Kinome RNAi Screens Reveal Synergistic Targeting of MTOR and FGFR1 Pathways for Treatment of Lung Cancer and HNSCC

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Abstract

The FGFR1 is a therapeutic target under investigation in multiple solid tumors and clinical trials of selective tyrosine kinase inhibitors (TKI) are underway. Treatment with a single TKI represents a logical step toward personalized cancer therapy, but intrinsic and acquired resistance mechanisms limit their long-term benefit. In this study, we deployed RNAi-based functional genomic screens to identify protein kinases controlling the intrinsic sensitivity of FGFR1-dependent lung cancer and head and neck squamous cell cancer (HNSCC) cells to ponatinib, a multikinase FGFR-active inhibitor. We identified and validated a synthetic lethal interaction between MTOR and ponatinib in non–small cell lung carcinoma cells. In addition, treatment with MTOR-targeting shRNAs and pharmacologic inhibitors revealed that MTOR is an essential protein kinase in other FGFR1-expressing cancer cells. The combination of FGFR inhibitors and MTOR or AKT inhibitors resulted in synergistic growth suppression in vitro. Notably, tumor xenografts generated from FGFR1-dependent lung cancer cells exhibited only modest sensitivity to monotherapy with the FGFR-specific TKI, AZD4547, but when combined with the MTOR inhibitor, AZD2014, significantly attenuated tumor growth and prolonged survival. Our findings support the existence of a signaling network wherein FGFR1-driven ERK and activated MTOR/AKT represent distinct arms required to induce full transformation. Furthermore, they suggest that clinical efficacy of treatments for FGFR1-driven lung cancers and HNSCC may be achieved by combining MTOR inhibitors and FGFR-specific TKIs. Cancer Res; 75(20); 4398–406. ©2015 AACR.

Introduction

Our studies and those of others demonstrate that overexpressed, nonmutated FGFR1 participates as an oncogenic driver via autocrine FGFRs in cell lines derived from lung cancers of all histologies (1–5), head and neck squamous cell carcinomas (HNSCC, refs. 6, 7) and malignant pleural mesothelioma (8). As a result, multiple early-phase clinical trials of FGFR-targeting tyrosine kinase inhibitors (TKI) are now under way, including a study of the multitargeted TKI ponatinib (9) in lung cancer at our institution (NCT01933336).

The clinical efficacy of FGFR TKIs as single anticancer agents is not fully realized. Yet, the problem of intrinsic and acquired resistance to TKI monotherapy has emerged as a major limitation to long-term control or cure of solid tumors (10–13) and portends similar difficulties with single FGFR TKIs as therapeutics. Defining mechanisms of acquired resistance to targeted therapeutics is an ongoing subject of intense investigation and sets the stage for strategies to deploy inhibitors of the resistance mechanisms following treatment failure of the initial drug. Thus, "serial monotherapy" has emerged as a logical approach in clinical oncology for solid tumors, including lung cancer. In this regard, however, it is important to review the lessons learned from acquired resistance to antimicrobial and antiviral monotherapy over the past 60 years (reviewed in ref. 12). The present strategy to combat acquired resistance to monotherapy in cancer by deploying sequential therapies to block emergent resistance pathways (i.e., MET inhibitors after resistance to EGFR-specific TKIs) failed as a strategy to cure TB and HIV infections.

Importantly, therapeutic success in HIV and TB infections was only achieved when combinations of inhibitors were deployed that induced rapid and synergistic suppression of the infectious agent at the onset of therapy, thereby preventing the emergence of drug resistance (12). We hypothesize that the development of rational, mechanism-based combinations of inhibitors that simultaneously inhibit multiple elements within transforming receptor tyrosine kinase (RTK) coactivation networks (14) active in cancer cells may achieve a similar impact on cancer cure or control. In this study, we deployed functional genomics screens with a kinome-targeting shRNA library to identify auxiliary pathways that co-signal with FGFR1 in lung cancer and HNSCC cell lines. Our studies establish MTOR as a protein kinase with essential properties in some FGFR1-dependent cancer cell lines as well as auxiliary, synthetic lethal (SL) properties in the context of FGFR inhibitors in other cell lines. In sum, our findings identify
MTOR as a protein kinase that contributes to the intrinsic sensitivity of cancer cells to FGFR TKIs such that combined treatment with MTOR inhibitors and FGFR TKIs elicits synergistic growth inhibition. Thus, direct MTOR kinase inhibitors are attractive agents to consider combining with FGFR-specific TKIs for treatment of FGFR1-dependent lung cancers and HNSCCs.

