Growth inhibition and induction of apoptosis in acute myeloid leukemia cells by new indolinone derivatives targeting fibroblast growth factor, platelet-derived growth factor, and vascular endothelial growth factor receptors

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Abstract
In acute myeloid leukemia (AML), receptor tyrosine kinase ligands promote growth and survival and contribute to AML-associated marrow neoangiogenesis. We have tested simultaneous inhibition of vascular endothelial growth factor, fibroblast growth factor, and platelet-derived growth factor receptor signaling by novel indolinone derivatives using 14 myeloid, including 11 human leukemic, cell lines. Compounds inhibited colony formation of all cell lines in a dose-dependent fashion. Inhibitory concentrations for 50% of the colony formation/survival (IC50) for BIBF1000 were < 100 nmol/L for 3 of 11, 500 nmol/L for 6 of 11, and < 1,000 nmol/L for 10 of 11 leukemic cell lines, with one cell line being resistant in the dose range < 1,000 nmol/L. BIBF1120 was less effective with 4 of 11 leukemic cell lines being resistant within the dose range < 1,000 nmol/L. Testing of myeloid 32D cells transfected with empty vector, wild-type Flt3, or Flt3 carrying an internal tandem duplication mutation revealed higher resistance for the internal tandem duplication mutant. These effects of the compounds were associated with inhibition of tyrosine phosphorylation and the mitogen-activated protein kinase pathway. Furthermore, both compounds induced apoptosis in the sensitive cell lines. In Mono-Mac1 cells, the compounds had a direct proapoptotic effect that was increasing the proapoptotic effect of 1-beta-D-arabinofuranosylcytosine. The data provide a rationale for clinical evaluation of these tyrosine kinase inhibitors in AML. [Mol Cancer Ther 2006;5(12):3105 – 12]

Introduction
Acute myeloid leukemia (AML) represents a group of malignant diseases still leading to the death of the majority of patients. Thus, innovative treatment approaches are urgently needed. Recent evidence suggests that bone marrow neoangiogenesis plays an important role in the pathogenesis of AML (1–4). Experimental models have shown that a paracrine feedback loop between AML blasts and endothelial cells involving vascular endothelial growth factor (VEGF) is operational (4). Furthermore, stromal secretion of platelet-derived growth factor (PDGF) may also be important in the biology and progression of AML (5), and we have recently reported that basic fibroblast growth factor (bFGF) can also act as an autocrine cytokine in AML (6). As c-kit, the receptors for VEGF (Flk1/KDR), PDGF (PDGFR), and bFGF are all members of the split kinase domain family of receptor tyrosine kinases (RTK).

Small-molecule tyrosine kinase inhibitors represent a new class of targeted drugs. As an outstanding example of their therapeutic potential, imatinib (Gleevec, formerly STI571) is remarkably effective in the treatment of chronic myeloid leukemia by inhibition of the Abl kinase that is deregulated as a consequence of the oncogenic Bcr/Abl gene fusion (7). Related compounds, designated RTK inhibitors (RTKI), hold promise as antineoplastic agents by interference with receptor-mediated extrinsic cellular growth and survival signals. RTKIs with different receptor specificities are currently under investigation in various malignancies.

Recent evidence suggests that RTKIs with different target specificities show clinical activity in AML, leading to complete and partial remissions in some patients (8–10). Two novel RTKIs were used for this study: the indolinone derivatives BIBF1000 and BIBF1120. Both compounds competitively bind to the ATP-binding sites within the kinase domains of VEGFR1 through VEGFR3, FGFR1,
FGFR3, and PDGFRα and PDGFRβ. Preclinical analysis showed good antitumor efficacy in several tumor xenograft models in nude mice and strong inhibition of tumor angiogenesis using both compounds (11, 12). The importance of these target receptors for AML and the lack of data concerning activity of both indolinone derivatives against AML prompted us to analyze BIBF1000 and BIBF1120 in AML in vitro.

Materials and Methods

Cells
The cell lines were purchased from the American Type Culture Collection (Manassas, VA) or from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Bone marrow cells from AML patients in complete remission were Ficoll separated for obtaining normal mononuclear bone marrow precursor cells according to standard procedures. Patients’ informed consent was obligatory.

