Published OnlineFirst January 11, 2012; DOI: 10.1158/1535-7163.MCT-11-0450

Preclinical Development

Molecular Cancer Therapeutics

Ponatinib (AP24534), a Multitargeted Pan-FGFR Inhibitor with Activity in Multiple FGFR-Amplified or Mutated Cancer Models

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Abstract

Members of the fibroblast growth factor receptor family of kinases (FGFR1–4) are dysregulated in multiple cancers. Ponatinib (AP24534) is an oral multitargeted tyrosine kinase inhibitor being explored in a pivotal phase II trial in patients with chronic myelogenous leukemia due to its potent activity against BCR-ABL. Ponatinib has also been shown to inhibit the *in vitro* kinase activity of all four FGFRs, prompting us to examine its potential as an FGFR inhibitor. In Ba/F3 cells engineered to express activated FGFR1–4, ponatinib potently inhibited FGFR-mediated signaling and viability with IC_{50} values <40 nmol/L, with substantial selectivity over parental Ba/F3 cells. In a panel of 14 cell lines representing multiple tumor types (endometrial, bladder, gastric, breast, lung, and colon) and containing FGFRs dysregulated by a variety of mechanisms, ponatinib inhibited FGFR-mediated signaling with IC_{50} values <40 nmol/L and inhibited cell growth with GI_{50} (concentration needed to reduce the growth of treated cells to half that of untreated cells) values of 7 to 181 nmol/L. Daily oral dosing of ponatinib (10–30 mg/kg) to mice reduced tumor growth and inhibited signaling in all three tumor models examined. Importantly, the potency of ponatinib in these models is similar to that previously observed in BCR-ABL–driven models and plasma levels of ponatinib that exceed the IC_{50} values for FGFR1–4 inhibition can be sustained in patients. These results show that ponatinib is a potent pan-FGFR inhibitor and provide strong rationale for its evaluation in patients with FGFR-driven cancers. *Mol Cancer Ther; 11(3); 690–9. ©2012 AACR.*

Introduction

The fibroblast growth factor receptor (FGFR) family of tyrosine kinase receptors comprises 4 highly conserved members (FGFR1–4; ref. 1). Germ line gain-of-function mutations in FGFRs have been linked to various human diseases, most commonly FGFR2 in craniosynostosis syndromes and FGFR3 in chondrodysplasia syndromes (1, 2). Many of these same mutations, and a variety of other genetic alterations that affect expression or activity of all 4 FGFRs, have been identified in multiple tumor types suggesting FGFRs as potential therapeutic targets in cancer (1–3).

FGFR1 is amplified in 22% of squamous cell lung cancers (4) and 10% of breast cancers (5–7). FGFR2 is amplified in 4% of breast cancers (5, 8), 3% to 25% of gastric cancers (9–12) and in colon cancer (13, 14). In addition, activating mutations in FGFR2 and FGFR3 have been found in 10% of endometrial cancers (15–17) and about 60% of non–muscle-invasive bladder tumors (18, 19), respectively. FGFR3 is aberrantly expressed in the 15% of multiple myelomas that carry the t(4;14) translocation (20, 21) and is mutated in approximately 5% of these cases (22). Finally, activating mutations in FGFR4 have been identified in 7.5% of primary rhabdomyosarcoma tumors (23) and FGFR4 is overexpressed and has been shown to play a role in prostate, colon, and liver cancers (24–26).

Several multitargeted tyrosine kinase inhibitors (TKI) in clinical development, typically first identified as ATP-competitive inhibitors of the structurally related VEGF receptor (VEGFR) family, have been shown to have activity against one or more FGFRs in preclinical models (1, 27, 28). BIBF 1120 (intedanib) inhibits FGFR1–4 (29) and cediranib (AZD2171) has activity against FGFR1 and FGFR2 (30, 31), which translated to antitumor effects in gastric cancer cells carrying activating FGFR2 amplifications (31). Brivanib (BMS-540215) targets FGFR1 (32) and selectively inhibits growth of breast cancer cell lines with *FGFR1* gene amplification (33). Finally, dovitinib (TKI258) inhibits the kinase activity of FGFR1, FGFR2, and FGFR3 and the cellular activity of FGFR3 in models of multiple myeloma (34, 35).

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-11-0450

Published OnlineFirst January 11, 2012; DOI: 10.1158/1535-7163.MCT-11-0450

Ponatinib (AP24534) is an oral multitargeted kinase inhibitor that potently inhibits native and mutant forms of BCR-ABL (36–38) and is currently being investigated in a phase II pivotal trial in patients with chronic myelogenous leukemia (CML; Clinicaltrials.gov: NCT01207440). Initial characterization of the kinase selectivity profile of ponatinib showed that it exhibits potent *in vitro* biochemical activity, with IC_{50} values <20 nmol/L, against 40 additional kinases including all 4 FGFRs (37). These data suggest that ponatinib may have clinical potential as a pan-FGFR inhibitor in molecularly targeted patient populations. Here, we evaluated this potential by systemically exploring the *in vitro* and *in vivo* anti-FGFR activity of ponatinib, using a broad panel of engineered cell lines and cell lines derived from a variety of cancer types.

Materials and Methods

Cell lines, antibodies, and reagents

The following cell lines were purchased: AN3CA, RL95-2, SNU1, KATO III, SNU16, MDA-MB-134, T47D, T24, MDA-MB-231, H1581, H520, HCC827, H716, and Colo205 from the American Type Culture Collection; MFE-296 and MFE-280 cells from DSMZ; SUM 52PE from Asterand; and MFM-223 and UMUC14 from the Health Protection Agency Culture Collection. MGH-U3 cells were provided by Dr. Yves Fradet (Centre Hospitalier Universitaire de Quebec, Quebec, Canada). Further cell line authentication was not conducted by the authors. Ponatinib was synthesized at ARIAD Pharmaceuticals. Cediranib, dovitinib, and BIBF 1120 were purchased from Selleck Chemicals and brivanib from American Custom Chemical Corporation (Supplementary Fig. S1). All compounds were prepared as 10 mmol/L stock solutions in dimethyl sulfoxide (DMSO). The following antibodies were used in this study: FGFR1–3, FRS2α, and GAPDH from Santa Cruz Biotechnology; and phospho-FRS2α, phospho-FGFR (Tyr653/654), and FGFR1 from Cell Signaling Technology.