**Materials and Methods**

**Cell culture**

All cancer cell lines used in this study were submitted to fingerprint analysis by the University of Colorado Cancer Center DNA Sequencing and Analysis Core to confirm their authenticity. Cell lines were routinely cultured in RPMI-1640 growth medium (Invitrogen) supplemented with 10% FBS with 1% penicillin-streptomycin (Sigma-Aldrich) at 37°C in a humidified 5% CO2 incubator.

**Lentivirus preparation**

The Human Kinase TRC shRNA library (obtained from the Functional Genomics Shared Resource within the University of Colorado Cancer Center) was packaged in 293T cells as follows: 293T cells were incubated overnight at 37°C in a 5% CO2 humidified incubator with Turbofect transfection reagent, 5.0 μg pS8.9, 5.0 μg pcMV-VSV-G and 3.0 μg kinase library. The virus-containing media from the 293T cells was then diluted through a 0.45-μm filter after adding 1 μg/mL polybrene and either used immediately as described or stored at 4°C until ready for use. In experiments where MTOR was validated as an essential or SL kinase, two MTOR shRNAs in the pLKO.2 lentiviral vector (TRCN0000332888 and TRCN0000363672) distinct from those shRNAs included in the library or an shRNA-targeting GFP as a negative control were packaged with the pcMV-VSV-G and pΔ8.9 component vectors. The virus was titered on NIH3T3 fibroblasts and the effect on cell growth was measured by clonogenic growth assay as described previously.

**Functional genomics screens**

For details on functional genomics screens, see Supplementary Information.

**In vitro and in vivo growth assays**

**Clonogenic and anchorage-independent growth assays.** To measure the effect of inhibitors or shRNA-mediated knockdown on cell growth, cells were seeded at 100 cells per well in 6-well tissue culture plates in full media. After 24 hours, cells were treated as indicated and cultured for 14 days with the addition of fresh media containing inhibitors every 7 days. Plates were stripped and probed for the corresponding total protein using antibodies obtained from Cell Signaling Technology, Inc. Aliquots of cell extracts prepared in lysis buffer (0.5% Triton X-100, 50 mmol/L β-glycerophosphate (pH 7.2), 0.1 mmol/L Na4VO4, 2 mmol/L MgCl2, 1 mmol/L EGTA, 1 mmol/L DTT, 0.3 mmol/L NaCl, 2 μg/mL leupeptin, and 4 μg/mL aprotinin) were submitted SDS-PAGE. After electrophoretic transfer to nitrocellulose, filters were blocked in 3% BSA (Cohn Fraction V; ICN Biomedicals, Inc.) in TBS with 0.1% Tween 20 (TTBS). The filters were then incubated overnight at 4°C with antibodies, washed three times in TTBS, and incubated for 1 hour at room temperature with alkaline phosphatase–coupled goat anti-rabbit antibodies. The filters were developed using Lumina Classic Substrate (Millipore Corporation) according to the manufacturer’s instructions. When blotting phosphorylated proteins, the filters were stripped and probed for the corresponding total signaling enzyme level or Na/K-ATPase α-subunit (Santa Cruz Biotechnology, Inc.) as a loading control.

**Caspase-3 assay**

H1581 cells were plated at 200,000 cells per well in 6-well tissue culture plates in full media. After 24 hours, cells were treated with DMSO, 300 mmol/L AZD4547, 100 mmol/L AZD8055, or combination in triplicate and cultured for 3 days. Cells were harvested and caspase-3 activity was assessed using the Caspase-3 Direct Cell Proliferation Assay Kit PLISS (Enzo Life Sciences, Inc.) according to the manufacturer’s instructions.

