mTOR Kinase Inhibition Causes Feedback-Dependent Biphasic Regulation of AKT Signaling

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ABSTRACT
mTOR kinase inhibitors block mTORC1 and mTORC2 and thus do not cause the mTORC2 activation of AKT observed with rapamycin. We now show, however, that these drugs have a biphasic effect on AKT. Inhibition of mTORC2 leads to AKT serine 473 (S473) dephosphorylation and a rapid but transient inhibition of AKT T308 phosphorylation and AKT signaling. However, inhibition of mTOR kinase also relieves feedback inhibition of receptor tyrosine kinases (RTK), leading to subsequent phosphoinositide 3-kinase activation and rephosphorylation of AKT T308 sufficient to reactivate AKT activity and signaling. Thus, catalytic inhibition of mTOR kinase leads to a new steady state characterized by profound suppression of mTORC1 and accumulation of activated AKT phosphorylated on T308, but not S473. Combined inhibition of mTOR kinase and the induced RTKs fully abolishes AKT signaling and results in substantial cell death and tumor regression in vivo. These findings reveal the adaptive capabilities of oncogenic signaling networks and the limitations of monotherapy for inhibiting feedback-regulated pathways.

SIGNIFICANCE: The results of this study show the adaptive capabilities of oncogenic signaling networks, as AKT signaling becomes reactivated through a feedback-induced AKT species phosphorylated on T308 but lacking S473. The addition of RTK inhibitors can prevent this reactivation of AKT signaling and cause profound cell death and tumor regression in vivo, highlighting the possible need for combinatorial approaches to block feedback-regulated pathways.

INTRODUCTION
Dysregulation of phosphoinositide 3-kinase (PI3K) signaling is a common event in human cancer, and mutations in multiple components of the pathway have been identified (1–3). Activation of PI3K signaling in tumors is responsible for key features of the transformed phenotype, suggesting that inhibition of the pathway could be a useful therapeutic strategy (1, 4). The mTOR protein kinase is an important component of the PI3K/AKT pathway, which controls cell proliferation, size, and metabolism by integrating the effects of growth factors and the availability of nutrients and energy. mTOR exists in 2 complexes: mTORC1 and mTORC2. mTORC1 contains the mTOR, Raptor, mLST8/GβL, and PRAS40 proteins and controls cell size and protein translation via its 2 major substrates, p70S6K and 4E-BP1 (5, 6). Activated S6 kinase causes feedback inhibition of insulin-like growth factor 1 (IGF-1)/insulin signaling by phosphorylating insulin receptor substrate 1 (IRS-1) and causing its degradation (7). The mTORC2 complex contains mTOR, Rictor, mSin1, Protor, and mLST8/GβL and is also activated in response to growth factor stimulation (8). mTORC2 has been shown to phosphorylate AKT and SGK1 in a conserved hydrophobic domain (9, 10). Phosphorylation of AKT at the S473 site by mTORC2 enhances the catalytic activity of AKT already phosphorylated on threonine 308 (T308; ref. 11). Thus, mTOR complexes function both upstream and downstream of AKT (12).

Inhibitors of PI3K, AKT, and mTOR are currently being developed as potential therapeutic agents for tumors in which the pathway is dysregulated (13, 14). Initial studies have focused on inhibition of mTORC1 with the natural product rapamycin. Rapamycin binds to FKBP-12; this complex then binds to and causes the allosteric inhibition of mTORC1, suppressing CAP-dependent protein translation and, in model systems, inhibition of cell proliferation and tumorigenesis (15–17). In patients, rapamycin has been shown to have therapeutic activity in renal cell carcinoma, neuroendocrine tumors, and other cancers (18). However, significant therapeutic responses rarely occur in tumors in which mutations that activate PI3K/AKT signaling are prevalent, such as in prostate and breast cancer and glioblastoma (19, 20).

We and others have observed that although rapamycin effectively blocks S6K phosphorylation, it also induces AKT S473 phosphorylation and AKT activity in tumors in model systems and in patients, as well (16, 21, 22). Physiologic activation of PI3K/AKT signaling is regulated by mTOR-dependent feedback inhibition of IRS expression and, consequently, IGF-1 receptor (IGF-1R)/insulin receptor signaling (7). Rapamycin relieves this feedback and induces AKT S473 phosphorylation in an mTORC2-dependent manner, leading to AKT activation, which may attenuate its therapeutic effects (16, 23). In response to this problem, ATP-competitive inhibitors of mTOR kinase that potently inhibit both mTORC1 and mTORC2 complexes have now been developed. It has been hypothesized that such inhibitors will have greater antitumor activity than does rapamycin because they inhibit mTORC2 complexes.
and will therefore prevent feedback induction of AKT, which may also directly affect its activity against certain substrates (14). Furthermore, this class of compounds has been shown to inhibit mTORC1 more potently than does rapamycin (24).