Kinase assay. Kinase inhibition assays were conducted at Reaction Biology Corporation. Compounds were tested at 10 μmol/L ATP. Mean data from 2 assays are shown.

Ba/F3 TEL-FGFR cell viability assay

Ba/F3 cell lines expressing the recombinant TEL/kinase domain fusion protein for FGFR1–4 were purchased from Advanced Cellular Dynamics. The parental Ba/F3 cell line transduced with an empty vector lacking a recombinant kinase domain was included as a negative control and was grown in the presence of 10 ng/mL IL-3 (R&D Systems). Cell viability and growth were assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega; ref. 39).

Cell growth assay

Cells were plated into 96-well plates and were treated the next day with compound or vehicle (DMSO) for 72 hours. MFE-280 cells were assayed in FBS-free medium in the presence of 25 ng/mL FGF2 (R&D Systems) and 10 μg/mL Na heparin (Sigma). To differentiate between a cytostatic and cytotoxic drug effect, the concentration that causes 50% growth inhibition (GI_{50}) was determined by correcting for the cell count at time zero (time of treatment; ref. 40) and plotting data as percentage of growth relative to vehicle-treated cells.

Immunoblot analysis

Cells were treated with ponatinib or vehicle for 1 hour. Cellular lysates (50 μg) were resolved by electrophoresis and membranes immunoblotted with antibodies against phospho and total proteins (39). IC_{50} values were calculated by plotting percentage of phospho-protein normalized to total protein, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), of ponatinib-treated cells relative to vehicle-treated cells.

Subcutaneous xenograft model

All animal experiments were carried out under a protocol approved by the Institutional Animal Care and Use Committee. Tumor xenografts were established by subcutaneous implantation of SNU16 (5×10^6 cells per mouse), UMUC14 (2.5×10^6 cells per mouse), and AN3CA (1×10^7 cells per mouse) into the right flank of female CB.17/SCID mice, 8 to 9 weeks of age (Charles River Laboratories) with Matrigel (BD Biosciences). For analysis of efficacy, once daily oral dosing of ponatinib (10 mice per group) was initiated when the average tumor volume reached about 200 mm^3 (39). Tumor volume data were analyzed with a one-way ANOVA test (GraphPad Prism). Each ponatinib treatment group was further compared with the vehicle control group for statistical significance using Dunnett multiple comparison test.

Pharmacodynamic and pharmacokinetic analyses

Following xenograft tumor establishment, mice were treated with a single oral dose of either vehicle (5 mice per group) or ponatinib (3 mice per group) and tumors harvested 6 hours later. Tumors were homogenized in Phospho-safe (Novagen) and immunoblotted as described above. Ponatinib concentrations in plasma were determined by an internal standard liquid chromatography/tandem mass spectrometry method using protein precipitation and calibration standards prepared in blank mouse plasma (39).

Results

Activity of ponatinib and other TKIs against FGFR1–4-mediated survival and cell signaling

Ponatinib has been shown to potently inhibit the *in vitro* kinase activity of FGFR1–4 with IC_{50} values of 2, 2, 18, and 8 nmol/L, respectively (Supplementary Table S1; ref. 37). To assess its activity in cells, we used a panel of Ba/F3 cell lines transformed to interleukin (IL)-3 independence by expression of constitutively activated versions of each of

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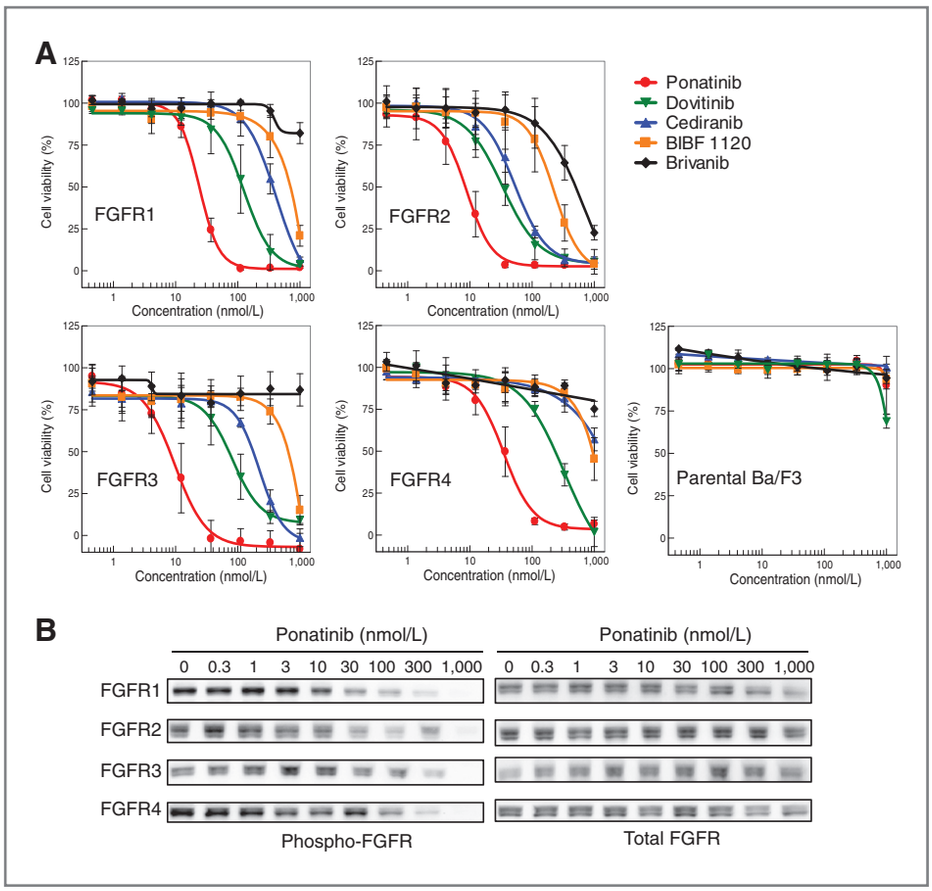