**Cell proliferation assay.** Cells were plated at 100 cells per well in 96-well tissue culture plates and treated with inhibitors at various doses. When the DMSO-treated control wells became confluent (1.2 weeks), cell numbers were assessed using a CyQUANT Direct Cell Proliferation Assay (Invitrogen) according to the manufacturer’s instructions.

**Xenograft tumor studies.** H1581 and Colo699 cells were suspended in 50% Matrigel/PBS at 10 million cells per mL and 1 million cells were injected s.c. in both flanks of female nu/nu mice according to protocols approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC). When at least one of the tumors reached a volume of 100 mm3, the mice were randomized into treatment groups (n = 9,10/group) of diluent control (1% Polysorbate 80), AZD4547 (12.5 mg/kg), AZD2014 (10 mg/kg) or both AZD4547 and AZD2014. Drugs were delivered daily by oral gavage (~0.25 mL/mouse). Peak plasma levels of AZD4547 following similar dosing are approximately 1 to 2 μmol/L (15). Also, a total Cmax of 11 μmol/L (free Cmax of 0.51 μmol/L) is achieved 30 minutes after a single 10 mg/kg dose of AZD2014 (S. Cosulich, AstraZeneca; unpublished data). Tumor volumes were determined by caliper measurements of the long and short diameter performed twice per week using the modified ellipsoid formula for volume, V = 1/2(length x width2). Mice were euthanized when tumors reached a volume greater than 2 cm3 or they exhibited signs of morbidity specified in the IACUC protocol.

**Immunoblot analyses**

Phospho-ERK, total ERK, phospho-AKT S473, total AKT, p-70S6K T389, total 70S6K, p-Stat3 Y705, Stat3, p-Rictor T1135, total Rictor, MTOR, and PARP1 were measured by immunoblotting using antibodies obtained from Cell Signaling Technology, Inc. Aliquots of cell extracts prepared in lysis buffer (0.5% Triton X-100, 50 mmol/L β-glycerophosphate (pH 7.2), 0.1 mmol/L Na4VO4, 2 mmol/L MgCl2, 1 mmol/L EGTA, 1 mmol/L DTT, 0.3 mmol/L NaCl, 2 μg/mL leupeptin, and 4 μg/mL aprotinin) were submitted SDS-PAGE. After electrophoretic transfer to nitrocellulose, filters were blocked in 3% BSA (Cohn Fraction V; ICN Biomedicals, Inc.) in TBS with 0.1% Tween 20 (TTBS). The filters were then incubated overnight at 4°C with antibodies, washed three times in TTBS, and incubated for 1 hour at room temperature with alkaline phosphatase–coupled goat anti-rabbit antibodies. The filters were developed using Lumina Classic Substrate (Millipore Corporation) according to the manufacturer’s instructions. When blotting phosphorylated proteins, the filters were stripped and probed for the corresponding total signaling enzyme level or Na/K-ATPase α-subunit (Santa Cruz Biotechnology, Inc.) as a loading control.
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sensitivity of lung cancer cell lines to FGFR inhibitors, we per-
To screen for signal pathways whose activity reduces intrinsic
predicted to limit the clinical response of FGFR inhibitors as well.

Results
A functional genomics-based SL screen with a kinome shRNA
library identifies MTOR as a collaborator with FGFR1

