Activity of the Fibroblast Growth Factor Receptor Inhibitors Dovitinib (TKI258) and NVP-BGJ398 in Human Endometrial Cancer Cells

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Abstract

The recent identification of activating fibroblast growth factor receptor 2 (FGFR2) mutations in endometrial cancer has generated an opportunity for a novel target-based therapy. Here, we explore the therapeutic potential of 2 FGFR inhibitors, the multikinase inhibitor dovitinib (TKI258) and the more selective FGFR inhibitor NVP-BGJ398 for the treatment of endometrial cancer. We examined the effects of both inhibitors on tumor cell growth, FGFR2 signaling, cell cycle, and apoptosis using a panel of 20 molecularly characterized human endometrial cancer cell lines. Anchor-independent growth was studied using soft agar assays. In vivo studies were conducted using endometrial cancer xenograft models. Cell lines with activating FGFR2 mutations (S252W, N550K) were more sensitive to dovitinib or NVP-BGJ398 when compared with their FGFR2 wild-type counterparts (P = 0.073 and P = 0.021, respectively). Both agents inhibited FGFR2 signaling, induced cell-cycle arrest, and significantly increased apoptosis in FGFR2-mutant lines. In vitro, dovitinib and NVP-BGJ398 were both potent at inhibiting cell growth of FGFR2-mutant endometrial cancer cells, but the activity of dovitinib was less restricted to FGFR2-mutant lines when compared with NVP-BGJ398. In vivo, dovitinib and NVP-BGJ398 significantly inhibited the growth of FGFR2-mutated endometrial cancer xenograft models. In addition, dovitinib showed significant antitumor activity in FGFR2 wild-type endometrial cancer xenograft models including complete tumor regressions in a long-term in vivo study. Dovitinib and NVP-BGJ398 warrant further clinical evaluation in patients with FGFR2-mutated endometrial cancer. Dovitinib may have antitumor activity in endometrial cancer beyond FGFR2-mutated cases and may permit greater flexibility in patient selection. Mol Cancer Ther; 12(5); 632–42. ©2013 AACR.

Introduction

Endometrial cancer is the most common gynecologic malignancy in industrialized countries. Early diagnosis of endometrial cancer contributes to the relatively good overall long-term survival. However, for women who present with late-stage disease or who suffer recurrences, outcomes are poor (1). Despite recent advances in the molecular characterization of endometrial cancer, current treatment strategies do not include target-based therapies (2). As such, fibroblast growth factor receptor 2 (FGFR2) may represent a novel molecular target for the treatment of endometrial cancer (3, 4). Earlier studies show somatic FGFR2 mutations in 10% to 16% of primary endometrial cancer specimens with a particularly high occurrence of N550K mutations in the FGFR2 kinase domain and S252W and P253R mutations in the extracellular ligand-binding domain (3–6). The kinase domain mutations such as N550K are likely to result in receptor activation, as an identical mutation in FGFR3 occurs in patients with hypochondroplasia (7). The mutations in the ligand-binding domain such as S252W and P253R activate FGFR2 in a manner that is unique for the FGFR family (FGFR1, 2, 3, and 4; ref. 8). Members of the FGFR tyrosine kinase family are differentially activated by binding to a subset of 18 FGF) in conjunction with heparan sulfate proteoglycan, which stabilizes and sequesters FGFs (9). Mutations in the ligand-binding region may confer a gain in ligand-binding promiscuity such that ligands expressed by endothelial cells that normally only bind to FGFR2 isoforms expressed on mesenchymal cells can now bind to FGFR2 isoforms expressed on epithelial cells leading to an
autocrine stimulation loop (8). Preclinical studies confirm the transforming capacity of such mutations in the ligand-binding domain of FGFR2 in human endometrial cancer models (4). Importantly, the role of FGFR2 mutations as oncogenic drivers and as potential therapeutic target in endometrial cancer has been underscored by findings from a recent clinical study in which FGFR2 mutations were found to be of independent prognostic relevance in early-stage primary endometrial cancer (6).

Dovitinib is a nonspecific FGFR1/2/3 inhibitor, which also targets VEGF receptor (VEGFR)-1/2/3, platelet-derived growth factor receptor (PDGFR)-α/β, c-Kit, and FMS-like tyrosine kinase 3 (FLT3; ref. 10; Fig. 1A). In tumors, dovitinib may act via 2 mechanisms: (i) direct antitumor activity mediated by FGFRs, PDGFRα, and c-Kit, and (ii) antiangiogenesis mediated by VEGFR2, FGFRs, and PDGFRβ. In contrast, NVP-BGJ398 is a selective inhibitor of the FGFR family (ref. 11, Fig. 1B). Selectivity of NVP-BGJ398 was evaluated in biochemical and cell-based assays, where NVP-BGJ398 inhibited FGFR1, 2, and 3 at low nanomolar concentrations, but VEGFR2 and c-Kit at micromolar concentrations (11). Here, we study and compare the antiproliferative effects dovitinib and NVP-BGJ398 using a panel of 20 endometrial cancer cell lines, which represent all histologic subtypes of the disease. To better understand their antiproliferative activity, we also studied the effects of both inhibitors on FGFR2 activity and its downstream signaling intermediates AKT and extracellular signal-regulated kinase (ERK) as well as their effects on both cell cycle and apoptosis. Furthermore, we explored the in vivo growth inhibitory effects using multiple representative endometrial cancer xenograft models. These studies were conducted to assess and compare the therapeutic potential of a nonspecific multi-kinase inhibitor and a specific FGFR kinase inhibitor in endometrial cancer cell line models with or without mutational activation of FGFR2.