Figure 1. Ponatinib inhibits FGFR1–4-dependent cell growth in engineered Ba/F3 cells. Ba/F3 cells expressing a recombinant TEL/kinase domain fusion protein for FGFR1–4 or empty vector (parental Ba/F3) were used to evaluate compound selectivity and potency. A, cells were incubated with the indicated concentrations of compound for 72 hours and cell viability assessed. Data are presented as means (±SD) from 3 experiments. B, Ba/F3 cells were treated for 1 hour, lysates immunoblotted for phospho-FGFR (left), and then reprobed for total levels (right) of the respective FGFR. Similar results were obtained in 2 independent experiments.

the 4 FGFRs (via fusion to the TEL dimerization domain). As shown in Fig. 1A, in the absence of IL-3, ponatinib potently inhibited viability of cells expressing FGFR1–4, with IC_{50} values of 24, 8, 8, and 34 nmol/L, respectively (Supplementary Table S1). Ponatinib did not affect the viability of parental Ba/F3 cells grown in the presence of IL-3 (IC_{50} > 1,000 nmol/L; Fig. 1A), suggesting that the effect was mediated by inhibition of each FGFR. To confirm target inhibition, we assessed the effects of ponatinib on FGFR phosphorylation in each cell line and found that ponatinib inhibited phosphorylation of FGFR1–4 with IC_{50} values of 39, 29, 32, and 39 nmol/L, respectively (Fig. 1B).

For comparison, the *in vitro* kinase and cellular activities of 4 other multitargeted TKIs that have been reported to have anti-FGFR activity (and whose structures were published at the time of these studies), dovitinib, cediranib, BIBF 1120, and brivanib (29–35), were also examined. By both measures, ponatinib was found to be the most potent inhibitor of each of the 4 FGFRs (Fig. 1A; Supplementary Table S1). Overall, dovitinib was the next most potent inhibitor, with IC_{50} values 3- to 43-fold greater and 4- to 9-fold greater than ponatinib in the kinase and cell viability assays, respectively. Cediranib had somewhat further reduced potency and BIBF 1120 and brivanib were considerably less potent.

Method of analysis of the anti-FGFR activity of ponatinib in cancer cell lines

To further investigate the anti-FGFR activity of ponatinib, a series of studies was conducted using 14 cell lines, representing a variety of cancer types, which had previously been shown to overexpress an FGFR or express an activated mutated form. Growth of all 14 cell lines has previously been shown to be sensitive to FGFR knockdown or inhibition (see citations in Table 1). For each indication, a cell line that does not express activated FGFR was also examined for comparison. Because inhibition of FGFR activity could potentially lead to inhibition of cell growth (i.e., a cytostatic effect) or cell killing (i.e., a cytotoxic effect), depending on the relative contribution of the FGFR to cell proliferation or survival in each cell line, we conducted cell growth assays in a manner that could distinguish between such effects. Because inhibition of FGFR activity had a cytostatic effect in most cases, the concentration that inhibited growth by 50% (GI_{50}; ref. 40) was reported for consistency. In cases in which there was evidence of a cytotoxic effect, effects of ponatinib on markers of apoptosis were also examined. In ponatinib-sensitive cell lines, effects on signaling were examined by measuring levels of phosphorylated FGFR and its substrate FRS2α by immunoblot analysis. Finally, the *in vivo* activity of ponatinib was examined in 3 cell lines

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Table 1. Activity of ponatinib in a panel of FGFR-amplified or -mutated cancer cell lines

			Phospho-FGFR IC_{50}, nmol/L	Cell growth GI_{50}, nmol/L				
Cancer	**Cell line**	**FGFR status (ref.)**	**Ponatinib**	**Ponatinib**	**Dovitinib**	**Cediranib**	**BIBF 1120**	**Brivanib**
Endometrial	AN3CA	FGFR2 N549K (16)	4	14	112	40	886	>1,000
	MFE-296	FGFR2 N549K (16)	3	61	359	210	980	>1,000
	MFE-280[a]	FGFR2 S252W (16)	13	35	350	84	258	197
Bladder	MGH-U3	FGFR3 Y375C (41)	40[b]	181	204	>1,000	>1,000	>1,000
	UMUC14	FGFR3 S249C (41–42)	33[b]	103	182	168	625	>1,000
Gastric	SNU16	FGFR2 amp (43, 45)	20	25	99	142	473	>1,000
	KATO III	FGFR2 amp (43)	21	10	64	86	233	865
Breast	MDA-MB-134	FGFR1 amp (7)	7	23	186	297	226	>1,000
	SUM 52PE	FGFR2 amp (8)	6	14	63	76	364	>1,000
	MFM-223	FGFR2 amp (8)	7	69	411	416	>1,000	>1,000
Lung	H1581	FGFR1 amp (4)	13	32	216	168	427	>1,000
	DMS-114	FGFR1 amp (4)	30	108	818	911	>1,000	>1,000
	H520	FGFR1 amp (4)	7	155	>1,000	>1,000	>1,000	>1,000
Colon	H716	FGFR2 amp (14)	9	7	33	49	178	598

Abbreviations: amp, amplification; ref, reference for cell line FGFR genetic alteration and dependence for cell growth.
[a]Tested in serum-free media plus FGF2.
[b]FRS2α phosphorylation.

by assessing effects on growth of tumor xenografts and FGFR signaling. Plasma levels of ponatinib were also determined.