Our recent studies demonstrate through molecular and phar-
macologic approaches an autocrine role for nonmutated FGFR1
in multiple cancers, including lung cancer, malignant mesoth-
lioma, and HNSCC (1,2,6,8). On the basis of precedent from
monotherapy with TKIs in other oncogene-driven lung cancers,
including those bearing mutant EGFR or rearranged ALK
(10,11,13), intrinsic and/or acquired resistance mechanisms are
predicted to limit the clinical response of FGFR inhibitors as well.
To screen for signal pathways whose activity reduces intrinsic
sensitivity of lung cancer cell lines to FGFR inhibitors, we per-
formed functional genomics-based SL screens with a kinome-
targeting shRNA library (see Materials and Methods) and lung
cancer cell lines exhibiting high sensitivity (Colo699, H520, and
H1703) and moderate/low sensitivity (H1299 and H157) to
ponatinib (Table 1; ref. 2). Although ponatinib is a multikinase
inhibitor with IC50 values ranging from 0.2 to 8 nmol/L on ABL
and SRC family kinases, FGFR1, 2 and 4, PDGFRs, VEGFRs and
RET (16), our recent studies in lung cancer and mesothelioma cell
lines indicate that sensitivity to the TKI is closely associated with
FGFR1 expression and function (2,8). The cell lines were trans-
duced with lentiviruses encoding the kinome-targeting shRNAs in
the pLKO.1 vector at a multiplicity of infection <1. Following
selection for puromycin resistance, which eliminates nontrans-
duced cells and cells expressing an shRNA-targeting an essential
gene, the cells were treated with or without ponatinib for 3 days
followed by an additional 3 days of culture in medium lacking the
drug. The shRNAs were amplified from genomic DNA by PCR and
submitted to massively parallel deep sequencing, and the reads
were analyzed by BiNGS!SL-seq (17) to determine the count
frequency of the individual shRNAs in the control and treated
samples. Kinase genes that are SL with respect to ponatinib
are defined herein by significantly decreased shRNA
counts in the treated samples with at least two independent
shRNAs.
The functional genomics analysis identified MTOR as the top-
ranking SL hit in both H157 and H1299 cells, but not in the more
ponatinib-sensitive H520 and H1703 cells (Supplementary Table
S2) or Colo699 cells (data not shown). To validate MTOR as an SL
gene with respect to ponatinib in H157 and H1299 cells, lentivi-
ernal-encoded shRNAs distinct from those used in the kinome
shRNA library were transduced into H157 and H1299 cells. As
shown in Fig. 1A, both MTOR-targeting shRNAs reduced MTOR
protein levels relative to a nonsilencing control shRNA-targeting
GFP. Although MTOR silencing, alone, exerted little or no effect
on clonogenic growth of H157 or H1299 cells, enhanced growth
inhibition by ponatinib upon MTOR knockdown was observed in
both cell lines (Fig. 1B-D). Finally, treatment of H157 cells with
ponatinib and the MTOR inhibitor, AZD8055 (18), yielded
significantly greater clonogenic growth inhibition than ponatinib
alone (Supplementary Fig. S3). These results validate MTOR as an
SL gene with ponatinib.

MTOR is an essential gene in multiple FGFR1-dependent cancer
Cell lines

H520 Lung squamous cell carcinoma 53 57 23 21
Colo699 Lung adenocarcinoma 14 6 61 4
H1703 Lung adenosquamous carcinoma 24 460 60 8
CCL30 Nasopharynx, squamous cell carcinoma ND 9 66 ND
H581 Lung large cell carcinoma 1 2 78 40
H1299 Lung large cell carcinoma 140 >1,000 178 25
H157 Lung squamous cell carcinoma 119 >1,000 632 210
S84-A2 Larynx, squamous cell carcinoma ND 6 ND ND

NOTE: The tissue source and histology of the cancer cell lines used in the studies is summarized. The sensitivity of the cell lines to ponatinib is from ref. 2. The sensitivities of the cell lines to the FGFR inhibitor, AZD4547, and the MTOR inhibitor, AZD8055, were determined with clonogenic or anchorage-independent growth assays using drug concentrations from 0 to 1 µmol/L. The sensitivity of the cell lines to AZD4024 was measured using cell proliferation assays and the CyQUANT reagent over drug concentrations from 0 to 1 µmol/L. The primary data for AZD8055 sensitivity on six of the cell lines are shown in Fig. 2C and the IC50 values were calculated with the Prism software program.