Novel Indolinone Derivatives and Other Reagents
The small-molecule inhibitors BIBF1000 and BIBF1120 were provided by one of the authors (F.H., Boehringer Ingelheim Austria, Vienna, Austria; refs. 11, 12). The structure of BIBF1000 is shown in Fig. 1. A 0.2 mol/L stock solution was prepared in DMSO. To obtain adequate working concentrations, the stock solution was diluted with double-distilled water containing at least 0.005% DMSO. The inhibitory activity and specificity of the compounds was determined in biochemical assays measuring autophosphorylation of RTKs. The assays were done using recombinant clones of baculovirus constructs containing GTS fusion proteins of the cytoplasmatic kinase domains of these receptors (11, 12). Inhibitory concentrations for 50% (IC50) for various RTKs using BIBF1000 as an example are 40 nmol/L for VEGFR1, 28 nmol/L for VEGFR2, 142 nmol/L for VEGFR3, 43 nmol/L for FGFR1, 52 nmol/L for FGFR3, and 35 nmol/L for PDGFRα. The human epidermal growth factor receptor-2 (HER-2), insulin-like growth factor receptor, and the insulin receptor were among the insensitive receptors with IC50 values >10,000 nmol/L.

BIBF1000 (WO 01/27081)

Recombinant human VEGF165, VEGF121, and PDGF were purchased from R&D Systems (Wiesbaden, Germany). bFGF was from Boehringer (Mannheim, Germany). Annexin V-FITC and propidium iodide were purchased from BD PharMingen (BD Biosciences, Heidelberg, Germany). Anti–phospho-extracellular signal-regulated kinase 1/2 (T202/ Y204) antibody was purchased from BD PharMingen (clone 20a, mouse IgG1, conjugated to phycocerythrin), monoclonal anti-phosphotyrosine antibody was from Sigma-Aldrich (Taufkirchen, Germany; clone PT-66, mouse IgG1, conjugated to FITC). Antibodies used for immunocytochemistry and Western blotting/immunoprecipitation were anti–phospho-p44/p42 mitogen-activated protein kinase (MAPK; Thr202/ Tyr204) from Cell Signalling Technology (New England Biolabs, Frankfurt am Main, Germany), rabbit immunoaffinity purified IgG and anti–MAPK 1/2 (extracellular signal-regulated kinase 1/2) from Upstate (Biomol, Hamburg, Germany), and rabbit immunoaffinity purified IgG. For secondary antibody, peroxidase-conjugated AffiniPure donkey anti-rabbit IgG (H+L) antibody from Jackson ImmunoResearch (Dianova, Hamburg, Germany) was used.

Human Tumor Cloning Assay
The assay for anchorage-independent clonal leukemia cell growth was done in a mixture of methylcellulose and agar as described before (13–15). Briefly, 3,000 viable (trypan blue dye exclusion) cells were suspended in 1 mL of a mixture containing 8.35 mg/mL methylcellulose (Sigma, St. Louis, MO) and 1.78 mg/mL agar noble (Difco, Detroit, MI), both previously dissolved in double-distilled water by boiling; 30% (v/v) FCS (Biochrom KG, Seromed, Berlin, Germany); 40% (v/v) Iscove’s modified Dulbecco’s medium; 14% (v/v) RPMI 1640; and 55 μmol/L β-mercaptoethanol. Cells were preincubated with the experimental drugs or control vehicle for 4 h at 37°C and 5% CO2 and transferred into 35-mm Petri dishes again containing experimental drugs or control vehicles as indicated to give the final concentrations in the assay mixture as stated in Results. Human tumor cloning assay (HTCA) within the dishes were incubated at 37°C and 5% CO2 with saturated humidity for 10 to 12 days. Colonies (>50 cells) were counted with an inverted microscope on day 1 to exclude preexisting cell clumping and after the incubation period. Results are presented as IC50 values indicating the molar concentration of the drug necessary to inhibit 50% of the colony growth when compared with the controls (means of six dishes). Some data are given by showing the absolute colony numbers per dish, where each data point represents the mean ± SD of counts from six dishes.