Activity of ponatinib in endometrial cancer models with FGFR2-activating mutations

We evaluated the effects of ponatinib in endometrial cancer cell lines that express FGFR2 with either an activating mutation in the kinase domain (N549K; AN3CA and MFE-296 cells) or a mutation that increases ligand binding (S252W; MFE-280 cells; refs. 16, 17). Ponatinib inhibited growth of AN3CA and MFE-296 cells with GI_{50} values of 14 and 61 nmol/L, respectively (Fig. 2A). The S252W mutation, in the extracellular region of FGFR2, does not lead to constitutive FGFR2 phosphorylation but does increase ligand-binding affinity (28); therefore, we evaluated the effects of ponatinib on MFE-280 cells grown in the presence of FGF2. Under these conditions, ponatinib inhibited growth with a GI_{50} value of 35 nmol/L (Fig. 2A). In contrast, ponatinib did not potently inhibit growth of endometrial cancer cells (RL95-2) that do not contain mutated FGFR2 (GI_{50} > 300 nmol/L; Fig. 2A; ref. 17). The constitutive phosphorylation of FGFR2 in both AN3CA and MFE-296 cells was potently inhibited by ponatinib in a dose-dependent manner with an IC_{50} value of approximately 4 nmol/L, with a concomitant inhibition of FRS2α phosphorylation also observed (Fig. 2B and data not shown). Similar effects were observed under FGF2-stimulated conditions in MFE-280 cells, with FGFR2 phosphorylation inhibited with an IC_{50} value of 13 nmol/L (Supplementary Fig. S2).

To examine the effect of ponatinib on growth of $FGFR2^{N549K}$ mutant cells *in vivo*, ponatinib or vehicle was administered orally once daily for 11 days to mice bearing AN3CA xenografts. As shown in Fig. 2C, tumor growth was not substantially inhibited in mice dosed with 3 mg/kg ponatinib. In contrast, growth was inhibited by 49% in mice dosed with 10 mg/kg and by 82% ($P < 0.05$) in mice dosed with 30 mg/kg. To confirm target modulation *in vivo*, mice bearing AN3CA xenografts were treated with a single oral dose of ponatinib or vehicle and tumors harvested 6 hours later. Consistent with the effects of ponatinib on tumor growth, dose-dependent effects on target phosphorylation were observed and were associated with increasing plasma levels of ponatinib. While partial inhibition of FGFR2 and FRS2α phosphorylation was observed after a 3 mg/kg dose, more than 80% inhibition was observed after doses of 10 or 30 mg/kg (Fig. 2D). Mean plasma levels of ponatinib at these dose levels were 27, 262, and 316 ng/mL, respectively.

Activity of ponatinib in bladder cancer models with FGFR3-activating mutations

We next evaluated the activity of ponatinib in bladder cancer cell lines that express FGFR3 with an activating mutation in the extracellular domain (S249C or Y375C) that leads to constitutive dimerization (41). Ponatinib inhibited growth of UMUC14 ($FGFR3^{S249C}$) and MGH-U3 ($FGFR3^{Y375C}$) cells with GI_{50} values of 103 and 181 nmol/L, respectively, but had no effect on growth of T24 cells that express wild-type FGFR3 (GI_{50} > 1,000 nmol/L; Fig. 3A; ref. 41). Inhibition of FGFR3 signaling in UMUC14 and MGH-U3 cells was evidenced by a dose-dependent

Published OnlineFirst January 11, 2012; DOI: 10.1158/1535-7163.MCT-11-0450

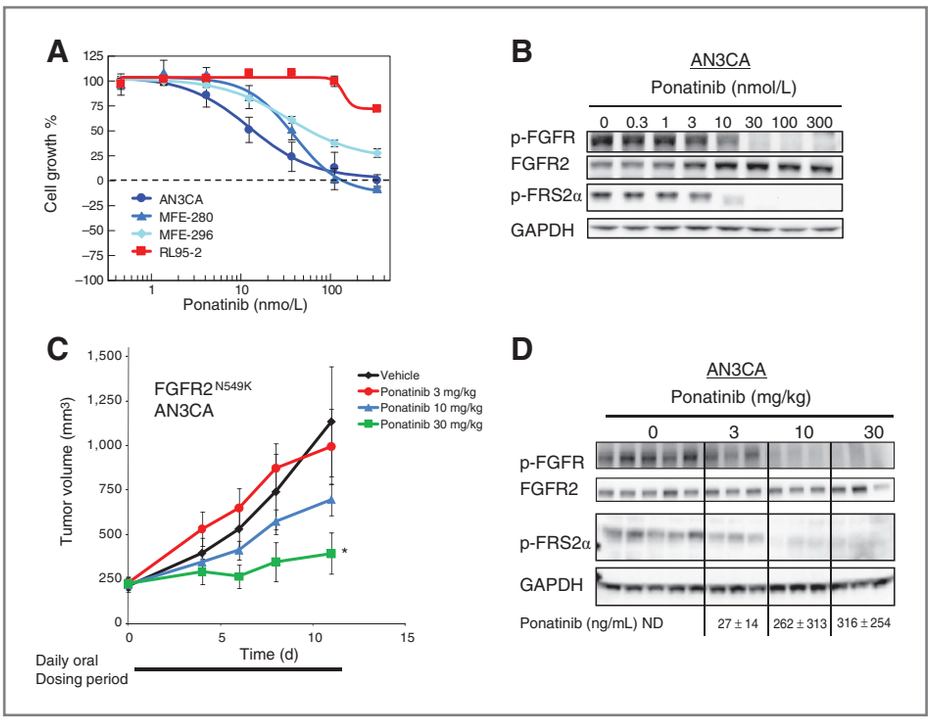

Figure 2. Ponatinib inhibits mutant FGFR2 in endometrial cancer models. A, endometrial cancer cells were incubated with ponatinib for 72 hours and cell growth assessed (MFE-280 cells were assayed in FBS-free media in the presence of 25 ng/mL FGF2 and 10 μg/mL heparin). Data are presented as means (±SD) from 3 experiments. B, AN3CA cells were treated for 1 hour and lysates immunoblotted for phospho and total protein levels. Similar results were obtained in 2 independent experiments. C, AN3CA xenografts were established and mice dosed for 11 days. Mean tumor volumes (±SEM) are plotted. D, pharmacodynamic effect of ponatinib in AN3CA tumor xenografts. Each lane represents a separate animal. Mean plasma ponatinib levels (±SD) are shown. ND, not determined.