We have now tested these assertions with the selective ATP-competitive mTOR kinase inhibitor AZD8055 (25). This drug inhibits 4E-BP1 phosphorylation more effectively than does rapamycin. It also inhibits mTORC2 and AKT S473 phosphorylation, which leads to AKT T308 dephosphorylation and suppression of AKT activity and downstream signaling. However, these latter effects are transient. In addition, mTOR kinase inhibition causes marked activation of receptor tyrosine kinase (RTK) signaling, which induces PI3K signaling and reinduction of T308 phosphorylation and, despite persistent inhibition of mTORC2 activity and AKT S473 phosphorylation, which reactivates AKT activity and signaling.

**RESULTS**

**AZD8055 Is a Potent Inhibitor of mTORC1 and mTORC2 Complexes**

mTOR kinase inhibitors have now been developed and shown to effectively inhibit mTORC1 and mTORC2 (12, 24–27). AZD8055 is an ATP-competitive inhibitor of mTOR kinase that blocks the enzyme with a $K_i$ of 1.3 nM in vitro (25) and inhibits S6K and 4E-BP1 phosphorylation in cells with IC$_{50}$ values of 10 nM and 100 nM, respectively (data not shown). AZD8055 is selective, in that it displayed a potency $>1,000$-fold against all related kinases (25). In Fig. 1A, the effects of AZD8055 on mTORC1 and mTORC2 signaling were compared with those elicited by rapamycin in 3 breast cancer cell lines with different mechanisms of activation of the PI3K pathway—BT-474 (HER2 amplified, PI3K mutant), MCF-7 (PI3K mutant), and MDA-MB-468 [epidermal growth factor receptor (EGFR) amplified, PTEN deficient]. Inhibition of mTORC1 with rapamycin potently restricts the phosphorylation of p70S6 kinase and its substrate S6, but only poorly restricts 4E-BP1 phosphorylation, as has been previously described (28, 29). In contrast, AZD8055 potently inhibits phosphorylation of both S6K and 4E-BP1, although more drug (Fig. 1A) and time (Fig. 1A) are required to restrict the latter. As reported earlier, rapamycin does not inhibit mTORC2; instead, it induces AKT S473 phosphorylation owing to relief of feedback of IGF-1R signaling (16, 22). In contrast, AZD8055 potently and rapidly inhibits S473 phosphorylation and, therefore, despite restricting S6K phosphorylation, prevents the induction of S473 phosphorylation that results from relief of mTORC1-dependent negative feedback. Blocking the phosphorylation of these mTORC1 and mTORC2 substrates with AZD8055 was sustained for 24 hours (Fig. 1B). We conclude that AZD8055 is a strong inhibitor of both mTORC1 and mTORC2.

**mTOR Kinase Inhibition Transiently Inhibits AKT T308 Phosphorylation and AKT Function**

PI3K activation causes phosphatidylinositol-3,4,5-trisphosphate (PIP3)-dependent membrane localization of AKT and PKD1, in which the latter is responsible for phosphorylation of AKT T308 (30). AKT T308 phosphorylation is required for AKT kinase activity, which is further enhanced by the phosphorylation of S473 by mTORC2 (11). It has been proposed that phosphorylation of S473 stabilizes that of T308 and thereby enhances AKT catalytic activity (9, 31). In BT-474, MDA-MB-468, and MCF-7 cells, AZD8055 inhibits AKT T308 phosphorylation within 1 hour of treatment (Fig. 2A and B, panel 1; Supplementary Fig. S1 for MCF-7 cells). Phosphorylation of T308 falls in parallel with that of the mTOR substrates AKT S473, S6K, and 4E-BP1. These findings are consistent with data obtained with other mTOR kinase inhibitors (12, 24, 27). The phosphorylation of AKT substrates S6K, AKT S473, and 4E-BP1 at S65 and T70 remains blocked for 24 hours after addition of the drug, showing that mTOR kinase inhibition persists over this period. However, phosphorylation of AKT at the T308 site and of the AKT substrates S6K-β, FOXO1/3, and PRAS40 declines 4 hours after addition of the drug and reaches pretreatment levels 8 to 16 hours later (Fig. 2A and B, panel 1). The phosphorylation of FOXO is markedly enhanced, compared with pretreatment levels. These data imply that inhibition of AKT in response to mTOR kinase inhibition is transient, despite continued blocking of S473 phosphorylation. 4E-BP1 phosphorylation on T37/T46 also rises slightly in comparison with its nadir, reaching a new steady state between 8 and 24 hours after addition of the drug. Another mTOR kinase inhibitor, PP242, also caused transient suppression of the phosphorylation of AKT T308 and AKT substrates, suggesting that this is a general property of these drugs (Supplementary Fig. S2; ref. 32).