Materials and Methods

Cell lines, cell culture, and reagents

The effects of dovitinib and NVP-BGJ398 on malignant cell growth were studied in a panel of 20 established human endometrial cancer cell lines. Individuality of each cell line was checked by mitochondrial DNA sequencing immediately after receipt from the respective cell line repository or collaborating research laboratory. Cell lines were passaged for less than 3 months after authentication.
The established human endometrial carcinoma cell lines, KLE, RL952, AN3CA, HEC1A, HEC1B, and ECC1, were obtained from American Type Culture Collection. The established human endometrial cancer cell lines, MFE280, MFE296, MFE319, EFE184, and EN, were obtained from the German Tissue Repository DSMZ. Ishikawa cells were obtained from the European Collection of Animal Cell Cultures. The established human endometrial carcinoma cell lines HEC155, SNGII, and SNGM were obtained from the Japanese Health Science Research Resources Bank (Osaka, Japan; ref. 12). The cell lines, SPAC1S and SPAC1L, were provided by the laboratory of Dr. Y. Hirai from the Department of Gynecology, Cancer Institute Hospital (Tokyo, Japan; ref. 13). The cell line EN1 was provided by Dr. V. Möbus from Department of Gynecology at the University of Ulm (Ulm, Germany). USPC1 and USPC2 cells were provided by Dr. A. Santin from the Department of Obstetrics and Gynecology, Division of Gynecologic Oncology at the University of Arkansas (Arkansas, Little Rock, AR; ref. 14). USPC1, USPC2, RL952, SPAC1L, SPAC1S, and HEC1B cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mmol/L glutamine, and Penicillin-Streptomycin-Fungizone (PSF; Irvine Scientific). HEC1A cells were cultured in McCoy’s medium supplemented with 10% heat-inactivated FBS and PSF (Irvine Scientific). SNGII and SNGM cells were cultured in Ham’s F-12 supplemented with 10% heat-inactivated FBS and PSF (Irvine Scientific). The remaining cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mmol/L glutamine, and PSF (Irvine Scientific). Dovitinib and NVP-BGJ398 were provided by Novartis (Novartis Oncology).

**Proliferation assays**

Cells were plated into 24-well tissue culture plates at a density of $2 \times 10^3$ to $5 \times 10^5$ and grown without or with increasing concentrations of dovitinib or NVP-BGJ398 (ranging between 0.001 and 10 μmol/L). Cells were harvested by trypsinization on day 7 and counted using a particle counter (Z1; Beckman Coulter Inc.). Experiments were carried out at least 3 times in duplicate for each cell line. Growth inhibition (GI) was calculated as a function of the number of generations. As such, the percentage inhibition was calculated as 1 - (cell count divided by cell count of untreated controls). The log of the fractional GI was then plotted against the log of the drug concentration, and the IC₅₀ values were interpolated from the resulting linear regression curve fit (CalcuSyn; Biosoft).

**Cell-cycle analysis**

The effects of dovitinib and NVP-BGJ398 on the cell cycle were assessed using Nim-DAPI staining (NPE Systems). Cells were allowed to grow to log phase and were then treated with dovitinib and NVP-BGJ398 for 72 hours. Samples were analyzed using a Cell Lab Quanta SC flow cytometer (Beckman-Coulter Inc.) according to the manufacturer’s protocol.

**Annexin V and propidium iodide flow cytometry**

The effects of dovitinib and NVP-BGJ398 on apoptosis were studied using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (MBL) and flow cytometry. Cells were exposed to dovitinib and NVP-BGJ398 for 72 hours. Samples were analyzed using the Cell Lab Quanta SC flow cytometer (Beckman-Coulter Inc.).

**Clonogenic colony count assay**

To study the inhibition of anchorage-independent colony formation, soft agar assay was conducted. A 0.5% agar dilution (Difco Agar Nobel) was placed on the bottom of a 24-well plate. Cells were seeded in quadruplicates of $5 \times 10^3$ and mixed into the 0.3% agar top layer that had been prepared with or without 1 μmol/L dovitinib or NVP-BGJ398. Culture plates were stored at 37°C, 5% CO₂ for up to 5 weeks. Colonies were stained with Neutral Red solution (Sigma-Aldrich) and counted by visual inspection. All assays were conducted at least 3 times in duplicate for each cell line. USPC1, USPC2, EFE184, HEC155, and SPAC1S cells did not form reproducible colonies so soft agar assays.