Flow Cytometry
Annexin V and Propidium Iodide Staining for Apoptosis and Cell Death Analysis. For assaying the influence of BIBF1000, BIBF1120, and combinations of these indolinone derivatives with cytosine arabinoside [1-β-D-arabinofuranosylcytosine (ara-C)] on induction of cellular apoptosis and cell death, 3 × 104 cells per 1 mL were incubated (37°C, in an atmosphere of 5% CO2 and high humidity) either with 100 or 500 nmol/L BIBF1000, BIBF1120, or control medium with only DMSO for 1 h (basis value for
spontaneous apoptosis) and 48 h (2 days). Assessment of apoptotic and dead cells was carried out by Annexin V and propidium iodide staining. After incubation, cells were washed twice with PBS, and cell pellet was resuspended with Annexin V binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl₂] followed by incubation with Annexin V conjugated to FITC (BD PharMingen) and propidium iodide staining solution (BD PharMingen) for 15 min at room temperature in the dark. Cells were then analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) as soon as possible (within 1 h).

Figure 2. Influence of BIBF1000 and BIBF1120 on anchorage-independent clonal growth (HTCA) of human myeloid leukemia cell lines in vitro. Mean colony numbers per 3 x 10³ cells of 6-fold assays from representative experiments are given per cell line. Three groups of cell lines could be classified: (A) one with high (Mono-Mac1 and Kasumi), (B) the second with intermediate (Meg 01 and K 562), and (C) the third with lower (UT-7 and TF-1) sensitivity to the drugs. Note the different dose ranges on the x-axis. P < 0.05 when colony numbers surviving concentrations higher than the IC₅₀ were compared with controls not containing drugs (Mann-Whitney test).
Flow Cytometric Analysis of Phosphotyrosine and Phospho-Extracellular Signal-Regulated Kinase 1/2. Concentration of the cell suspension was adjusted to 3 × 10^4 cells per mL of medium and incubated either with 100 nmol/L BIBF1000, BIBF1120, or DMSO control condition for 4 h at 37°C in a fully humidified atmosphere and 5% CO₂. Collected experimental and control cells were washed twice with wash buffer (PBS, 1% bovine serum albumin, 0.1% sodium azide), fixed in 2% paraformaldehyde solution for 10 min at 37°C, and then permeabilized in 90% methanol overnight at −20°C. After this preparation, cells were washed twice with wash buffer and stained either with anti-phospho-extracellular signal-regulated kinase 1/2 conjugated to phycoerythrin (BD PharMingen) or anti-phosphotyrosine conjugated to FITC (Sigma-Aldrich) and with fluorochrome-conjugated isotype-matched control antibodies (BD Biosciences) for 1 h at room temperature in the dark. After incubation, cells were washed with wash buffer, and flow cytometric analysis was done by using a FACSCalibur flow cytometer (Becton Dickinson).

Statistics
Statistical significance of overall differences between multiple groups was analyzed by the Kruskal-Wallis one-way ANOVA. If the test was significant, pairwise comparisons were done by the multiple-comparison criterion. Differences between two independent groups were analyzed by the Mann-Whitney rank sum test. The Wilcoxon matched-pair signed rank test was used for comparison of differences within pairs. P ≤ 0.05 was considered significant.

Results
Inhibitory Profile of BIBF1000 and BIBF1120 in the HTCA
In a first set of experiments, we have tested the activity of the two novel indolinone derivatives BIBF1000 and BIBF1120 in inhibiting the colony formation of human myeloid leukemic cell lines in an anchorage-independent colony growth assay using semisolid media. We have used such an assay because its in vitro results strongly predict the in vivo tumorigenicity of a given cell type (16, 17) and the in vivo growth-modulating effects of cytokines (13, 14). This type of assay is also used to predict clinical responsiveness of human tumors to cytokotoxic drugs, with good in vitro-to-in vivo predictability (18, 19). Thus, this colony-based assay seems to have high clinical relevance. Eleven human leukemic cell lines have been tested. Both indolinone derivatives inhibited colony growth of all cell lines in a dose-dependent fashion. Three groups of leukemic cell lines exhibiting high, intermediate, and low sensitivity