Reactivation of AKT signaling could be due to a fall in drug concentration in the cell or to establishment of a new steady state of the signaling network with higher levels of AKT activity. To distinguish between these possibilities, either AZD8055 or a selective allosteric inhibitor of AKT1 and 2 was added to BT-474 (Fig. 2A, panels 2 and 3) and MDA-MB-468 cells (Fig. 2A, panels 2 and 3) 8 hours after exposure of the cells to AZD8055. Readdition of AZD8055 had essentially no effect; phosphorylation of AKT T308, AKT substrates, and 4E-BP1 T37/46 remained elevated. In contrast, phosphorylation of AKT T308, S6K-β, FOXO1/3, and PRAS40 were all sensitive to the AKT inhibitor. This finding suggests that the increased phosphorylation of AKT substrates is due to reactivation of AKT. The residual phosphorylation of 4E-BP1 T37/46 was also sensitive to AKT, but not to mTOR kinase inhibition, indicating that AKT-dependent, but mTOR-independent, signals may regulate phosphorylation of this site. These data and the persistent suppression of AKT S473 and S6K phosphorylation suggest that the reinduction of phosphorylation of AKT substrates is not due to decreased levels of drug in the cells. Furthermore, these data may show that reinduction is due to reactivation of AKT and not another kinase.

To confirm that the rapid inhibition and subsequent reinduction of phosphorylation of AKT substrates is due to changes in AKT activity, we performed *in vitro* AKT kinase assays on immunoprecipitates from cells treated with AZD8055 for up to 24 hours. We found that AKT kinase activity declines within 1 hour of addition of the drug, reaches a nadir of 15% of baseline at 8 hours, and then rises to 60% of baseline by 24 hours after addition of the drug (Fig. 2C).
The biphasic inhibition and subsequent mTOR-independent reactivation of AKT is likely due to parallel changes in T308 phosphorylation. To determine whether the initial rapid decline in T308 phosphorylation resulted from the inhibition of mTORC2-dependent S473 phosphorylation, we used the AKT S473D mutant, which mimics constitutive phosphorylation of the site. BT-474 cells transfected with either AKT wild-type (WT) or AKT S473D were treated with AZD8055 for 1 or 4 hours. Phosphorylation of endogenous AKT S473 (lower band) falls within 1 hour of drug treatment in both transfectants (Fig. 2A). As expected, binding of the anti-phospho 473 antibody to the S473D mutant (upper band) is unaffected by the drug treatment, confirming that the aspartate substitution is phosphomimetic. Drug treatment also caused the rapid inhibition of T308 phosphorylation of endogenous WT AKT in both transfectants. However, T308 phosphorylation of the AKT S473D mutant (upper band) does not decline; in fact, it increases after drug treatment. These data support the work of others that suggests inhibition of AKT S473 phosphorylation causes a decline in T308 phosphorylation (9, 27, 32). The rapid induction of T308 phosphorylation in mutant S473D confirms the conclusion that this induction is not due to declining intracellular drug concentrations. The rapid loss of T308 phosphorylation in WT AKT and the rise in the AKT S473D mutant suggest that, in these cells, 2 separate processes account for the decline and subsequent reinduction of T308 phosphorylation and AKT activity after mTOR kinase inhibition.

**mTOR Kinase Inhibition Leads to Activation of PI3K**

Phosphorylation of T308 is caused by PI3K-dependent localization of PDK1, the T308 kinase, to the membrane. We asked whether the initial loss of T308 phosphorylation is counteracted by PI3K activation (Fig. 3a). The p85

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**Figure 1.** AZD8055 is a potent inhibitor of mTORC1 and mTORC2 signaling. **A,** immunoblot analysis performed on mTORC1 and mTORC2 effectors after treatment with increasing concentrations of AZD8055 or rapamycin for 4 hours in MCF-7, BT-474, and MDA-MB-468 breast cancer cells. **B,** the same cell lines treated with 500 nM of AZD8055 and collected at indicated times and analyzed by immunoblotting.
regulatory subunit of class 1 PI3K was immunoprecipitated from lysates of cells treated for 4 hours with drug, and \textit{in vitro} PI3K assays were performed on the precipitates in the presence of \textsuperscript{32}P-gamma-labeled ATP and phosphatidylinositol. Phosphatidylinositol 3-phosphate (PI3P; a product of PI3K) was greatly induced by IGF-1 and inhibited by the PI3K inhibitor wortmannin. Rapamycin and AZD8055 both substantially induced PI3K activity by >2-fold (Fig. 3A, lower panel). To investigate further whether the increased \textit{in vitro} kinase activity is associated with increased intracellular levels of PI3P, we used an intracellular reporter assay in HeLa cells. The reporter is a fusion protein comprising the AKT PH domain–binding proteins to the membrane.