loss of FRS2α phosphorylation, with IC_{50} values of 33 and 40 nmol/L, respectively (Fig. 3B and data not shown).

To evaluate the activity of ponatinib in a bladder cancer model *in vivo*, mice bearing UMUC14 xenografts were dosed orally with ponatinib once daily for 21 days (Fig. 3C). The UMUC14 cell line has previously been shown to exhibit FGFR-dependent tumor growth using either a selective FGFR inhibitor (41) or a specific FGFR3 antibody (42). Administration of 10 and 30 mg/kg ponatinib inhibited tumor growth by 33 and 80%, respectively, whereas administration of 3 mg/kg had no effect. An association between ponatinib dose and inhibition of FGFR3 and FRS2α phosphorylation in the tumors was observed 6

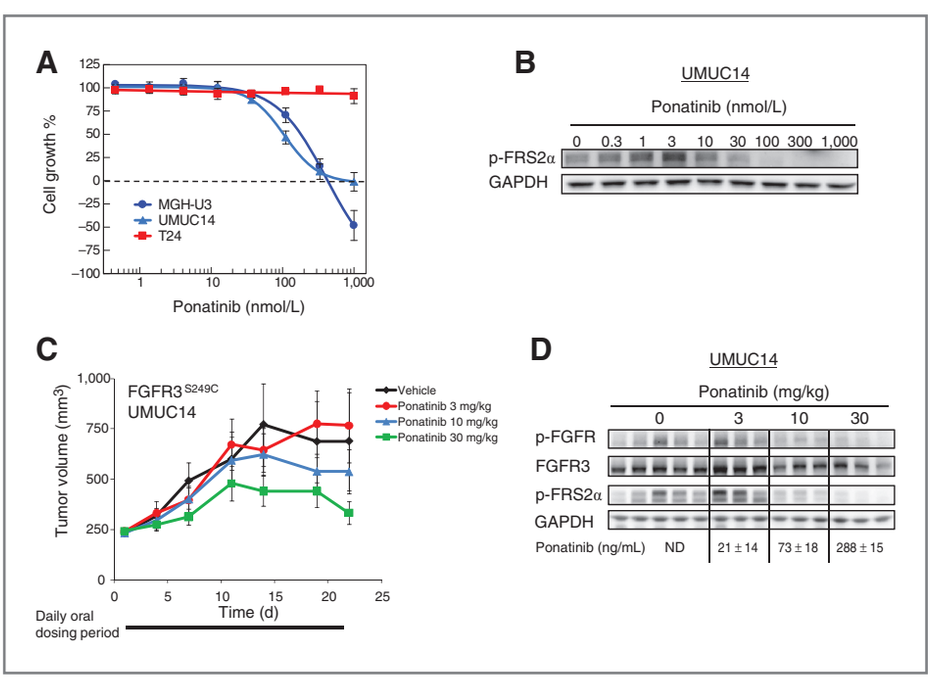

Figure 3. Ponatinib inhibits mutant FGFR3 in bladder cancer models. A, bladder cancer cells were incubated with ponatinib for 72 hours and cell growth assessed. Data are presented as means (±SD) from 3 experiments. B, UMUC14 cells were treated for 1 hour and lysates immunoblotted for phospho-FRS2α and GAPDH. Similar results were obtained in 2 independent experiments. C, UMUC14 xenografts were established and mice dosed for 21 days. Mean tumor volumes (±SEM) are plotted. D, pharmacodynamic effect of ponatinib in UMUC14 tumor xenografts. Each lane represents a separate animal. Mean plasma ponatinib levels (±SD) are shown. ND, not determined.

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hours after dosing, with no inhibition observed after a 3 mg/kg dose and substantial inhibition observed after doses of 10 or 30 mg/kg (Fig. 3D). Mean ponatinib plasma levels at these doses were 21, 73, and 288 ng/mL, respectively.

Activity of ponatinib in gastric cancer models with FGFR2 amplification

We next investigated the effects of ponatinib in 2 gastric cancer cell lines, SNU16 and KATO III, which have high levels of FGFR2 activity due to genomic amplification (43). Exposure of SNU16 and KATO III cells to ponatinib resulted in potent inhibition of cell growth, with GI_{50} values of 25 and 10 nmol/L, respectively (Fig. 4A). In contrast, a gastric cancer cell line that does not express activated FGFR2, SNU1 (43), was much less sensitive to the antiproliferative effects of ponatinib (GI_{50} = 372 nmol/L). In both SNU16 cells (Fig. 4B) and KATO III cells (data not shown), ponatinib treatment inhibited phosphorylation of both FGFR2 and FRS2α with IC_{50} values of approximately 20 nmol/L.