**Efficacy of dovitinib and NVP-BGJ398 in an athymic mouse model of endometrial cancer xenografts**

We used FGFR2-mutated MFE296, AN3CA, and FGFR2 wild-type SNGM and HEC1A endometrial cancer cells, which spontaneously formed xenografts in athymic mice for our in vivo studies. Care of the mice was in accordance with Institutional Animal Committee Guidelines. Mice were maintained and handled under aseptic conditions, and animals were allowed access to food and water ad libitum. Female athymic mice (20.0–30.0 g) aged 4 to 6 weeks from an outbred strain (CD1 nu/nu; Charles River Laboratories) were injected subcutaneously (2 × 10⁷ cells per mouse) in the flank. AN3CA cells were suspended in Matrigel and DMEM (volume 1:1). A period of 7 days elapsed to allow formation of tumor nodules (mean xenograft volume = 105 ± 5.6 mm³). Mice were then stratified into treatment groups with one tumor per mouse on the basis of their weight and tumor volume at the start of the experiment, such that the starting weight and tumor volume in each group were uniform. Mice (10/group) were treated via oral gavage of (i) vehicle control (5 mmol/L sodium citrate and 1 μL 6N HCL/mL), (ii) NVP-BGJ398 30 mg/kg (6 mg in 0.5 mM EG300 and 0.5 mL acetic acid/acetate buffer, pH 4.68), (iii) dovitinib 30 mg/kg (1 mg in 1 mL of 5 mmol/L sodium citrate and 1 μL 6N HCL/mL), and (iv) dovitinib 50 mg/kg (1 mg in 1 mL of 5 mmol/L sodium citrate and 1 μL 6N HCL/mL). Treatment was continuous on a daily basis. Tumors were monitored by serial micrometer measurements made by a single observer with tumor volumes calculated length × width × depth. Differences in xenograft volume between groups were analyzed by Student t test of the tumor volume data.
Mutational analysis of PIK3CA, PTEN, KRAS, and FGRFR2

Relevant exons of PIK3CA (exons 9, 20), PTEN (exons 3, 5, 6, 7, and 8), and KRAS (exon 2) were PCR amplified in each cell line, sequenced, and assessed for potential sequence alterations using approaches previously described (15). The nucleotide sequences were analyzed using the Mutation Surveyor Program (Soft Genetics LLC) and through visual inspection. All somatic mutations were confirmed by independent PCR and sequencing reactions. FGFR2 mutations were assessed using mass spectrometric genotyping as previously described using the optimized mutation profiling platform OncoMap, which includes the FGFR2 mutations S252W, N550K, and K310R (16, 17).

CGH array

Genomic DNA was extracted from frozen cells using the DNeasy Blood and Tissue Kit (Qiagen). Labeling and hybridization of Agilent 105K oligonucleotide comparative genomic hybridization (CGH) arrays were conducted according to the manufacturer’s protocol for Human Genome CGH 105A Oligo Microarray Kit, Version 5.0 (Agilent Technologies) and have been described earlier (18). Log2-transformed ratios more than 1 were considered amplified (2-fold increase).

Western blot analysis

Following treatment with dovitinib or NVP-BGJ398, cells were washed in PBS and lysed at 4°C in lysis buffer. Insoluble material was cleared by centrifugation at 10,000 x g for 10 minutes. Protein was quantitated using BCA (Pierce), resolved by SDS-PAGE, and transferred to nitrocellulose membranes (Invitrogen Life Technologies). FGFR2 kinase activity was studied by assessing the phosphorylation of fibroblast receptor substrate alpha (FRSα), AKT, and ERK. Anti-p-FRS2α (Tyr196, #3864), anti-p-AKT (Ser473, #9271), anti-p-AKT (Thr308, #2965), and anti-p-ERK (Tyr202/Tyr204, #9101) antibodies were purchased from Cell Signaling. MFE280 cells, which harbor a S252W mutation in the ligand-binding domain of FGFR2, were pretreated with FGF7 30 ng/mL for 30 minutes (#k1757 Sigma-Aldrich). Detection was conducted using ECL Plus chemiluminescent reagent (Amersham Biosciences), and imaging of the resulting Western blot analysis was conducted using the chemiluminescence method by Typhoon 9400 (Amersham Biosciences).