\textbf{mTOR Kinase Inhibition Activates RTKs}

We have previously observed that mTORC1 inhibition leads to activation of upstream receptor tyrosine kinase signaling \cite{16}. Moreover, we and others have recently shown that PI3K and AKT inhibition induces expression and activation of multiple RTKs \cite{34-36}. Therefore, we hypothesized that induction of PI3K activation by AZD8055 is mediated in part by growth factor receptor activation. An array of 42 anti–phosphotyrosine receptor antibodies was used to assess whether RTK phosphorylation levels were induced in breast cancer cell lines after their exposure to the drug (Fig. 4A for BT-474 cells; Supplementary Fig. S3 for MDA-MB-468 and MCF-7 cells). As shown in Fig. 4A, phosphorylation of multiple RTKs was induced, including members of the HER kinase (EGFR, HER2, 3, 4), IGF-1R, insulin receptor, and FGFR1-3 families. Induction occurred in all 3 models—BT-474, MCF-7, and MDA-MB-468. To confirm this, AZD8055 rapidly initiates PI3K activity in cells, and this causes induction of PIP3 levels sufficient to translocate PH domain–binding proteins to the membrane.
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MDA-MB-468 express high levels of HER2 and EGFR, respectively, owing to gene amplification. The HER2-predominant HER kinase inhibitor lapatinib suppresses AKT signaling when added 8 hours after exposure of BT-474 cells to mTOR kinase inhibition (Fig. 5A, panel 3). Gefitinib, an EGR-predominant HER kinase inhibitor, has similar effects in MDA-MB-468 cells (Supplementary Fig. S4, panel 3). Thus, in breast tumor cells in which mTOR kinase is inhibited, AKT signaling is dependent on the activation of upstream RTKs. In the steady state >8 hours after mTOR kinase inhibition, breast tumor cells are characterized by high levels of RTK phosphorylation and PI3K activity; phosphorylation of AKT T308, but not S473; phosphorylation of AKT substrates; and profound mTORC1 inhibition. The data support the hypothesis that the effects of mTOR kinase on AKT signaling are dependent on the activation of upstream RTKs.

In the steady state >8 hours after mTOR kinase inhibition, breast tumor cells are characterized by high levels of RTK phosphorylation and PI3K activity; phosphorylation of AKT T308, but not S473; phosphorylation of AKT substrates; and profound mTORC1 inhibition. To model the consequences of mTOR kinase inhibition in cells in which relief of RTK feedback does not occur, we treated BT-474 cells with AZD8055 and lapatinib at the same time. We observed that the phosphorylation of EGFR, HER2, and HER3 was inhibited, and reinduction of AKT T308 and AKT substrate phosphorylation did not occur (Fig. 5B). In these cells, chronic mTOR kinase inhibition is characterized by potent inhibition of both mTORC1 and AKT signaling. The data support the hypothesis that the effects of mTOR kinase inhibition on AKT signaling are dependent on the activation of upstream RTKs.

Figure 3. mTOR kinase inhibition leads to PI3K activation. A, MCF-7 cells were treated with 500 nM of AZD8055, 50 nM of rapamycin, or 100 nM of wortmannin for 4 hours, or 60 ng/mL of IGF for 10 minutes, and a PI3K activity assay was performed. The product, PIP3, was resolved by thin-layer chromatography and detected by autoradiography. A lane with a purified PI3K enzyme was used as a positive control and a lane without enzyme was used as a negative control for the assay. The results were quantified by densitometry (lower panel). B, the pcDNA3-AKT-PH-GFP vector was transfected into HeLa cells. Twenty-four hours after the transfection, the cells were treated with either DMSO, 500 nM of AZD8055, 100 nM of wortmannin, or a combination of wortmannin and AZD8055 (wortmannin was added 30 minutes prior to AZD8055 for the combination treatment) for 4 hours or with 60 ng/mL of IGF for 10 minutes. The GFP signal was detected using confocal microscopy. Shown are representative cells.

the increase in levels of phosphorylated receptor, lysates of BT-474 and MDA-MB-468 cells treated with AZD8055 were analyzed by immunoblotting. The phosphorylation of EGFR family members and IGF-1R/insulin receptor kinases was induced within 1 hour of exposure of cells to AZD8055 and persisted for 24 hours (Fig. 4A and C). In BT-474 cells, in which HER2 is expressed at very high levels, we observed induction of both expression and phosphorylation of RTKs, with greater induction of phosphorylation than expression (Fig. 4A). A similar effect was observed in MDA-MB-468 cells, with levels of P-HER3 increasing 5-fold by 24 hours after addition of the drug (P-EGFR, 2-fold increase; P-HER4, 2-fold, and P-IGF-1R/insulin receptor, 6-fold; Fig. 4A).

AKT Reactivation Is Dependent on HER Kinase Activation of PI3K

Reinduction of AKT signaling after its initial inhibition in AZD8055-treated cells is accompanied by an increase in both PI3K and RTK activity. Addition of a class I PI3K inhibitor (PI-103) blocks reinduction of AKT T308 and AKT substrate phosphorylation in BT-474 (Fig. 5A, panel 2) and MDA-MB-468 cells (Supplementary Fig. S4, panel 2) that had been pretreated with AZD8055 for 8 hours. BT-474 and MDA-MB-468 express high levels of HER2 and EGFR, respectively, owing to gene amplification. The HER2-predominant HER kinase inhibitor lapatinib suppresses AKT signaling when added 8 hours after exposure of BT-474 cells to mTOR kinase inhibition (Fig. 5A, panel 3). Gefitinib, an EGR-predominant HER kinase inhibitor, has similar effects in MDA-MB-468 cells (Supplementary Fig. S4, panel 3). Thus, in breast tumor cells in which mTOR kinase is inhibited, AKT signaling is dependent on the activation of upstream RTKs.
inhibition will vary as a function of the degree of reactivation of upstream signaling.