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ (nmol/L)</th>
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<tr>
<td></td>
<td>BIBF1000</td>
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<tr>
<td>HL-60</td>
<td>500</td>
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<tr>
<td>Kasumi-1</td>
<td>8</td>
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<tr>
<td>K 562</td>
<td>600</td>
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<td>Meg01</td>
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<td>Moe 7</td>
<td>8</td>
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<tr>
<td>Mono-Mac1</td>
<td>8</td>
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<tr>
<td>NB-4</td>
<td>100</td>
</tr>
<tr>
<td>OCI-AML-2</td>
<td>700</td>
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<tr>
<td>OCI-AML-5</td>
<td>500</td>
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<tr>
<td>TF-1</td>
<td>&gt;1,000</td>
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<td>UT-7</td>
<td>800</td>
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<td>32D</td>
<td>400</td>
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<tr>
<td>32D-FLT3-ITD</td>
<td>&gt;1,000</td>
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<tr>
<td>32D-FLT3-WT</td>
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NOTE: IC₅₀ values were taken from more than two experiments.
towards the compounds could be characterized (for examples, see Fig. 2). IC_{50} values of the colony formation (see Table 1) for BIBF1000 were <100 nmol/L for 3 of 11, ≤500 nmol/L for 6 of 11, and <1000 nmol/L for 10 of 11 leukemic cell lines, with one cell line (TF1) being resistant in the dose range ≤1,000 nmol/L. BIBF1120 was comparatively less effective with 4 of 11 leukemic cell lines being resistant within the dose range <1,000 nmol/L (Table 1).

Furthermore, we have tested mouse myeloid 32D cells transfected with either empty vector (as control), wild-type Flt3, or Flt3 carrying an internal tandem duplication mutation cloned from an AML patient in the same assay. Cell lines and transfection methodology was published earlier (20, 21). These experiments revealed higher resistance for the internal tandem duplication mutant against both compounds (Table 1), indicating mutated Flt3 as not being a target for the activity of these indolinone derivatives.

In the HTCA, 50 ng/mL VEGF, bFGF, and PDGF induced significantly more colony growth of leukemic cell lines than medium in the controls. For example, in Mono-Mac1 cells, stimulation of colony growth was 2.4-fold of controls by bFGF, 3.3-fold by VEGF, and 3.0-fold by PDGF (P < 0.003 when compared with controls). Combinations of these three cytokines did not elicit stronger stimulation of colony growth than single factors alone (2.7-fold). On the other hand, the antileukemic activity of the BIBF compounds observed could not be reproducibly abrogated by addition of VEGF, bFGF, PDGF, or combination of these cytokines to the cultures (details not shown). This is compatible with a specific intracytosolic inhibitory effect of the BIBF compounds on receptor signaling.

Figure 4. Influence of BIBF1000 and BIBF1120 on MAPK phosphorylation/activation as measured by flow cytometry (see Materials and Methods). One of three experiments with similar results. Filled histograms, DMSO control condition; open histograms, fluorescence intensity of either BIBF1000 (A) or BIBF1120 (B) conditions. Shift of histograms to the left represents inhibition of MAPK phosphorylation/activation by the test compounds. Isotype controls are shown with dotted lines. The three cell lines as assayed in the HTCA either showed high (Mono-Mac1), intermediate (K562), or low (TF-1) sensitivity towards the colony growth inhibition by the compounds. Thus, there was a direct correlation between colony growth inhibition and inhibition of MAPK phosphorylation by the drugs, as the most sensitive cell lines in the HTCA showed quantitatively more inhibition of MAPK phosphorylation than cell lines of an intermediate or a low sensitivity. Isotype controls are shown with dotted lines.