Results

The effects of dovitinib and NVP-BGJ398 on human endometrial cancer cells were evaluated using a panel of 20 established endometrial cancer cell lines (Table 1). These cell lines were selected to be representative of a range of endometrial cancer subtypes. Five cell lines (USPC1, USPC2, HEC155, SPAC1L, SPAC1S) were obtained from patients with type II uterine serous papillary endometrial cancers (12–14). The remaining cell lines were derived from type I endometrioid endometrial cancers. To determine the relative effect of somatic gene mutations on dovitinib or NVP-BGJ398 response, FGFR2, PIK3CA, PTEN, and KRAS mutations were assessed in each cell line. In the current panel, 11 of 20 (55%) lines harbored PTEN mutations. PIK3CA mutations were present in 7 of 20 (35%) and 3 cell lines harbored KRAS mutations (Table 1). Consistent with earlier reports, activating FGFR2 mutations were found in AN3CA (N550K and K310R), MFE296 (N550K), MFE280 (S252W) and MFE319 (S252W) endometrial cancer cells (3, 4). All 4 cell lines with activating FGFR2 mutations also harbored either PTEN and/or PIK3CA mutations (Table 1). None of the cell lines used in our study had an amplification of FGFR1, FGFR2, FGFR3, or FGFR4. However, the type II endometrial cancer cell lines HEC155 and USPC1 were both found to have amplification of the gene encoding the FGFR1 ligand. Additional mutational data for selected endometrial cancer cell lines have been published as part of the Cancer Cell Line Encyclopedia (CCLE) database (19). As such, the endometrial cancer cell line EN was found to harbor a novel Y587H missense mutation in FGFR2, and SMG4 was found to harbor a V306I missense mutation in FGFR3 (19). However, to our knowledge, the clinical significance of these mutations is yet unknown, and their functional impact was deemed to be moderate using CCLE prediction software programs (19).

Activity of dovitinib and NVP-BGJ398 in endometrial cancer cells

Dovitinib inhibited the proliferation of all endometrial cancer cell lines investigated in a concentration-dependent fashion, however, sensitivity varied significantly between individual cell lines with up to a 7-fold difference in the IC50 values, ranging from 0.42 μmol/L in the FGFR2-mutated cell line MFE280 to 3.06 μmol/L in the FGFR2 wild-type cell line SPAC1L. Cell lines with activating FGFR2 mutations (S252W, N550K) had lower IC50 values compared with FGFR2 wild-type cell lines following treatment with dovitinib (mean IC50 0.86 vs. 1.60, P = 0.073). Of note, of the 4 cell lines harboring activating FGFR2 mutations, MFE319 was the one least sensitive to dovitinib (Fig. 1C). Importantly, CCLE describes a concurrent splice site mutation (pG478_splice) in BRAF in the MFE319 cell line, which can have a significant effect on splicing, which concurs with a truncated protein. This inactivating BRAF mutation may lead to a paradoxical activation of MAP–ERK kinase (MEK) signaling, accelerated cell growth, and ultimately cause resistance to an FGFR inhibitor (20, 21). Earlier studies have shown that inhibition of BRAF by chemical or genetic means in the presence of oncogenic or growth factor–activated RAS induces BRAF binding to CRAF, leading to CRAF hyperactivation and consequently elevated MEK and ERK signaling (22, 23). NVP-BGJ398, a selective inhibitor of the FGFR family of receptor tyrosine kinases (RTK), similarly inhibited proliferation of all endometrial cancer cell lines in a
Table 1. Mutational status of PIK3CA, PTEN, KRAS, FGFR2, and IC\textsubscript{50} values for dovitinib and NVP-BGJ398 in 20 endometrial cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PIK3CA</th>
<th>PTEN</th>
<th>KRAS</th>
<th>FGFR2</th>
<th>Dovitinib IC\textsubscript{50} ± SE in \textmu mol/L</th>
<th>NVP-BGJ398 IC\textsubscript{50} ± SE in \textmu mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFE280</td>
<td>H1047Y</td>
<td></td>
<td></td>
<td></td>
<td>0.60 ± 0.00</td>
<td>0.90 ± 0.00</td>
</tr>
<tr>
<td>AN3CA</td>
<td></td>
<td>R130fs</td>
<td></td>
<td></td>
<td>0.50 ± 0.10</td>
<td>1.00 ± 0.20</td>
</tr>
<tr>
<td>HEC155</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.66 ± 0.09</td>
<td>4.74 ± 1.09</td>
</tr>
<tr>
<td>MFE926</td>
<td>P539R, P120M\textsuperscript{b}</td>
<td>R130Q, V317fs</td>
<td></td>
<td></td>
<td>0.66 ± 0.19</td>
<td>2.86 ± 0.20</td>
</tr>
<tr>
<td>SPAC15</td>
<td></td>
<td></td>
<td></td>
<td>N550K\textsuperscript{a}</td>
<td>0.77 ± 0.08</td>
<td>3.19 ± 0.93</td>
</tr>
<tr>
<td>RL952</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.93 ± 0.01</td>
<td>3.41 ± 0.23</td>
</tr>
<tr>
<td>EN1</td>
<td>Q546E</td>
<td>K266fs, D301N</td>
<td></td>
<td></td>
<td>1.02 ± 0.25</td>
<td>4.75 ± 0.62</td>
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<tr>
<td>SNGII</td>
<td></td>
<td>E288fs</td>
<td>G12V</td>
<td></td>
<td>1.24 ± 0.28</td>
<td>4.29 ± 0.58</td>
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<td>ENSHIKAWA</td>
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<td>1.30 ± 0.11</td>
<td>5.48 ± 0.03</td>
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<tr>
<td>HEC1A</td>
<td>G1049R</td>
<td></td>
<td></td>
<td></td>
<td>1.34 ± 0.30</td>
<td>10.00 ± 1.00</td>
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<tr>
<td>KLE</td>
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<td></td>
<td></td>
<td></td>
<td>1.37 ± 0.02</td>
<td>3.03 ± 0.11</td>
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<tr>
<td>SNGM</td>
<td>R88Q\textsuperscript{b}</td>
<td>K163fs</td>
<td>G12V</td>
<td></td>
<td>1.42 ± 0.13</td>
<td>5.00 ± 0.41</td>
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<tr>
<td>USPC2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.62 ± 0.01</td>
<td>7.00 ± 0.21</td>
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<tr>
<td>EN</td>
<td>T1025A, T1031A</td>
<td>K266fs</td>
<td>V317fs</td>
<td></td>
<td>1.66 ± 0.01</td>
<td>6.03 ± 0.31</td>
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<tr>
<td>MFE309</td>
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<td></td>
<td></td>
<td>Y587H\textsuperscript{b}</td>
<td>1.87 ± 0.45</td>
<td>5.37 ± 0.03</td>
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<tr>
<td>EFE184</td>
<td></td>
<td></td>
<td></td>
<td>S252W\textsuperscript{c}</td>
<td>2.04 ± 0.13</td>
<td>8.04 ± 0.69</td>
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<tr>
<td>ECC1</td>
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<td>2.07 ± 0.01</td>
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<tr>
<td>HEC1B</td>
<td>G1049R</td>
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<td></td>
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<td>2.57 ± 0.23</td>
<td>6.45 ± 0.67</td>
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<tr>
<td>USPC1</td>
<td>E542K</td>
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<td></td>
<td>2.60 ± 0.13</td>
<td>5.75 ± 0.50</td>
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<tr>
<td>SPAC1L</td>
<td></td>
<td>E288fs, Y68fs</td>
<td></td>
<td></td>
<td>3.06 ± 1.14</td>
<td>4.92 ± 0.50</td>
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</table>