**Combined Inhibition of the mTOR and AKT Kinases Induces Tumor Cell Death**

Reinduction of AKT activity in tumors treated with mTOR kinase inhibitors may attenuate the biological and therapeutic effects of these drugs. To test this hypothesis, BT-474 cells were treated with AZD8055, an AKT inhibitor, or the combination for 48 hours. As seen in Figure 6A, the individual treatments had almost no effect on cell death (subG1 fraction) at 48 hours; however, the combination of both treatments greatly increased the level of apoptotic cells and the levels of cleaved PARP and cleaved caspase-3 (Fig. 6B). Furthermore, the combination of both treatments inhibited the reinduction of AKT substrates owing to mTOR kinase inhibition. These data support the hypothesis that restoration of AKT signaling helps to maintain cell survival under conditions in which mTOR kinase signaling is inhibited.

**HER Kinase Inhibition Enhances the Antitumor Activity of AZD8055 In Vivo**

We previously showed that reactivation of AKT signaling might be in part responsible for the modest antitumor activity of mTORC1 inhibitors in patients (16). This reactivation may be the case for mTOR kinase inhibitors as well, even though they potently inhibit mTORC1 and mTORC2 (25, 26). We found that the maximal tolerated dose of AZD8055 in mice is 150 mg/kg, twice per week (data not shown). To determine if the induction of upstream RTKs in vitro could be observed in vivo, mice bearing BT-474 xenografts were treated for 4 hours with different concentrations of AZD8055. The mTOR kinase substrates S6K, 4E-BP1, and AKT S473 were maximally dephosphorylated in response to 75 mg/kg of AZD8055 (Fig. 7A). At this dose, a concomitant induction of EGFR, HER2, HER3, and IGF-1/insulin receptor phosphorylation and extracellular signal–regulated kinase (ERK) phosphorylation occurred. In mice, we have found that the most effective regimen of AZD8055 for antitumor therapy is 75 mg/kg, 3 times per week (data not shown). In BT-474 xenografts treated with a single dose of 75 mg/kg of AZD8055 (Fig. 7B), we observed that AZD8055 effectively inhibited the phosphorylation of mTORC1 and mTORC2 substrates for ≥24 hours, but the effect was largely gone by 48 hours. As observed in tissue culture experiments (Fig. 2, panel 1); phosphorylation of AKT T308 and the AKT substrates GSK3-β, FOXO1/3, and PRAS40 was initially inhibited (within 4 hours of treatment) and falls in parallel with that of the mTOR kinase substrates. However, we observed a subsequent increase in phosphorylation 8 hours after addition.
of the drug. Induction of phosphorylation of EGFR, HER2, and HER3 also occurs in vivo at 4 hours. Phosphorylation of HER2 and EGFR, but not HER3, declines after 16 hours of drug exposure, after reactivation of AKT signaling. Of note, AKT T308 phosphorylation remains elevated at 24 hours, despite loss of HER2 phosphorylation. This finding suggests that PI3K activity remains elevated, perhaps via activation of other HER3 or other receptors. In sum, the data suggest that chronic inhibition of mTOR kinase in vivo leads to a new steady state with persistent inhibition of mTORC1; activated AKT phosphorylated on T308, but not S473; and enough PI3K activation to support T308 phosphorylation.

To test whether inhibition of reactivated HER kinases sensitized the tumors to mTOR kinase inhibition, we evaluated the effects of combining AZD8055 with lapatinib on the growth of BT-474 xenografts (Fig. 7C). We used a low dose of lapatinib, administered 3 times weekly, that had no antitumor activity when administered alone, to distinguish sensitization of the tumor to mTOR kinase inhibition from additive activity of the 2 drugs. Chronic AZD8055 treatment causes complete arrest of tumor growth with little or no evidence of regression. After 11 days of treatment, the tumors

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**Figure 5.** mTOR kinase inhibition–induced reactivation of AKT substrates is HER2 and PI3K dependent. **A**, BT-474 cells were treated with 500 nM of AZD8055 and collected at the indicated times, and lysates were immunoblotted with indicated antibodies (panel 1 is the same as Fig. 2A, panel 1). After 8 hours of AZD8055 treatment, the cells were treated with either 1 μM of PI-103 (panel 2), or 200 nM of lapatinib (panel 3 in A). Each inhibitor was added for 1 extra hour (indicated as time 9), 4 extra hours (indicated as time 12), or 16 extra hours (indicated as time 24), and the lysates were immunoblotted with the indicated antibodies (see also Supplementary Fig. S4). **B**, BT-474 cells were treated simultaneously with both 500 nM of AZD8055 and 1 μM of lapatinib and were collected at the indicated times, and lysates immunoblotted with indicated antibodies.

**Figure 6.** Addition of an AKT inhibitor to AZD8055 promotes apoptosis in BT-474 cells. **A**, BT-474 cells were treated with either 500 nM of AZD8055 or 2 μM of an AKT inhibitor or the combination for 48 hours. The fraction of apoptotic cells (sub-G1) was determined by flow cytometry. **B**, BT-474 cells were collected after 24 hours of treatment, and the lysates were analyzed by immunoblotting.
began to regrow, but more slowly than the controls. In contrast, combined treatment with AZD8055 and lapatinib caused persistent inhibition of growth over 3 weeks of treatment and was associated with 35% regression of the tumor.

