the last decade have clarified the mechanisms involved in cancer growth, invasion and metastasis, and enabled the development of molecular-targeted agents, best represented by trastuzumab for breast cancer, imatinib and rituximab for hematopoietic tumors, and gefitinib and erlotinib for lung cancer. These molecular-targeted agents are broadly classified into two categories; drugs targeting cancer cell-specific molecules, and non-specific molecular-targeted drugs for molecular biological abnormalities induced in the host stroma or blood vessels by the presence of cancer. Examples of the former approach include trastuzumab, which targets HER2, the expression of which is a poor prognostic factor for breast cancer; rituximab, which is used to treat B-cell lymphoma, targets CD20 expressed on normal and neoplastic mature B cells; while imatinib binds to the ATP-binding site of Bcr-abl, a protein that causes chronic myelogenous leukemia. However, no critical target molecules responsible for treatment response have been identified in hepatocellular carcinoma (HCC).

In recent years, clinical trials have been conducted for many agents that act on growth factor receptors (such as epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR)) and intra-

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cellular signaling pathways. In addition, multikinase inhibitors, including sorafenib, have emerged and evaluated. Clinical trials are now ongoing to compare drugs with the same mechanism of action and to test the combined efficacy and relative merits of these drugs with existing drugs for many cancers. Since the main treatment option for metastatic, advanced-stage cancers, such as breast and colorectal cancer, is systemic chemotherapy, clinical trials are ongoing to investigate how to combine molecular-targeted agents with standard therapies based on the results of long-term, large-scale clinical trials, and to identify which molecular-targeted agents should be used as initial or second-line therapy.

However, for HCC, background liver damage limits the indication for systemic chemotherapy and no anticancer drugs were found to be effective in large-scale randomized controlled trial except sorafenib. Now that the usefulness of sorafenib has been demonstrated in two large-scale randomized clinical trials, the development of new drugs that are effective for poor prognostic advanced HCC, who are resistant to a standard of care agent, sorafenib.

Signaling Pathways and Molecular-Targeted Agents in HCC

As in other cancers, the molecular mechanisms involved in the development and progression of HCC are complex. It has been shown that, after HBV/HCV infection and alcohol or aflatoxin B1 exposure, genetic and epigenetic changes occur, including oncogene activation and tumor-suppressor gene inactivation due to inflammation-induced increase in hepatocyte turnover and oxidative stress-induced DNA damage. Through apoptosis and cell proliferation, these changes lead to the multistep development and progression of a hyperplastic to dysplastic nodule, early HCC, and advanced HCC. A number of studies have reported changes in gene expression, chromosomal amplification, mutations, deletions and copy number alterations (gain/loss), somatic mutations, CpG hypermethylation, and DNA hypomethylation, as well as molecular abnormalities, which can constitute therapeutic targets [1–5].

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In general, the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt/mTOR, c-MET, IGF, Wnt-β-catenin and Hedgehog signaling pathways, and the VEGFR and PDGFR signaling cascades show altered activity in HCC, and agents targeting these pathways are under development (fig. 1, 2; table 1) [9–11]. Many molecular-targeted agents are now under development and the target signaling pathways and growth factors are outlined below.

MAPK Pathway (Ras/Raf/MEK/ERK)

The MAPK intracellular signaling pathway, which is mainly involved in cell growth and survival, and regulates cell differentiation, is upregulated in cancer cells. Therefore, this pathway has been extensively studied as a therapeutic target. The MAPK pathway is a common downstream pathway for the EGFR, PDGFR and VEGFR, and is universally used for signal transduction downstream of cytokine receptors, integrin complexes and G-protein receptors to Ras. The MAPK pathway also plays an important role in HCC in that its activation is reportedly involved in HCC growth and survival [5]. The downstream extracellular signaling-regulated kinase (ERK) is activated by two upstream protein kinases, which are coupled to growth factor receptors by Ras proteins. Ras, which is activated by ligand binding, activates Raf serine/threonine kinases and MEK (MAP kinase/ERK kinase), while MEK phosphorylates and activates ERK, which phosphorylates proteins involved in cell growth, apoptosis resistance, extracellular matrix production and angiogenesis [12–15].

Raf and Ras Inhibitors. Raf and Ras are proto-oncogenes. In particular, K-ras mutations are commonly observed in many cancers, including pancreatic and colorec-