To evaluate the activity of ponatinib in a gastric tumor model *in vivo*, mice bearing SNU16 xenografts were dosed orally with ponatinib once daily for 21 days (Fig. 4C). The SNU16 cell line has previously been shown to exhibit FGFR2-dependent tumor growth using either a specific FGFR2 antibody (44) or an inducible FGFR2-short hairpin RNA (45). Ponatinib exhibited a dose-dependent effect on tumor growth, with a dose of 3 mg/kg having no effect, 10 mg/kg inhibiting growth by 56% ($P < 0.05$), and 30 mg/kg inducing tumor regression by 49%. No inhibition of FGFR2 and FRS2α phosphorylation was observed 6 hours after a single 3 mg/kg dose, partial inhibition after a 10 mg/kg dose, and more than 95% inhibition after a 30 mg/kg dose (Fig. 4D). Mean ponatinib plasma levels at these doses were 17, 124, and 403 ng/mL, respectively. Importantly, analysis of 88 different phosphorylation sites across 78 human kinases showed that FGFR2 is the primary target of ponatinib in this model (Supplementary Fig. S3).

Activity of ponatinib in breast, lung, and colon cancer models with FGFR1 or FGFR2 amplification

To characterize the activity of ponatinib in estrogen receptor (ER)-positive breast cancer cells with dysregulated FGFR1, we studied MDA-MB-134 cells in which FGFR1 is amplified (7). Ponatinib potently inhibited MDA-MD-134 cell growth with a GI_{50} value of 23 nmol/L (Fig. 5A) and inhibited FGFR1 phosphorylation with an IC_{50} value of 7 nmol/L (Supplementary Fig. S4A). In contrast, growth of T47D cells, which lack an activated FGFR (7), was not affected (GI_{50} > 1,000 nmol/L; Fig. 5A).

We next evaluated the activity of ponatinib in ER-negative breast cancer cell lines, SUM 52PE and MFM-223, that overexpress FGFR2 due to genomic amplification (8). Ponatinib inhibited growth of SUM 52PE and MFM-223 cells with GI_{50} values of 14 and 69 nmol/L, respectively (Fig. 5B). In contrast, ponatinib had minimal effect on growth of the wild-type FGFR, ER-negative cell line MDA-MB-231 (GI_{50} = 518 nmol/L; Fig. 5B; ref. 8). Target inhibition was confirmed by the dose-dependent loss of FGFR2 phosphorylation in SUM 52PE cells (Supplementary Fig. S4B) and MFM-223 cells (data not shown) with IC_{50} values of 6 and 7 nmol/L, respectively.

FGFR1 amplification has recently been detected in squamous cell lung cancer and in the lung cancer cell

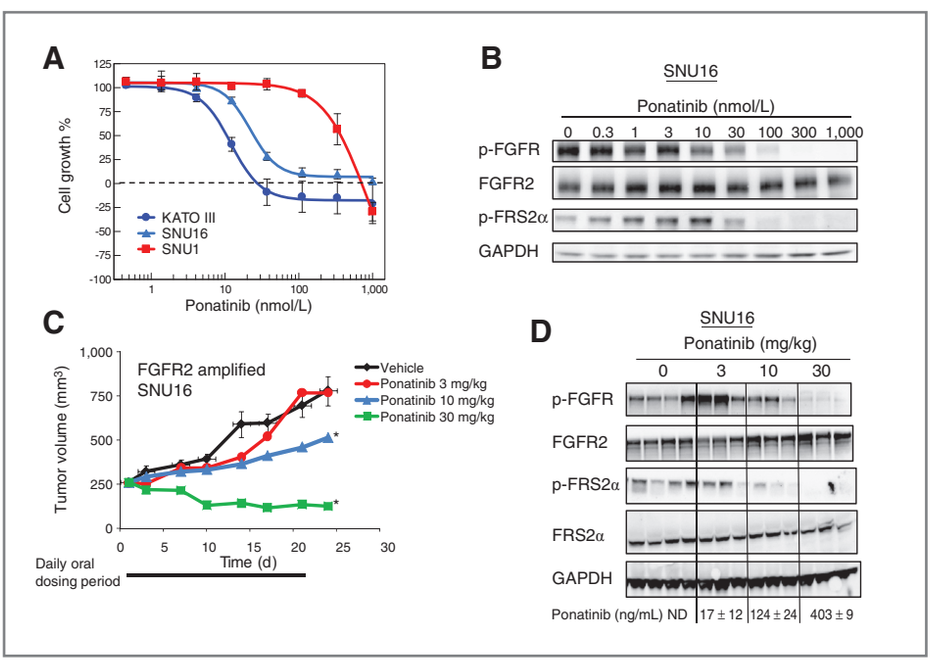

Figure 4. Ponatinib inhibits amplified FGFR2 in gastric cancer models. A, gastric cancer cells were incubated with ponatinib for 72 hours and cell growth assessed. Data are presented as means (±SD) from 3 experiments. B, SNU16 cells were treated for 1 hour and lysates immunoblotted for phospho-and total protein levels. Similar results were obtained in 2 independent experiments. C, SNU16 xenografts were established and mice were dosed for 21 days. Mean tumor volumes (±SEM) are plotted. D, pharmacodynamic effect of ponatinib in SNU16 tumor xenografts. Each lane represents a separate animal. Mean plasma ponatinib levels (±SD) are shown. ND, not determined.