### DISCUSSION

AKT and mTOR are key enzymes controlling major cellular processes, including cellular growth and metabolism; they each have been shown to regulate the activity of the other (12). We have now shown that the selective mTOR kinase inhibitor AZD8055 is an effective inhibitor of both mTORC1 and mTORC2 activity but has complex effects on AKT signaling. It potently inhibits both S6K and 4E-BP1 phosphorylation in cells, confirming that it is a better mTORC1 inhibitor than rapamycin; in addition, AZD8055 completely inhibits the phosphorylation of AKT S473, consistent with its efficient inhibition of mTORC2, as well. Loss of AKT S473 phosphorylation is accompanied by concomitant inhibition of AKT T308 phosphorylation and kinase activity and causes decreased phosphorylation of multiple AKT substrates. Some of these results were predicted from Rictor knockdown experiments, in which AKT T308 phosphorylation was shown to be inhibited, along with that of S473 (9), and have been obtained with other mTOR kinase inhibitors, as well (27, 32). They suggest that inhibition of mTORC2 will lead to dephosphorylation of AKT at the T308 site and would result in a more profound inhibition of AKT function than would be expected from dephosphorylation of AKT S473 alone. Thus, mTOR kinase inhibition should prevent the feedback activation of AKT signaling that has attenuated the response of patients with rapamycin therapy.

However, in tumor cells exposed to the drug, even though mTORC2 inhibition is potent and persistent, inhibition of AKT T308 and of AKT substrate phosphorylation is only transient, occurring very quickly and then, 4 to 8 hours after target inhibition, rising to baseline or higher than baseline levels. We show that this new steady state is caused by reactivation of AKT after initial inhibition, not by a decrease in drug concentration in the cells. Reinduction of phosphorylation of AKT T308 and of AKT substrates is sensitive to AKT inhibition, but not to readdition of the mTOR kinase inhibitor. Our data demonstrate that this reinduction results from hyperactivation of PI3K. The relief of feedback inhibition of RTK signaling brings about induction of PI3K activation. Although we have shown that AZD8055 activates RTK signaling more potently than does rapamycin, the increase in PI3K activity observed with the 2 drugs is equivalent. It is not clear whether other factors play a role in limiting PI3K activation or whether the in vitro kinase assays do not accurately reflect degree of induction of intracellular kinase activity. In tumors in which HER kinases are dysregulated, receptor blockade with tyrosine kinase inhibitors prevents reinduction of AKT T308 and AKT substrate phosphorylation. Taken together, our findings and those of others suggest the mechanisms that underlie the biphasic effects of mTOR kinase inhibitors (27, 32). Inhibition of mTORC2 leads to rapid inhibition of AKT S473 phosphorylation, with attendant destabilization of phosphorylation at the T308 site. Release of feedback inhibition of receptor tyrosine kinase signaling function leads to activation of PI3K with the release of PI3P, which increases both PDK1 and AKT partition to the membrane and thus raises the rate of AKT T308 phosphorylation (37). The loss and then the reinduction of T308 phosphorylation and AKT activity result from these 2 opposing effects. This observation is supported by our data; in cells expressing the AKT S473D mutant, AZD8055 causes a rapid monophasic rise in T308 phosphorylation that is not preceded by a decline (Fig. 2A). In contrast, in cells in which relief of RTK feedback is inhibited, AZD8055 causes stable inhibition of phosphorylation of T308 without rebound (Fig. 5A).

In cells in which mTOR kinase inhibitors relieve feedback inhibition of receptor tyrosine kinase, leading to activation of PI3K, the result is a new steady state in which mTORC1 is potently inhibited and AKT is phosphorylated on T308, but...
not on the S473 site. This AKT species is activated and able to phosphorylate key substrates in the cell (Supplementary Fig. S5). Whether the activity of AKT monophosphorylated on the T308 site differs from that of AKT phosphorylated on both residues, in the range or intensity of substrate phosphorylation, remains to be determined. Previously, selective deletion of mTORC2 activity in mouse embryonic fibroblasts with Rictor and mLST8 knockouts has been used to show that phosphorylation of most AKT substrates is mTORC2 independent but that phosphorylation of FOXO proteins depends on intact mTORC2 activity (38). Of note, we show in this article that phosphorylation of multiple AKT substrates, including FOXO, declines and then rises with phosphorylation of AKT T308, indicating that, in this system, AKT T308 phosphorylation is enough to activate phosphorylation of AKT substrates, including FOXO.

The basis for the different effects of pharmacologic and genetic ablation of mTORC2 inhibition on FOXO phosphorylation is unknown but could have to do with the different cell types used in the studies. Our data show that mTOR kinase inhibition does initially inhibit AKT activity, but this inhibition is limited by relief of feedback inhibition of receptor tyrosine kinases, leading to induction of PI3K activity. This induction of PI3K activation is likely to depend on which receptor tyrosine kinases are activated and whether their ligands are present. It is conceivable that in certain lineages, feedback reactivation of receptor tyrosine kinases is weak or occurs in contexts in which ligands are not available. In such cases, mTOR kinase inhibition will lead to inhibition of AKT activity, as well as inhibition of mTORC1 activity. In tumors in which mTORC1 inhibition leads to relief of RTK feedback, in the steady state, mTORC1 will be inhibited, but AKT, after initial inhibition, will be reactivated.