Abbreviation: ND, not determined.
Figure 1.
Validation of MTOR as a synthetic lethal gene with FGFR inhibition by RNAi-mediated knockdown. A, H157 and H1299 cells were transduced with either a negative control shRNA-targeting GFP or one of two independent MTOR targeting shRNAs (TRCN0000363722 or TRCN0000332888), henceforth abbreviated as MTOR 22 and MTOR 88, respectively. Cells were selected for resistance to puromycin and resulting colonies were harvested and immunoblotted for MTOR

As a pharmacologic approach to define the relative requirement of MTOR for growth and survival in FGFR1-dependent cancer cell lines, clonogenic cell growth was measured in a panel of cell lines in the presence of increasing concentrations of AZD8055. AZD8055, and the congener, AZD2014 are highly selective for MTOR in either the TORC1 or TORC2 complexes, with little activity on other members of the phosphatidylinositol kinase superfamily (18). In clonogenic/anchorage-independent growth assays, H157 and 584-A2 cells exhibited low sensitivity (IC50 > 600 nmol/L) and H1299 cells intermediate sensitivity (IC50 ~ 180 nmol/L) to AZD8055 (Fig. 2C; Table 1). By contrast, Colo699, H520, and H1703 as well as the FGFR1-dependent lung cancer cell line, H1581, and the HNSCC cell line, CCL30, were uniformly sensitive to AZD8055 with IC50 values <80 nmol/L. This rank order of sensitivity among the cancer cell lines to AZD8055 was confirmed with a cell proliferation assay (Supplementary Fig. S4) and was similar to that observed with AZD2014 (Table 1). The panel of cell lines was very sensitive to the TORC1-specific inhibitor, rapamycin, although little or no difference in relative sensitivity between the cell lines was observed with IC50 values ranging from 0.3 to 2 nmol/L (Supplementary Fig. S5). Together, the studies indicate that FGFR1-dependent lung cancer and HNSCC cell lines exhibit variable degrees of MTOR dependency for growth and survival and explain the observation that MTOR was not identified as SL with ponatinib in Colo699, H520, and H1703 cells because it exerts an essential function in these cell lines relative to H157 and H1299 cells.

Synergistic growth inhibition by FGFR and MTOR inhibitors in Colo699 and H1581 cells

Despite the range of MTOR dependencies measured in the panel of cancer cell lines, we tested whether FGFR and MTOR inhibitors might still yield additive or synergistic growth inhibition in cell lines even when MTOR exhibits an essential phenotype. As shown in Supplementary Fig. S6, combination treatment with either ponatinib or AZD4547, a specific FGFR1,2,3 inhibitor (15), and AZD8055 resulted in significantly greater clonogenic growth inhibition relative to FGFR inhibitor, alone, in Colo699, H1703, and H520 cells. To rigorously test for synergistic growth inhibition resulting from simultaneous blockade of FGFR1 and MTOR, FGFR1-dependent lung cancer cell lines (H1581 and Colo699) or an HNSCC cell line (CCL30) exhibiting high sensitivity to AZD8055 (Table 1) were treated in a 96-well plate format (see Materials and Methods) with multiple concentrations of AZD4547, alone and in combination with the MTOR inhibitor, AZD8055. The effects on cell growth measured by the CyQUANT assay are shown in Fig. 3 and Supplementary Fig. S7, and analysis of the resulting data by the method of Chou and Talalay (19) revealed synergistic growth inhibition over multiple concentrations of the two drugs. The greater than additive growth inhibition by combined AZD4547 and AZD8055 was confirmed with the distinct MTOR inhibitor, AZD2014 (18), in H1581, Colo699, and 584-A2 cells (Fig. 4) and with rapamycin and AZD4547 in Colo699 and H1581 cells (Supplementary Fig. S8). Thus, the identification of MTOR as an SL pathway with FGFR1 is observed pharmacologically in multiple FGFR1-dependent cancer cell lines.

To explore the mechanism by which simultaneous inhibition of FGFR1 and MTOR yield synergistic growth inhibition, the activity of signaling pathways known to be regulated by these protein kinases was monitored by immunoblot analyses. Figure 5A reveals that AZD4547 inhibited ERK phosphorylation, but had little or no effect on phosphorylation of the TORC2 site (S473) of AKT in H1581 and Colo699 cells. In addition, none of
the TORC1 targets (p70S6K, S6, and Rictor) showed altered phosphorylation by FGFR1 inhibition with AZD4547. By contrast, AZD8055 inhibited phosphorylation of AKT-Ser473, p70S6K, S6, and Rictor, but not phosphorylation of ERK (Fig. 5A). Finally, combined AZD4547 and AZD8055 treatment inhibited phosphorylation of ERKs and multiple targets of MTOR (Fig. 2).