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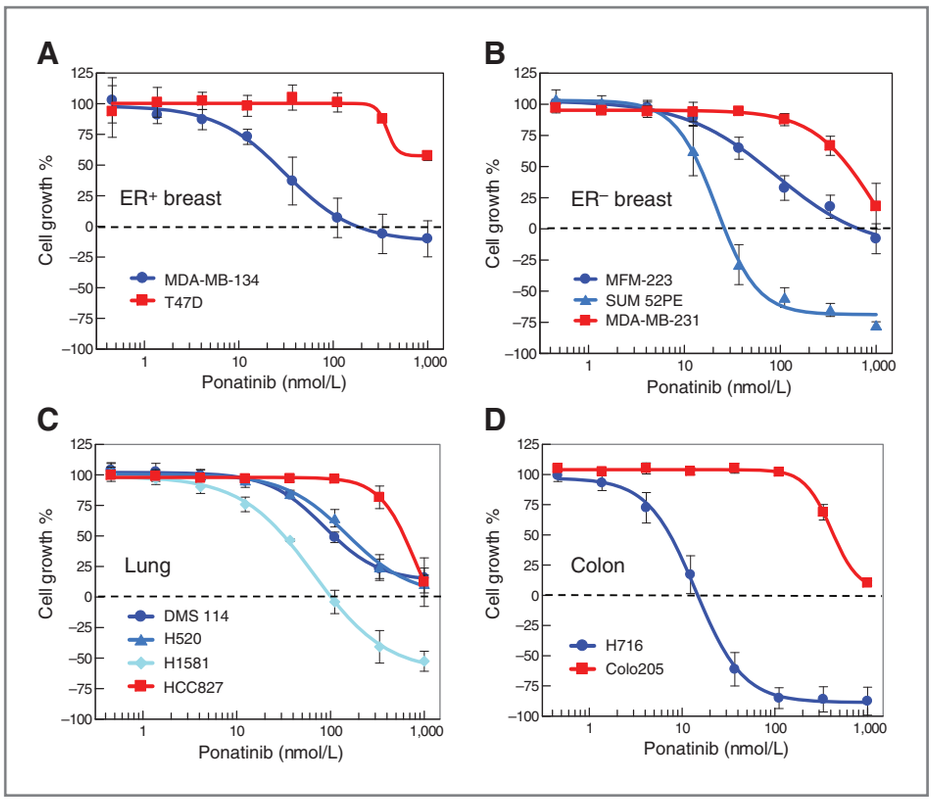

Figure 5. Ponatinib inhibits amplified FGFR1 and FGFR2 in breast, lung, and colon cancer models. Cancer cells were incubated with ponatinib for 72 hours and cell growth assessed. Data are presented as means (±SD) from 3 experiments. A, ER-positive breast cancer cells with and without FGFR1 amplification. B, ER-negative breast cancer cells with and without FGFR2 amplification. C, lung cancer cells with and without FGFR1 amplification. D, colon cancer cells with and without FGFR2 amplification.

lines H1581, H520, and DMS-114 (4). Ponatinib inhibited growth of H1581, H520, and DMS-114 cells with GI_{50} values of 32, 155, and 108 nmol/L, respectively (Fig. 5C), but had minimal effects on EGFR-mutant HCC827 lung cancer cells that lack constitutive FGFR phosphorylation (GI_{50} = 611 nmol/L; Fig. 5C; ref. 4). FGFR2 has been found to be amplified and overexpressed in the colon cancer cell line H716 (13). We found that ponatinib potently inhibited the growth of H716 cells with a GI_{50} value of 7 nmol/L but did not affect the growth of Colo205 cells (430 nmol/L; Fig. 5D) that lack constitutive FGFR2 phosphorylation (data not shown). In these 4 FGFR-amplified lung and colon cancer cell lines, ponatinib induced a dose-dependent decrease in FGFR phosphorylation with IC_{50} values of 7 to 30 nmol/L (Supplementary Fig. S4C and S4D and data not shown).

In SUM 52PE, H1581, and H716 cells, concentrations of ponatinib below 1,000 nmol/L (30, 300, and 30 nmol/L, respectively) induced a substantial (>25%) decrease in cell number after treatment relative to before treatment (negative cell growth in Fig. 5B–D). To determine whether ponatinib induced apoptosis, we measured levels of PARP cleavage and caspase-3/7 activity in these cell lines. Increases in both measures of apoptosis (Supplementary Fig. S5A–S5C) were detected at the same concentrations that led to a substantial decrease in cell number (Fig. 5B–D). In KATO III cells in which ponatinib did not induce a substantial decrease in cell number relative to baseline (Fig. 4A), no effect on markers of apoptosis was observed (Supplementary Fig. S5D).

Comparative activity of other TKIs in FGFR-driven cancer cell lines

The results described above, and summarized in Table 1, show potent inhibition of cell growth and FGFR-mediated signaling by ponatinib in models from a variety of tumor types that contain FGFR1–3 activated by multiple distinct mechanisms. During the course of this analysis, the cell growth inhibitory activity of 4 other multitargeted TKIs reported to have anti-FGFR activity was also examined in parallel (Table 1 and Supplementary Fig. S6). In all 14 cell lines examined, ponatinib had the most potent inhibitory effect on cell growth. Similar to the results observed in Ba/F3 cells transformed with individual FGFRs (Fig. 1A), ponatinib displayed greater potency compared with BIBF 1120 and brivanib, in particular, across all models. Ponatinib was also more potent than dovitinib and cediranib across all models, with the greatest differences (2- to 13-fold increased potency) observed in cell lines containing dysregulated FGFR1 or FGFR2.

Discussion

Here, we report a comprehensive set of preclinical studies describing the activity of the multitargeted kinase inhibitor, ponatinib, against the FGFR family of kinases.

Published OnlineFirst January 11, 2012; DOI: 10.1158/1535-7163.MCT-11-0450

Using an isogenic cell system in which the survival of Ba/F3 cells is made dependent on the activity of a constitutively activated FGFR, we show that ponatinib potently inhibits the activity of all 4 FGFRs in cells, inhibiting cell survival with IC_{50} values between 8 and 34 nmol/L. Consistent with the effects being mediated by inhibition of each FGFR, phosphorylation of the receptor was inhibited in all 4 cell lines with similar IC_{50} values (29–39 nmol/L). These results suggest that ponatinib is a potent pan-FGFR inhibitor.