Figure 5. Influence of BIBF1000 and BIBF1120 on MAPK phosphorylation/activation in normal bone marrow precursor cells as measured by flow cytometry (see Materials and Methods). One of three experiments with similar results. Filled histograms, DMSO control condition; open histograms, fluorescence intensity of either BIBF1000 (100 nmol/L for 4 h, top) or BIBF1120 (100 nmol/L for 4 h, bottom) conditions. There was no visible shift of histograms.
Inhibition of Tyrosine Phosphorylation and MAPK Phosphorylation by BIBF1000 and BIBF1120

Because both test compounds act by inhibition of specific RTKs, we have measured total tyrosine phosphorylation of the cells using flow cytometry. Not only was there a decrease in total phosphorylation induced by BIBF1000 and BIBF1120, but we have also found a direct correlation between in vitro sensitivity of the cell lines against the compounds in the HTCA and inhibition of tyrosine phosphorylation by these compounds (for examples, see Figs. 2 and 3). Cell lines with a high inhibition of tyrosine phosphorylation also showed lower colony growth/survival under the influence of BIBF1000 and BIBF1120 and vice versa.

Because MAPKs are important effectors of receptor signaling to the nucleus elicited by binding of growth factors, such as VEGF, bFGF (22), and PDGF, effects of BIBF1000 and BIBF1120 on MAPK (p44/42) phosphorylation in sensitive leukemic cells were evaluated next. Performing immunocytochemistry, we could show considerable decrease of MAPK phosphorylation (p42 and p44) induced by both drugs after a 4-h incubation period (details not shown). This effect, however, was not complete, indicating other activated RTKs signaling via this pathway under the conditions of the experiments. Inhibition of MAPK phosphorylation could also be shown using Western blotting/immunoprecipitation (details not shown). Next, we did flow cytometry to assay possible correlations between the sensitivity of the cell lines towards colony growth inhibition by the study drugs and their MAPK phosphorylation status. Interestingly, again, there was a direct correlation, as the most sensitive cell lines in the HTCA showed quantitatively more inhibition of MAPK phosphorylation by the drugs than cell lines of an intermediate or a low sensitivity (see Figs. 2 and 4).

In contrast to this inhibition of signaling pathways in sensitive leukemic cells, normal Ficoll-separated bone marrow precursor cells from AML patients in remission were resistant to the antiproliferative activity of the compounds when tested at the same concentrations (Fig. 5).

Induction of Apoptosis

To elucidate further antileukemic effects besides inhibition of colony growth by the indolinone derivatives, we have tested the sensitive cell lines for induction of apoptosis by the two compounds as measured by Annexin V/propidium iodide staining. Figure 6 shows for the Mono-Mac1 cell line an example that both BIBF1000 and BIBF1120 induced cellular apoptosis during a 2-day incubation period. This effect was significant when compared with the controls using DMSO or media only instead of the drugs, and the effect was dose dependent (Fig. 6). Experiments showed BIBF1120 as being the compound with higher efficacy.

Combination of the Indolinone Derivatives with Ara-C

In a next set of experiments, we have investigated the induction of apoptosis in Mono-Mac1 cells by ara-C, BIBF1000, BIBF1120, or combinations of these indolinone derivatives with ara-C as measured by Annexin V/propidium iodide staining. Figure 7 shows for the Mono-Mac1 cell line an example that both BIBF1000 and BIBF1120 induced cellular apoptosis during a 2-day incubation period. This effect was significant when compared with the controls using DMSO or media only instead of the drugs, and the effect was dose dependent (Fig. 6). Experiments showed BIBF1120 as being the compound with higher efficacy.
In three different experiments, the effects were greater than the respective addition of the effects induced by the indolinone derivatives or ara-C when given alone.