NOTE: Cell lines are ordered in ascending order according to the IC\textsubscript{50} for dovitinib. For PIK3CA, exons 9 and 20 and for KRAS, exon 2 were sequenced. For PTEN, exons 3, 5, 6, 7, and 8 were sequenced. FGFR2 mutations were assessed using Sequenom OncoMap as described in Materials and Methods.

\textsuperscript{a}N to K mutation numbering at position 550 relative to the FGFR2 (NP-075259.2) reference.

\textsuperscript{b}Data for other PIK3CA and FGFR2 exons were added from the Broad Institute/Novartis Cell Line Encyclopedia database (19). The functional impact of the Y587H variant described in the CCLE database is described to be moderate and its clinical significance is unknown to our knowledge.

\textsuperscript{c}Broad Institute/Novartis Cell Line Encyclopedia database reports a concurrent BRAF (G478, splice) mutation in MFE319 cells (19).

concentration-dependent fashion. However, sensitivity varied significantly between individual cell lines with up to a 10-fold difference in the IC\textsubscript{50} values and ranged from 1.0 \textmu mol/L in the FGFR2-mutated AN3CA cell line to 10.0 \textmu mol/L in the KRAS-mutated HEC1A endometrial cancer cell line. Cell lines with activating FGFR2 mutations (S252W, N550K) had significantly lower IC\textsubscript{50} values when compared with FGFR2 wild-type endometrial cancer cell lines (mean IC\textsubscript{50} 2.96 vs. 5.55, P = 0.021). Again, the BRAF-mutated MFE319 cell line was the FGFR2-mutated cell line least sensitive to NVP-BGJ398 (Fig. 1D). There was no association between PIK3CA, PTEN, or KRAS mutational status and \textit{in vitro} response to either NVP-BGJ398 or dovitinib (data not shown) using the current endometrial cancer cell line panel.