Emerging evidence suggests that dysregulated activation of oncoproteins leads to extensive feedback throughout the signaling network. We and others have partially characterized the relief of negative feedback induced by modest mTORC1 inhibition with rapamycin or the potent and selective inhibition of AKT (16, 35, 36). The results are consistent with a model in which activation of AKT by receptors causes the coordinate feedback inhibition of receptor tyrosine kinase signaling and expression by mTOR and FOXO-dependent mechanisms (35). mTOR activation causes the downregulation of IRS-1 and other signaling intermediates and inhibition of the HER and IGF-1R/insulin receptor tyrosine kinases, as well (7). Inhibition of FOXO transcription factors by AKT-dependent phosphorylation downregulates the expression of HER3, IGF-1R, and insulin receptors (35).

AKT inhibition coordinate relieves this feedback, inhibits mTOR, activates FOXO function, and causes induction of the expression and activity of HER3, IGF-1R/insulin receptor, and other receptors. Rapamycin relieves feedback differently; inhibition of mTORC1 also induces receptor activation and IRS-1 expression and activates signaling. However, by further activating AKT, FOXO remains inhibited, and the receptor mRNAs are not induced (35). We show in this article that mTOR kinase inhibition leads to a third and more complex pattern of effects on these feedback pathways, with initial inhibition of AKT activity, which then recovers. This pattern is caused by a more highly marked reinduction of the phosphorylation of multiple HER kinases, IGF-1R, insulin receptor, and other receptors than is seen with rapamycin. The effect is likely due to a more complete inhibition of mTORC1 and to the transient potent inhibition of AKT activity by mTOR kinase inhibitors, which leads to an initial induction of both receptor expression and activity by these drugs, but only the latter by rapamycin.

These findings have important implications for the biology of tumors with deregulated PI3K/AKT/mTOR signaling and for their treatment with inhibitors of components of the pathway. One prediction from the data is that certain receptor tyrosine kinases are likely to be downregulated in these tumors unless feedback inhibition by AKT or mTOR has been altered by other genetic lesions. These tumors are unlikely to be dependent on these receptors. This observation is especially true for IGF-1R, because IGF-1 signaling is powerfully downregulated by multiple AKT- or mTOR-dependent feedback mechanisms, including downregulation of the expression of IGF-1R, insulin receptor, and their prime substrates, IRS-1 and IRS-2. In tumors treated with inhibitors of the pathway, the tumor cell reactivates IGF-1 signaling and may survive in an IGF-1R–dependent fashion (39).

This reactivation may be a general feature of these tumors; feedback reactivation of receptor tyrosine kinase signaling may significantly reduce their sensitivity to mTOR kinase inhibitors. This reduction could occur via activation of PI3K/AKT alone or, more likely, together with activation of other downstream players of the signaling pathway. mTOR kinase inhibitors and rapamycin have been noted to be predominantly cytostatic and to prominently induce autophagy, with only modest induction of apoptosis (24, 25, 40). AKT activation has been shown to prevent apoptosis by multiple mechanisms, including phosphorylation of BAD (41) and activation of NF-kB signaling (42). It is plausible that the reinduction of AKT signaling noted here plays an important role in suppressing apoptosis in tumors exposed to mTOR kinase inhibitors. Our finding that the AKT and mTOR kinase inhibitors induce synergistic apoptosis in the breast cancer cell line BT-474 is consistent with this hypothesis.

The idea that relief of feedback inhibition of receptor tyrosine kinases lessens the efficacy of PI3K pathway inhibition in patients is probable, but not yet proven. It does provide a framework for the rational design of therapeutic strategies that combine these drugs with inhibitors of reactivated pathways. The results of these trials will test the hypothesis. It is not yet clear whether mTOR kinase, AKT, or PI3K inhibitors will provide the greatest therapeutic index or whether they will need to be combined with inhibitors of individual receptors (e.g., IGF-1R, HER kinase) or of common downstream targets of these pathways (e.g., AKT, mitogen-activated protein/ERK kinase). The answer will probably vary as a function of tumor lineage and genotype, as well as the therapeutic index of the combinations. Our studies do reveal that rapamycin, mTOR kinase inhibitors, and AKT inhibitors relieve different aspects of PI3K pathway–dependent feedback, and this may be important in differentiating their clinical effects (16, 35). We show in this article that combined inhibition of mTOR and HER kinase activity causes significant regression of a breast tumor xenograft model, compared with the response elicited by the mTOR kinase inhibitor alone. These results and those of
others with similar combinations chosen on an empirical basis suggest that such a therapeutic strategy may be effective (36).