At least 3 classes of mechanisms have been described that can lead to dysregulation of FGFR activity in solid tumors (1, 3). First, FGFRs can be dysregulated by overexpression, for example, via gene amplification, as detected in gastric, breast, lung, and colon cancers (1, 4, 8). Second, mutations in FGFR leading to increased ligand-binding affinity (e.g., $FGFR2^{S252W}$), or upregulation of ligand expression, can lead to ligand-dependent dysregulated activity (1, 28). Third, mutations in FGFRs can lead to ligand-independent activation through several mechanisms (1, 28). For example, mutations in the kinase domain (e.g., $FGFR2^{N549K}$) and mutations that allow formation of disulfide bonds (e.g., $FGFR3^{Y375C}$ and $FGFR3^{S249C}$) lead to constitutive receptor activation. Significantly, we show that ponatinib can potently inhibit *in vitro* cell proliferation and signaling in cell lines containing FGFRs dysregulated by all of these mechanisms (summarized in Table 1). In addition, using 3 mouse xenograft models, we show that daily oral administration of 10 or 30 mg/kg ponatinib leads to substantial inhibition of tumor growth and FGFR-mediated signaling. These cell lines contained an amplified FGFR (SNU16) or mutations that confer ligand independence by 2 different mechanisms (AN3CA and UMUC14), further supporting the contention that ponatinib can potently inhibit the activity of FGFRs regardless of the mechanism of activation. Although there is a close association between antitumor activity and FGFR inhibition in these models, we cannot rule out the possibility that other activities of ponatinib [e.g., antiangiogenic activities that could be mediated by inhibition of VEGFR2 or platelet-derived growth factor receptor (PDGFR)β; ref. 46] could be contributing to the efficacy observed.

Across all 14 cancer cell lines tested, ponatinib inhibited FGFR signaling with IC_{50} values between 3 and 40 nmol/L (Table 1). In most cases, there was a close association between inhibition of FGFR signaling and inhibition of cell proliferation, with discrepancies possibly explained by the contribution of additional signaling pathways to cell proliferation in certain cell lines. In a subset of the cell lines examined (e.g., UMUC14, SNU16, and H716 cells), there was an apparent increase in phosphorylation of the FGFR substrate FRS2α at low doses, although the significance of this effect is unclear as FRS2α was inhibited at higher doses of ponatinib. In 3 cell lines, SUM 52PE, H1581, and H716, which contain amplified FGFR1 or FGFR2, FGFR inhibition induced apoptosis and resulted in overall decreased cell survival. However, in most cell lines, FGFR inhibition resulted in the complete inhibition of cell proliferation, without evidence of cell killing (see Supplementary Fig. S6). Similar results were seen with 4 other TKIs indicating that the effects were due to the intrinsic role of the activated FGFRs in each cell line. The molecular basis for the dependency of certain cell lines on an activated FGFR for cell proliferation versus cell survival remains to be determined.

Multiple kinase inhibitors with anti-FGFR activity have been described. These include 4 ATP-competitive VEGFR2 inhibitors that are in various stages of clinical development as antiangiogenic agents, dovitinib, cediranib, BIBF 1120, and brivanib, which have also been shown to have activity against FGFRs in preclinical models (29–35). Here, using a Ba/F3 system transduced with activated forms of FGFR1–4, we show that ponatinib was at least 4- to 29-fold more potent than dovitinib and cediranib and at least 25-fold more potent than BIBF 1120 and brivanib. Similar results were observed in the panel of 14 cancer cell lines that contained FGFRs dysregulated by a variety of mechanisms. Since the onset of this work, several additional FGFR-targeted agents have been reported, including NVP-BGJ398 (47), AZD4547 (48), and LY2874455 (49), that have potent pan-FGFR activity in preclinical models. It remains to be shown which, if any, of these agents can be safely administered to patients at levels sufficient to inhibit FGFRs.

In an ongoing phase I trial (Clinicaltrials.gov: NCT-00660920), ponatinib has shown an acceptable safety profile with evidence of anti-leukemic activity in patients with refractory CML that express native or mutant forms of BCR-ABL, including T315I (50). In preclinical studies, dose levels of ponatinib shown here to be active in mouse models of FGFR-amplified or -mutated tumors (10–30 mg/kg; once daily oral dosing) are equivalent to those previously shown to be active in models of T315I-mutant BCR-ABL–driven tumors (37). In addition, preliminary analysis of the clinical pharmacokinetic properties of ponatinib shows that well-tolerated oral daily doses lead to trough plasma drug levels exceeding 40 nmol/L (i.e., 24 hours post-dose) and peak levels that are several fold higher (50). Such levels exceed the IC_{50} for inhibition of FGFR signaling in all models tested here (Table 1 and Supplementary Table S1). These data suggest that the potency and pharmacologic properties of ponatinib may allow substantial inhibition of FGFR activity in patients.

In summary, ponatinib is a multitargeted kinase inhibitor that displays potent pan-FGFR activity and selectively inhibits the growth of cell lines containing an FGFR activated by multiple mechanisms. Several cancer indications contain genomic aberrations in FGFRs and patients with these diseases tend to lack effective targeted therapies. These data strongly support the investigation of ponatinib in patients with FGFR-driven cancers.

Published OnlineFirst January 11, 2012; DOI: 10.1158/1535-7163.MCT-11-0450

Disclosure of Potential Conflicts of Interest

V.M. Rivera is employed by and has ownership interest in ARIAD Pharmaceuticals, Inc. All authors are full-time employees of and have ownership interest in ARIAD Pharmaceuticals, Inc.

Grant Support

Research was supported by ARIAD Pharmaceuticals, Inc.

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Received June 20, 2011; revised December 8, 2011; accepted December 21, 2011; published OnlineFirst January 11, 2012.

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Published OnlineFirst January 11, 2012; DOI: 10.1158/1535-7163.MCT-11-0450

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Published OnlineFirst January 11, 2012; DOI: 10.1158/1535-7163.MCT-11-0450

Molecular Cancer Therapeutics

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Mol Cancer Ther 2012;11:690-699. Published OnlineFirst January 11, 2012.