Combined SFK/mTOR Inhibition Prevents Rapamycin-Induced Feedback Activation of AKT and Elicits Efficient Tumor Regression

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Abstract
Resistance to receptor tyrosine kinase (RTK) blockade in breast cancer is often mediated by activation of bypass pathways that sustain growth. Src and mammalian target of rapamycin (mTOR) are two intrinsic targets that are downstream of most RTKs. To date, limited clinical efficacy has been observed with either Src or mTOR inhibitors when used as single agents. Resistance to mTOR inhibitors is associated with loss of negative feedback regulation, resulting in phosphorylation and activation of AKT. Herein, we describe a novel role for Src in contributing to rapalog-induced AKT activation. We found that dual activation of Src and the mTOR pathway occurs in nearly half of all breast cancers, suggesting potential cross-talk. As expected, rapamycin inhibition of mTOR results in feedback activation of AKT in breast cancer cell lines. Addition of the Src/c-Abl inhibitor, dasatinib, completely blocks this feedback activation, confirming convergence between Src and the mTOR pathway. Analysis in vivo revealed that dual Src and mTOR inhibition is highly effective in two mouse models of breast cancer. In a luminal disease model, combined dasatinib and rapamycin is more effective at inducing tumor regression than either single agent. Furthermore, the combination of dasatinib and rapamycin delays tumor recurrence following the cessation of treatment. In a model of human EGFR–positive (HER2+) disease, dasatinib alone is ineffective, but potentiates the efficacy of rapamycin. These data suggest that combining mTOR and Src inhibitors may provide a new approach for treating multiple breast cancer subtypes that may circumvent resistance to targeted RTK therapies. Cancer Res; 74(17); 4762–71. ©2014 AACR.

Introduction
Breast cancers that respond to receptor-targeted therapies and phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) inhibition often develop resistance and recur. This is because of feedback activation of AKT, enhanced receptor tyrosine kinase (RTK) signaling, or cross-talk with other growth-promoting pathways (1–3). Growth factor signaling through PI3K/AKT results in activation of mTOR (4), the catalytic subunit of both the mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). These complexes activate downstream substrates such as the ribosomal protein S6 kinase (S6K), to regulate cellular metabolism, proliferation, and motility (5). S6K also has a negative feedback function, whereby it phosphorylates insulin receptor substrate 1 and 2 (IRS1/2) and uncouples PI3K from RTKs. This results in decreased AKT activity (6). S6K also directly phosphorylates the mTORC2 complex, an activating kinase for AKT, further inhibiting AKT phosphorylation and mTOR signaling (7). Consequently, inhibiting mTORC1 with rapamycin or its analogs (rapalogs) causes loss of negative feedback pathways, resulting in sustained AKT activation. This has provided a molecular rationale for the development and use of dual mTORC1/2 inhibitors, however, these second generation inhibitors initiate an RTK-driven feedback loop that leads to a new steady state, with rebound accumulation of activated AKT (8). It has also been proposed that combined mTOR/PI3K inhibitors would block rapalog-induced feedback activation of this pathway; however, one such inhibitor (BEZ235) has already been reported to increase signaling from the epidermal growth factor receptor (EGFR) family, resulting in ERK activation (9). Thus, it is unlikely that restricted focus on the AKT/mTOR pathway will be sufficient to produce sustained tumor regression.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-13-3627
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Growing evidence suggests that the non-RTK Src, and other Src-family kinases (SFK) may potentiate PI3K/AKT/mTOR signaling (10–12) to mediate cell-cycle progression and survival (13, 14). In breast cancer, Src is an important mediator of the same RTKs that activate mTOR, including insulin-like growth factor 1 receptor (IGF1R) and the EGFR family, as well as hormone receptors (15, 16). Src directly interacts with, and activates AKT (17), the primary activator of mTOR. Src kinase activity is also essential for the activity of the mTORC1 complex, independent of AKT activity (10). S6K activity and localization can also be regulated by Src (18). Taken together, the potential crosstalk between these pathways, in addition to the established roles that both Src and mTOR play in breast cancer, suggests that dual inhibition of Src and mTOR may circumvent several mechanisms of resistance to single-agent therapies by intercepting multiple signaling/feedback pathways.

Dasatinib, a selective Src/c-Abl, multitkine inhibitor, is currently approved for second line treatment of CML and Ph+ ALL, and several other Src inhibitors are in clinical trials for solid tumors, both as single agents or in combination, with preliminary data demonstrating tolerability. However, dasatinib showed limited activity as a single agent in human EGFR-2–positive (HER2+ and/or hormone receptor–positive (HR+; ref. 19) and triple-negative (TN) breast cancer (TNBC; ref. 20) in phase II trials. Given the high frequency of activation of each pathway in breast cancer (21, 22), we hypothesized that dual inhibition of Src and mTOR may overcome the limited clinical efficacy when targeting either pathway alone.

In this study, we show that Src and mTOR are both active in ~50% of human breast cancers representing all 4 receptor-defined tumor subtypes. Inhibition of SFKs with dasatinib blocks the rebound activation of AKT that occurs with mTOR inhibition in breast cancer cell lines representative of HR+, HER2+, and TNBC. We further demonstrate a synergistic effect on tumor regression when combining dasatinib with rapamycin in 2 mouse models of breast cancer. Finally, we show that tumor regression is also accompanied by a reduction in pulmonary metastasis as well as an increase in time to tumor recurrence. These results support the use of dasatinib, in combination with mTOR inhibition, as a rational approach for enhancing the efficacy of mTOR inhibitors in the treatment of breast cancer. Moreover, these data provide a novel mechanism whereby Src cooperates with mTOR signaling to regulate breast cancer survival and growth.