**Activity of dovitinib and NVP-BGJ398 on anchorage-independent growth**

Next, we compared the antitumor activity of dovitinib with NVP-BGJ398 by assessing the inhibition of anchor-age-independent growth using soft agar assays using a clinically achievable drug concentration for both dovitinib and NVP-BGJ398 (24, 25). At a concentration of 1 \textmu mol/L, dovitinib showed pronounced GI (≥50%) in all FGFR2-mutated cell lines, but also in 8 of 11 (73%) endometrial cancer cell lines with wild-type FGFR2. Likewise, at a concentration of 1 \textmu mol/L, NVP-BGJ398 showed pronounced GI (≥50%) in 3 of the 4 FGFR2-mutated cell lines, but in only 3 of 11 (27%) cell lines with wild-type FGFR2. These findings indicate that response to NVP-BGJ398 was more tightly correlated with the presence of FGFR2 mutations when using the clinically achievable concentration of 1 \textmu mol/L, which may be consistent with it being a more selective FGFR2 inhibitor (Fig. 2).

**FGFR2 pathway signaling**

As a measure of functional activity of FGFR2, we assessed the phosphorylation of FRS2\alpha and its downstream signaling intermediates AKT and ERK using immunoblotting techniques. Dovitinib and NVP-BGJ398 inhibited FRS2\alpha and ERK phosphorylation in a time-dependent fashion in AN3CA and MFE280 cells, which...
both carry the activating kinase domain mutation N550K (Fig. 3). In MFE280 cells, which contain a mutation in the ligand-binding domain of FGFR2 (S252W), dovitinib and NVP-BGJ398 similarly blocked basal (data not shown) and more so FGF7-stimulated FRS2 and ERK phosphorylation (Fig. 3). However, restoration of ERK signaling was seen within 24, 48, or 72 hours in MFE280, MFE296, and AN3CA cells, respectively (Fig. 3). Dovitinib and NVP-BGJ398 treatment led to a modest inhibition of AKT phosphorylation in AN3CA and MFE296 cells and to a compensatory increase of AKT phosphorylation in MFE280 cells in vitro (Fig. 3). In contrast to the pathway inhibition seen in cell lines with FGFR2 mutations, neither dovitinib nor NVP-BGJ398 treatment altered ERK or AKT signaling in the FGFR2 wild-type endometrial cancer cell lines SNGM or HEC1A (Fig. 3).

**Effects of dovitinib and NVP-BGJ398 on endometrial cancer cell cycling and survival**

Earlier reports have suggested that inhibition of the FGFR2 kinase through gene silencing induces cell-cycle arrest and apoptosis (4). Here, we confirm and extend these findings to the multikinase inhibitor dovitinib and the FGFR-selective inhibitor NVP-BGJ398. Exposure of AN3CA, MFE296, and MFE280 cells to either inhibitor led to a significant increase in the fraction of cells in G0–G1 arrest (Fig. 4A) and to a significant increase in the fraction of cells undergoing apoptosis, when compared with untreated controls (Fig. 4B). In contrast, neither dovitinib nor NVP-BGJ398 treatment altered the fractions of cells in G0–G1 arrest in the FGFR2 wild-type endometrial cancer cell lines SNGM or HEC1A in vitro (Fig. 4A). Moreover, dovitinib and NVP-BGJ398 treatment had no effect on apoptosis in the FGFR2 wild-type endometrial cancer cell line HEC1A, and dovitinib only led to a relative small increase in the fraction of SNGM cells undergoing apoptosis in vitro (Fig. 4B).

**In vivo effects of dovitinib and NVP-BGJ398 on human endometrial cancer xenografts**

Given the pronounced in vitro activity of dovitinib and NVP-BGJ398 in FGFR2-mutated cell lines, we evaluated and compared the potential antitumor activity of both agents in vivo. In preliminary experiments, we determined the in vivo growth properties of all of the present endometrial cancer cell lines as xenograft models. AN3CA, MFE296, SNGM, and HEC1A, but not MFE280 cells, formed xenografts in nude mice. To evaluate the antitumor efficacy of dovitinib and NVP-BGJ398, daily oral doses of 30 or 50 mg/kg (dovitinib) were given to mice bearing subcutaneous AN3CA, MFE296, SNGM, or HEC1A tumor xenografts. In vivo, both dovitinib and NVP-BGJ398 significantly delayed the growth of FGFR2-mutated endometrial cancer xenograft tumors (Fig. 5A and B). Next, we evaluated the activity of dovitinib and NVP-BGJ398 in mice bearing FGFR2 wild-type SNGM and HEC1A human endometrial cancer xenograft tumors. In vivo administration of the multikinase inhibitor dovitinib resulted in a significant dose-dependent GI in both FGFR2 wild-type models (Fig. 5C and D), and even led to tumor regressions in a long-term study using the FGFR2 wild-type endometrial cancer cell line SNGM (Fig. 5D). Of note, complete tumor regressions were observed in the majority of tumors at a dovitinib dose of 50 mg/kg in the SNGM xenograft model (Fig. 5D). As such, in vivo activity of dovitinib was seen in endometrial cancer cell lines where dovitinib had little or no activity in vitro. In contrast, NVP-BGJ398 had no in vivo inhibitory effects in the long-term study using the FGFR2 wild-type endometrial cancer cell line SNGM, but surprisingly did show in...
vivo activity in HEC1A cells by delaying tumor growth in these cells (Fig. 5C).