Figure 2. MTOR is an essential kinase in Colo699 cells. An essential kinase screen was performed with the kinome shRNA lentiviral library as described in Materials and Methods. In A, the count frequencies following Illumina sequencing of six MTOR targeting shRNAs is presented for Colo699 and 584-A2 cells. The findings show loss of five of the six MTOR shRNAs at 7 days of culture in Colo699 cells, but not 584-A2 cells. B, Colo699 cells were transduced with either a negative control shRNA targeting GFP or one of two independent MTOR-targeting shRNAs (MTOR 22 or MTOR 88). Cells were selected for resistance to puromycin, and resulting colonies following 2 weeks of culture were stained with crystal violet and total colony area was quantified. C, the indicated cell lines and others noted in Table 1 were submitted to clonogenic growth assays (see Materials and Methods) with 0 to 1 μmol/L AZD8055. After approximately 2 weeks, the colonies were fixed and stained with crystal violet and total colony area was quantified. The IC50 values were calculated with the Prism software program and are presented in Table 1.

Figure 3. Synergistic growth inhibition of H1581 and Colo699 cells by AZD4547 and AZD8055. The indicated cell lines were seeded at 100 cells per well in 96-well plates. The next day, the growth medium was replaced with 100 μL medium containing the indicated combinations of AZD4547 and AZD8055 in triplicate and incubation was continued for approximately 10 to 14 days. The medium and drugs were replaced every 7 days. Cell growth was assessed with the CyQUANT reagent as described in Materials and Methods and the mean fluorescence (n = 3) for each treatment is plotted on the y-axis. The data were submitted to further analysis with the Calcusyn program for determination of the degree of synergy achieved by the combinations relative to the monotherapy treatments. The resulting combination index (CI) values are tabulated below and the degree of synergy indicated.
TORC1 and TORC2, consistent with the functioning of an RTK coactivation network (14) in which MTOR is regulated in parallel, not distal to FGFR1. These results are also observed with rapamycin and AZD4547 except that no inhibition of AKT Ser473 phosphorylation is observed (Supplementary Fig. S9). Dual blockade of FGFR1 and MTOR, but not inhibition of either target alone, induced PARP1 cleavage in H1581 cells as measured by immunoblot analysis, suggesting the induction of apoptosis with the combination inhibitor treatment (Fig. 5B). As an independent biochemical measure of apoptosis, the activity of caspase-3 was assayed in extracts from H1581 cells similarly treated. Figure 5C shows an approximately 5-fold increase in caspase-3 activity in cells treated with combined AZD4547 and AZD8055 relative to control cells or cells treated with the single agents.

It is noteworthy that neither AKT1, 2, nor 3 were identified as high-ranking hits in the SL screens (Supplementary Table S2), despite the findings in Fig. 5 showing marked inhibition of AKT phosphorylation by MTOR kinase inhibitors. It is possible that functional redundancy occurs among the distinct AKT gene products such that silencing of any one AKT gene fails to exert a phenotype. We tested the ability of two AKT inhibitors, MK2206 (20) and GSK690693 (21) to exert synergistic growth inhibition with FGFR1 inhibitors. The sensitivity of Colo699 cells to these AKT inhibitors is shown in Supplementary Fig. S10A and indicates only modest sensitivity to the single agents. As shown in Supplementary Figs. S10B and S11, strong synergy of MK2206 and ponatinib in Colo699 cells and GSK690693 and ponatinib in H1581 cells was observed. Thus, the findings in Figs. 3–5 and Supplementary Figs. S10 to S11 support the parallel activation of the MTOR-AKT-signaling pathway as a modulator of the intrinsic sensitivity of multiple FGFR1-dependent cancer cell lines to FGFR TKIs.