**Discussion**

BIBF1000 and BIBF1120 are novel indolinone-derived RTKIs primarily targeting VEGFR1 through VEGFR3, FGFR1 and FGFR3, and PDGFRα. The data presented in this report show that both indolinone derivatives can inhibit anchorage-independent colony growth of all leukemic cell lines in a dose-dependent fashion (Table 1; Fig. 2). IC_{50} for BIBF1000 was ≤500 nmol/L for 6 of 11 and <1,000 nmol/L for 10 of 11 leukemic cell lines, with one cell line being resistant within the dose range <1,000 nmol/L. BIBF1120 was less effective with 4 of 11 leukemic cell lines being resistant within the dose range <1,000 nmol/L. These in vitro effects of the compounds were associated with inhibition of tyrosine phosphorylation and the MAPK pathway (Figs. 3 and 4) and could not be found in normal bone marrow precursor cells at the same concentration (Fig. 5). Additionally, both BIBF compounds induced apoptosis in the sensitive cell lines as measured by Annexin V/propidium iodide (PI) staining (Table 2). IC_{50} for BIBF1000 was ≤500 nmol/L for 6 of 11 and <1,000 nmol/L for 10 of 11 leukemic cell lines, with one cell line being resistant within the dose range <1,000 nmol/L. BIBF1120 was less effective with 4 of 11 leukemic cell lines being resistant within the dose range <1,000 nmol/L. These in vitro effects of the compounds were associated with inhibition of tyrosine phosphorylation and the MAPK pathway (Figs. 3 and 4) and could not be found in normal bone marrow precursor cells at the same concentration (Fig. 5). Additionally, both BIBF compounds induced apoptosis in the sensitive cell lines as measured by Annexin V/propidium iodide fluorescence-activated cell sorting staining (Fig. 6). In Mono-Mac1 cells, the BIBFs had a direct proapoptotic effect that was increasing the proapoptotic effect of ara-C when used in combination (Fig. 7).

Testing myeloid 32D cells transfected with empty vector, wild-type Flt3, or Flt3 carrying an internal tandem duplication mutation cloned from an AML patient revealed higher resistance for the internal tandem duplication mutant against both compounds. Thus, whereas the presence of wild-type Flt3, as described for the majority of AML, does not compromise the activity of the compounds, these data hint to the possibility of higher resistance of internal tandem duplication mutation carrying leukemias, which define a cohort of ~25% among the patients with AML with less favorable prognosis. However, whereas mutated Flt3 certainly represents an important target for new molecules in AML, the majority of cases do not carry activating mutations, and the AML phenotype is certainly a result of a multistep leukemogenic process involving activation of different signaling cascades. Therefore, the search for drugs targeting additional receptor pathways is of utmost importance.

In this respect, the comparatively high activity of both BIBF compounds with IC_{50} values within the ≤500 nmol/L range for >50% of randomly tested samples is remarkable. The primary targets of these structures are VEGFRs, FGFRs, and PDGFRs. Recent experimental evidence suggests that bone marrow neoangiogenesis plays an important role in the pathogenesis of AML (1–4). In addition, some models have shown that a paracrine feedback loop between AML blasts and endothelial cells involving VEGF is operational (4). Furthermore, besides the role of PDGFR receptors in TEL-PDGFRβ–positive chronic myelomonocytic leukemia (23) and FIP1L1-PDGFRα–positive hypereosinophilic syndrome (24, 25), stromal secretion of PDGF has been reported as being important also in the biology and progression of AML (5). Finally, we have recently described...
bFGF acting as an autocrine cytokine in AML (6). This observation is supported by the findings of this study with Mono-Mac1 cells, which can be stimulated to higher colony growth by bFGF. Thus, all three major targets for the indolinone derivatives tested in this report play a role in AML. However, we cannot exclude additional effects of the compounds on other target structures adding to the biological effects observed.

Clinical evidence accumulated in AML over the past years, that RTKIs with different target specificities, including autocrine/paracrine mechanisms for the leukemic cell itself and for supporting microvessel formation in the marrow, show clinical activity leading to complete and partial remissions in some patients (8–10). Remarkably, the indolinone derivatives reported here are among the first RTKIs with FGFRs among their primary targets investigated against AML (26). In a clinical phase I trial in advanced cancer patients, BIBF1120 showed good tolerability, a clinically significant number of patients with stable disease and objective responses (27). This profile makes them particularly interesting.

Taken together, our data provide the strong rationale for evaluation of this novel class of indolinone derivatives with FGFR, PDGFR, and VEGFR signaling within the scope of activity in clinical trials with patients suffering from AML. They also suggest that patients might benefit from combination therapies using standard antileukemic drugs, such as ara-C, together with the new type of RTKIs.

References

Molecular Cancer Therapeutics

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