Discussion

Multikinase inhibition directed against FGFR and VEGFR kinases is an attractive potential treatment approach for endometrial cancer, as FGFR2 and VEGFR signaling have both been reported to be deregulated in endometrial cancer (2–4, 6, 26). Dovitinib is a multikinase inhibitor, which has been shown to inhibit VEGFR, PDGFR, FGFR, c-Kit, and FLT3 in biochemical- and cell-based kinase assays at nanomolar concentrations (10). Our hypothesis was that the multikinase inhibitor dovitinib may act via direct antitumor activity mediated by FGFRs, PDGFRα, or c-Kit and via antiangiogenesis mediated by VEGFR2, FGFRs, and PDGFRβ. In vitro, we show that endometrial cancer cell lines with activating FGFR2 mutations were more sensitive to dovitinib when compared with their counterparts. Nonetheless, selected FGFR2 wild-type endometrial cancer cell lines (e.g., RL952, HEC155, or SPAC1S) showed in vitro sensitivity to dovitinib similar to that seen in FGFR2-mutated lines. In contrast to these effects on signaling in cell lines with FGFR2 mutations, neither dovitinib nor NVP-BGJ398 treatment altered ERK or AKT signaling in the FGFR2 wild-type endometrial cancer cell lines SNGM and HEC1A.
while dovitinib targets FGFR2, it may have other direct antitumor activity because of its multitarget specificity with respect to other kinases such as c-Kit or PDGFR. Moreover, in vivo administration of the multikinase inhibitor dovitinib resulted in significant dose-dependent tumor GI in FGFR2 wild-type endometrial cancer xenograft models, where little or no in vitro efficacy was seen. In fact, dovitinib given over 77 days at doses of 50 mg/kg daily completely inhibited the growth of FGFR2 wild-type human endometrial cancer cells in vivo and even led to complete tumor regressions. Earlier studies show a decrease of both number and size of CD31-positive blood vessels in colon cancer xenograft models following dovitinib treatment (10). Moreover, dovitinib has been shown to inhibit the proliferation and motility of endothelial cells, pericytes, and fibroblasts in angiogenesis and FGFR signaling, each known to be vital for the role of endothelial cells, pericytes, and fibroblasts in angiogenesis (29).

Notably, all FGFR2-mutated endometrial cancer cell lines in our study also carried mutations affecting either PTEN or PIK3CA (Table 1), suggesting that the activation of this pathway does not confer resistance to an FGFR inhibitory therapy in this cancer type. However, we were able to identify an association between a potentially inactivating BRAF mutation (pG478-splice) in the MFE319 cell line and resistance to FGFR inhibitor therapy. Inhibition of BRAF by genetic means, in the presence of oncogenic or growth factor–activated RAS, has been shown to induce BRAF binding to CRAF and lead to CRAF hyperactivation and consequently elevated MEK and ERK signaling (22). Importantly, however, further studies will be necessary to determine whether the pG478 splice mutation in BRAF can lead to paradoxical activation of MEK signaling in the context of FGFR2-driven Ras activation.

Rational drug design has led to the optimization of FGFR-targeting compounds and the identification of NVP-BGJ398, a highly selective inhibitor of the FGFR family of RTKs (11). The selectivity of NVP-BGJ398 for FGFR1/2/3 has recently been confirmed using Ba/F3 differential cytotoxicity assays where NVP-BGJ398 inhibited cells constitutively expressing FGFR1/2/3 at a concentration of 10 to 14 nmol/L, and FRGR4 or VEGFR2 at a concentration of 400 and 1020 nmol/L, respectively (27). Importantly, a more selective inhibitor could become a superior therapeutic agent owing to a potentially more favorable toxicity profile when compared with an unselective agent. Considering the prominent role of FGFR2 mutations in endometrial cancer, we evaluated the activity of NVP-BGJ398 in our in vitro and in vivo endometrial cancer models and compared its activity with that seen for dovitinib. In vitro response to NVP-BGJ398 was more tightly correlated with the presence of FGFR2 mutations...