Enhanced tumor growth inhibition by combination AZD4547 and AZD2014 in flank xenograft assays

H1581 and Colo699 cells were implanted in the flanks of female nu/nu mice as described in Materials and Methods. When the tumors reached approximately 100 mm3, the mice were randomized into treatment groups; diluent control, AZD4547 (12.5 mg/kg), AZD2014 (10 mg/kg), or the combination of both drugs. As shown in Fig. 6B and C, treatment of flank H1581 tumors with either AZD4547 or AZD2014 alone yielded little growth inhibition relative to diluent control (Fig. 6A). In fact, the modest effect of AZD4547 monotherapy is surprising, considering the potency with which this cell line is inhibited by AZD4547 in vitro (Fig. 3 and Table 1). However, in combination, AZD4547 and AZD2014 yielded significant tumor growth inhibition (Fig. 6D and E) and significantly prolonged survival (Fig. 6F), consistent with the synergistic growth inhibition observed in vitro. Similar relative activities of AZD4547 and AZD2014 as monotherapies, and in combination were observed following treatment of flank Colo699 xenografts (Supplementary Fig. S12A–S12F), although the survival benefit afforded by the combination therapy was not statistically significant (P = 0.06).

Discussion

Using an unbiased RNAi screen, our study highlights MTOR as an actionable protein kinase that can be targeted in combination with FGFR1 to achieve synergistic growth inhibition in FGFR1-dependent cancer cell lines. The molecular basis for the synergism appears to involve the collapse of a greater signaling network by the combination therapy than that achieved by either FGFR1 or MTOR inhibition alone. The findings in Fig. 5 support the dominant regulation of the ERK pathway downstream of FGFR1 and activation of TORC1 targets (p70S6K and S6) and TORC2 targets (pAKT S473) by MTOR. Moreover, this rationally derived combination of MTOR inhibitors with FGFR inhibitors is consistent with an extensive literature demonstrating benefit of adding MTOR inhibitors to various targeted therapeutics. For example, a rapamycin analogue increased growth inhibition by ponatinib in FGFR2-driven endometrial cancer cell lines (22), and combining a dual PI3K–MTOR inhibitor with TKIs active on BCR-ABL yielded increased growth inhibition of CML cell lines (23). Combination of a BTK inhibitor with the MTOR inhibitor, AZD2014, induced synergistic killing of diffuse large B-cell lymphoma cells (24), and the benefit of combining IGF inhibitors with MTOR inhibitors for reducing growth of Ewing sarcoma cell lines has been documented (25). The generality of the synergistic growth suppression achieved with addition of MTOR inhibitors...
indicates the degree to which MTOR participates in cancer signaling networks.

The identification of MTOR as an SL protein kinase in the setting of ponatinib treatment of H157 and H1299 cells as well as the ability of AZD8055 and AZD2014 to synergize with AZD4547 in Colo699 and H1581 cells supports a model where MTOR signals in parallel with, not downstream of FGFR1. In this regard, our results are consistent with the existence of RTK coactivation networks as reviewed by Xu and Huang (14) where the ERK pathway distal to FGFR1 and MTOR represents distinct fragile points. The ability of the MEK inhibitor, selumetinib, to synergize with ponatinib for growth inhibition of multiple cell lines (Supplementary Figs. S10 and S11), providing support for AKT as at least one important target of MTOR in these cancer cells. Also, rapamycin strongly inhibited growth (Supplementary Figs. S5 and S8), supporting the critical involvement of TORC1. It is noteworthy that AKT1, 2, or 3 were not high-ranking hits in the screens, although redundancy among the three gene products could preclude identification of any with our RNAi-based approach. Like MTOR inhibitors, two independent AKT inhibitors synergized with ponatinib for growth inhibition of multiple cell lines (Supplementary Fig. S10 and S11), providing support for AKT as at least one important target of MTOR in these cancer cells.