METHODS

Cell Culture and Reagents

Human breast cancer cell lines were obtained from the American Type Culture Collection and maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DME) and Ham’s F-12 supplemented with 4 mM glutamine, 100 units/mL each of penicillin and streptomycin, and 10% heat-inactivated FBS, and incubated at 37°C in 5% CO2. DNA fingerprinting was used for authentication of the MDA-MB-468 cell line; no further validation was performed. AZD8055 and gefitinib were obtained from AstraZeneca Pharmaceuticals; rapamycin, PI-103, wortmannin, AKT1/2 inhibitor, IGF, and EGF were purchased from EMD Bioscience; and purified PI3K was purchased from Millipore. Laptatinib was provided by Tonia Gilmer at GlaxoSmithKline and was dissolved in dimethylsulfoxide (DMSO) for in vitro studies and 0.5% hydroxypropylmethylcellulose/0.1% Tween 80 for in vivo studies.

Immunoblot Analysis

Cells were washed with PBS once, disrupted on ice for 30 minutes in Nonidet P-40 (NP-40, 50 mM Tris (pH 7.4), 1% NP-40, 150 mM NaCl, 40 mM NaF) or radioimmunoprecipitation assay lysis buffer supplemented with protease and phosphatase inhibitors (Pierce Chemical) and cleared by centrifugation. Protein concentration was determined with BCA Reagent from Pierce. Equal amounts of protein (10–50 μg) in cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore), immunoblotted with specific antibodies, and detected by chemiluminescence with the ECL Detection Reagents from Amersham Biosciences. Antibodies used for P-AKT (S473), P-AKT (T308), P-GSK3α (S9), P-FOXO1 (T24)/FOXO3A(T32), P-p70S6K (T389), P-S6 (S240/244 and S235/236), P-4EBP1 (T37/46), P-4EBP1 (S65), P-4EBP1 (T70), P-EGFR (Y1068), P-HER3 (Y1289), P-HER4 (Y1284), P-IGF-1R/insulin receptor (Y1135/1136), c-PARP, caspase-3, and P-ERK (T202/Y204) were purchased from Cell Signaling Technology. The agonist-conjugated PI3K p85, p85, and P-Her2 (Y1248) antibodies were obtained from Millipore. Antibodies against HER3, insulin receptor, IGF-1R, cyclin D1, cyclin D2, cyclin D3, and HER2 were from Santa Cruz Biotechnology. The β-actin antibody was obtained from Sigma, and antibody against P-PRAS40 (T246) was from Biosource International.

In Vitro AKT Kinase Assay

Kinase activity was assayed using a Cell Signaling AKT kinase kit, as described in ref. 16. The full method is explained in Supplementary Materials and Methods.

Mutagenesis

The HA-tagged AKT1 WT plasmid was used as template to create the HA-tagged S473D AKT plasmid, with standard cloning methods. A point mutation in AKT1 WT (S473) converting serine to aspartate was introduced using the Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer’s instructions. See Supplementary Materials and Methods for the transfection procedure.

PI3K Activity Assay

PI3K activity was determined as described previously (43). See Supplementary Materials and Methods for additional information.

Immunofluorescence

Cells were plated on fibronectin-coated Lab-Tek chamber slides (VWR) and treated as described. After the treatment, cells were washed with PBR and fixed in paraformaldehyde. Cells were incubated with primary antibody, followed by fluorescein-conjugated secondary antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were visualized using confocal microscopy. Confocal images were taken with Leica TCS AOB8 SP2, using 63×/1.2 numerical aperture objective. Laser lines of 405 nm and 488 nm were used to excite DAPI and Alexa 488 dyes, respectively. The same settings were used to take images of cells in different conditions.

RTK Arrays

Phospho-RTK arrays (R&D Systems) were used according to the manufacturer’s instructions. Cells were washed with cold PBS and lysed in NP-40 lysis buffer, and 400 μg of cell lysates was incubated with blocked membranes overnight. Membranes were subsequently washed and exposed to chemiluminescent reagent and developed by autoradiography. The RTK coordinates are listed in Supplementary Materials and Methods.

Analysis of Cell Cycle and Apoptosis

Cells were plated in 10-cm dishes and treated with drug or vehicle (DMSO) the following day for 48 hours. Both adherent and floating cells were harvested, and the cell nuclei were prepared as described previously (44).

Animal Studies

Six-week-old nu/nu athymic female mice (National Cancer Institute–Frederick Cancer Center) were maintained in pressurized ventilated cages. Experiments were carried out under an Institutional Animal Care and Use Committee–approved protocol, and institutional guidelines for the proper and humane use of animals in research were followed as described previously (35). See Supplementary Materials and Methods for additional information.

Statistical Analysis

Results are mean values ± standard error. Statistical analyses were performed by an unpaired, 2-tailed Student t-test.

Disclosure of Potential Conflicts of Interest

S. Guichard is an employee of AstraZeneca and has declared that no competing interest exists. The other authors disclosed no potential conflicts of interest.

The Editor-in-Chief of Cancer Discovery is an author of this article. In keeping with the AACR’s Editorial Policy, the paper was peer reviewed and a member of the AACR’s Publications Committee rendered the decision concerning acceptability.

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