Figure 4. A, cell-cycle analysis of cells treated with dovitinib and NVP-BGJ398. Cells were allowed to grow to log phase and were then treated with dovitinib and NVP-BGJ398 for 72 hours. Cells were analyzed by flow cytometry after propidium iodide staining. The figure depicts the fraction of cells in G0–G1 cell-cycle arrest. B, detection of apoptotic subpopulations was achieved by labeling phosphatidylserine residues of the cell surface with Annexin V–FITC and staining cells with propidium iodide. Cells were exposed to dovitinib and NVP-BGJ398 for 72 hours. Cells were analyzed by flow cytometry after propidium iodide staining. The figure depicts the fraction of cells in G0–G1 cell-cycle arrest.
consistent with it being a more selective FGFR2 inhibitor when compared with dovitinib. Moreover, NVP-BGJ398 did not inhibit growth in the \( \text{FGFR2} \) wild-type SNGM endometrial cancer model \textit{in vivo}, as was seen for dovitinib. Surprisingly, however, NVP-BGJ398 did delay tumor growth in \( \text{FGFR2} \) wild-type HEC1A cells, where \textit{no in vitro} efficacy was seen. In cellular assays, the most potently inhibited kinase by NVP-BGJ398, in addition to the FGFRs, was found to be VEGFR2, displaying 70- to 100-fold reduced potency as compared with FGFR1, FGFR2, and FGFR3 (27). Therefore, although NVP-BGJ398 is a selective, pan-FGFR kinase inhibitor, with predominant activity on FGFR1, FGFR2, and FGFR3, it may nonetheless have some effect on VEGFR2 \textit{in vivo}. Further studies will be necessary to better understand the effects of NVP-BGJ398 at inhibiting VEGFR2 \textit{in vivo}.

Sequence analyses of primary tumors and cell lines have uncovered somatic \( \text{FGFR2} \) mutations in endometrial cancer in (i) the extracellular ligand-binding domain (e.g., S252W), which increase the binding affinity of the receptor for multiple FGFs from 2- to 8-fold and confer a gain in the ligand-binding promiscuity (8), (ii) in the transmembrane domain (e.g., C383R), which may cause constitutive receptor dimerization (30), and (iii) in the intracellular kinase domain (e.g., N550K), which can lead to constitutive activation of the kinase domain (4). The endometrial cancer cell line EN was found to harbor a Y587H missense mutation in \( \text{FGFR2} \) located in the kinase domain. However, the clinical significance of this \( \text{FGFR2} \) mutation is yet unknown and the functional impact is deemed to be moderate using CCLE prediction software programs (19). Additional preclinical and clinical studies will help to fully understand the role the Y587H mutation may play in endometrial cancer and drug response. The future clinical relevance of an FGFR2 inhibitor in endometrial cancer will to some extent also depend on the frequency of activating \( \text{FGFR2} \) mutations in endometrial cancer. Recent studies indicate that only 10% of the tumors of patients diagnosed with primary endometrial cancer harbor activating \( \text{FGFR2} \) mutations (5, 6). However, this number may be an underestimation of its expected frequency in recurrent disease. To date, all studies on the frequency of
FGFR2 mutations in endometrial cancer have only included patients diagnosed with primary endometrial cancer. Because FGFR2 mutations in primary endometrial cancer have been associated with recurrence, the frequency of FGFR2 mutations is expected to be higher in patients with recurrent or metastatic disease. In conclusion, the current studies show a comparable preclinical activity of the nonspecific multikinase inhibitor dovitinib and the FGFR-specific inhibitor NVP-BGJ398 against endometrial cancer cells harboring activating FGFR2 mutations. However, we were also able to show a potential therapeutic advantage of dovitinib over NVP-BGJ398 through its broader in vitro activity in FGFR2 wild-type endometrial cancer cells. In addition, because dovitinib equally targets multiple RTKs expressed in tumor cells, stromal cells, endothelial cells, and pericytes, it is more likely to target angiogenesis when compared with NVP-BGJ398. Thus dovitinib may be a promising therapeutic agent for the treatment of both FGFR2-mutated and FGFR2 wild-type endometrial cancer as it may act via direct antitumor activity mediated by FGFR2, PDGFRs, and c-Kit and via antiangiogenesis mediated by VEGFR2, FGFRs, and PDGFRs. On the basis of the present preclinical data, we have launched a multicenter, open-label, nonrandomized phase II trial designed to evaluate the safety and efficacy of dovitinib as second-line therapy in patients with advanced and/or metastatic endometrial cancer (ClinicalTrials.gov, identifier NCT01379534). In this study, tumor tissue collection will be mandatory for correlative biomarker studies. Two groups of patients will be enrolled separately based on the presence (group 1) or absence (group 2) of FGFR2 mutations in tumor samples. Taken together, our findings provide a rationale for clinical trials of dovitinib and NVP-BGJ398 in patients with recurrent endometrial cancer whose tumors show FGFR2 mutations and suggest the potential of clinical activity of dovitinib in patients with endometrial cancer that harbors wild-type FGFR2.

Disclosure of Potential Conflicts of Interest
R.S. Finn is a consultant/advisory board member of Novartis. M.M. Shi is employed (other than primary affiliation; e.g., consulting) as a senior director and has ownership interest (including patents) in Novartis. A. Yovine is employed (other than primary affiliation; e.g., consulting) as a senior global clinical leader in Novartis. No potential conflicts of interest were disclosed by the other authors.

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