In light of the potent in vitro sensitivity of Colo699 and H1581 cells to FGFR TKIs (Figs. 3 and 4), we were surprised by the modest degree of growth inhibition achieved with AZD4547 alone when these cell lines were propagated as xenografts that are limited to combination treatment. The identifiability of MTOR as an SL protein kinase in the setting of ponatinib treatment of H157 and H1299 cells as well as the ability of AZD8055 and AZD2014 to synergize with AZD4547 in Colo699 and H1581 cells supports a model where MTOR signals in parallel with, not downstream of FGFR1. In this regard, our results are consistent with the existence of RTK coactivation networks as reviewed by Xu and Huang (14) where the ERK pathway distal to FGFR1 and MTOR represents distinct fragile points. The ability of the MEK inhibitor, selumetinib, to synergize with ponatinib for growth inhibition of multiple cell lines (Supplementary Fig. S10 and S11), providing support for AKT as at least one important target of MTOR in these cancer cells. Also, rapamycin strongly inhibited growth (Supplementary Figs. S5 and S8), supporting the critical involvement of TORC1. It is noteworthy that AKT1, 2, or 3 were not high-ranking hits in the screens, although redundancy among the three gene products could preclude identification of any with our RNAi-based approach. Like MTOR inhibitors, two independent AKT inhibitors synergized with ponatinib for growth inhibition of multiple cell lines (Supplementary Fig. S10 and S11), providing support for AKT as at least one important target of MTOR in these cancer cells.

In light of the potent in vitro sensitivity of Colo699 and H1581 cells to FGFR TKIs (Figs. 3 and 4), we were surprised by the modest degree of growth inhibition achieved with AZD4547 alone when these cell lines were propagated as flank xenografts in nu/nu mice (Fig. 6 and Supplementary Fig. S12). Still, this degree of growth inhibition is not inconsistent with the early results of clinical trials of AZD4547 and BGJ398 in squamous cell lung cancer where only partial responses have been observed thus far in less than 20% of patients (28,29). On the basis of our present studies showing strong in vivo responses with flank xenografts that are limited to combination treatment with AZD4547 and AZD2014, we wonder whether targeting auxiliary pathways such as MTOR will be required to observe
FGFR1 and MTOR Coinhibition Synergistically Suppresses Growth

significant clinical responses in FGFR1-dependent lung cancers and HNSCCs. If so, it is important that FGFR1 not be immediately abandoned as a therapeutic target in these settings based solely on a marginal clinical response to FGFR TKI monotherapy. Although the frequent and profound tumor shrinkage responses observed in lung cancers bearing mutant EGFR or rearranged ALK treated with TKI monotherapies have established a new expectation for clinical responsiveness, we hypothesize that cancers driven by nonmutated drivers like FGFR1 may inherently depend more on RTK coactivation networks for full-transforming potential (14). Admittedly, clinical investigation of combination therapies is unwieldy relative to monotherapy and bears increased concern of drug toxicity. Still, the fact that many lung cancers and the majority of HNSCC will present with nonmutated oncogene drivers necessitates careful consideration of combination therapies as a starting point in treatment design.

Disclosure of Potential Conflicts of Interest
L.E. Heasley reports receiving a commercial research grant from ARIAD. No potential conflicts of interest were disclosed by the other authors.

Figure 6. Combination AZD4547 and AZD2014 provides superior growth inhibition of H1581 xenografts. Flank xenografts derived from H1581 cells were generated as described in Materials and Methods. When the tumors reached 100 mm³, the mice were randomized to treatment by daily oral gavage with diluent (A), 12.5 mg/kg AZD4547 (B), 10 mg/kg AZD2014 (C), or combined AZD4547 and AZD2014 (D). The individual tumor volumes relative to their initial volumes are shown for the different treatment groups. The average fold change in tumor volume among the groups after 2 weeks of treatment is shown in E and reveals significantly greater growth inhibition by the combination relative to either monotherapy. F, Kaplan-Meier survival curve analysis of the mice in the four groups demonstrates significantly longer survival (P = 0.0002) with combination therapy relative to the mice treated with diluent or the monotherapies.

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Writing, review, and/or revision of the manuscript: K.R. Singleton, J. Kwak, J. Kim, A.C. Tan, L.E. Heasley
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.E. Heasley
Other [assisted with execution of experiments and maintenance of laboratory materials (making buffers, media, cleaning supplies, etc.) and presentation of the data collected (generation of graphs and images) for experiments]: T. Harp

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References

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Kinome RNAi Screens Reveal Synergistic Targeting of MTOR and FGFR1 Pathways for Treatment of Lung Cancer and HNSCC

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