Materials and Methods

Cell culture

All cell lines were obtained from the American Type Culture Collection (ATCC) and authenticated by STR profiling (BDC Molecular Biology Core Facility, University of Colorado, Boulder, CO). Cells were maintained at 37°C with 5% CO2. All lines were propagated in respective media: MCF7 [Dulbecco’s Modified Eagles Medium (DMEM)], BT474 (Hybri-Care Media, ATCC), and MDA-MB-231 (RPMI-1640), supplemented with 10% FBS. BT474 also received 10 ng/mL human insulin (Roche). Drug concentrations used for in vitro studies with dasatinib, rapamycin (LC Laboratories), and saracatinib (Selleckchem) were based on previously reported IC50 values in breast cancer cell lines (23–26). Retroviral vectors for wild-type Src and dasatinib-resistant Src (Addgene) were used to create stable populations of MDA-MB-231 cells as previously described (27).

Protein analysis

Cells and homogenized tissues were lysed in radioimmunoprecipitation assay buffer supplemented with complete protease inhibitors and PhosSTOP (Roche) and proteins were processed for Western blot analyses as described (28). Immunoblots were probed with antibodies that recognize phosphorylated (Ser473)-AKT, total AKT, phosphorylated (Thr24/32) FoxO1/3a, phosphorylated (Tyr-416)-Src, total Src, phosphorylated (Thr389) p70S6K, total p70S6K, phosphorylated (Ser235/236)-S6, total S6 (all from Cell Signaling), and β-actin (Sigma-Aldrich). Densitometry was performed using ImageJ (NIH, rsweb.nih.gov/ij/).

Cell-cycle and apoptosis analysis

Cell-cycle analysis was performed as previously described (28). Annexin V staining for apoptotic cells was completed per the manufacturer’s protocol. Briefly, cells were incubated with FITC-conjugated Annexin V (Molecular Probes). Following addition of propidium iodide (PI; Sigma), stained cells were analyzed by flow cytometry and percent apoptotic cells (Annexin V–positive, PI-negative) quantified.

Animal and drug trials

All animal work was approved by the Case Western Reserve University Institutional Animal Care and Use Committee. FVB/N-Tg(MMTV-PyVT)634Mul/J (MMTV-PyMT) mice, which overexpress the Polyoma Virus Middle-T Antigen (PyMT), and FVB-Tg(MMTV-Erbbb2)NK1Mul/J (MMTV-NeuT) mice, which express the activated rat neu (HER2+) oncogene, were purchased from the Jackson Laboratory and bred with FVB/N mice to generate cohorts of tumor-bearing females. For the MMTV-PyMT recurrence studies and the MMTV-NeuT cohort, established primary tumors were removed from transgenic mice and a piece of tumor smaller than 1 mm3 in size was implanted into the inguinal mammary fat pad of adult syngeneic FVB/N females [genetically engineered mouse model (GEMM)–derived allografts (GDA)]. Mice were palpated weekly for tumor formation and monitored for toxicity by assessing grooming and weight. Once tumor volumes reached ~300 mm3 for all models, mice were randomized to receive vehicle, dasatinib alone, rapamycin alone, or combined dasatinib and rapamycin. Doses were identified based on previous in vivo studies and the murine pharmacokinetics of dasatinib and rapamycin (29–31). Dasatinib was suspended in 50% propylene glycol/50% sterile water and administered by daily oral gavage at 15 mg/kg. Rapamycin was reconstituted with 5.2% Tween80/5.2% polyethylene glycol in 0.9% saline and injected i.p., every other day, at 7.5 mg/kg. Tumor measurements were recorded using calipers and volumes calculated using the formula (length x width2/2). For the MMTV-PyMT cohort, tumors from vehicle-treated mice were only measured at 4 and 15

Published OnlineFirst July 14, 2014; DOI: 10.1158/0008-5472.CAN-13-3627

www.aacrjournals.org Cancer Res; 74(17) September 1, 2014 4763

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days of treatment because the primary tumors were too large to continue this cohort for 30 days. All other treatment groups were analyzed for a period of 4, 15, or 30 days. Tumors from the MMTV-NeuT cohort were analyzed after 15 days of treatment. For the MMTV-PyMT recurrence study, single agent and combination-treated mice were monitored for 14 and 28 days, respectively, after last drug administration.

Response criteria
The percent change in tumor volume from baseline at 4, 15 and 30 days was used to quantify response. Progressive disease (PD), stable disease (SD), partial response (PR), and complete response (CR) were defined according to RECIST criteria (32). Ninety-five percent or greater decrease in tumor volume was used to define CR. This cutoff, based on histological evaluation, was chosen to account for the limited accuracy in caliper measurements of small masses. Residual masses of less than 5% were fibrotic tissue with little to no viable tumor.

Histology and immunohistochemistry
Mammary tumors and lungs were collected within 3 hours of the last treatment and placed in 4% paraformaldehyde in PBS, fixed for 4 hours at room temperature, and transferred to 1× PBS before paraffin embedding and sectioning. Immunohistochemistry was performed utilizing the Dako Envision Plus Rabbit HRP Kit. Antigen retrieval was achieved by incubating slides in a decloaking chamber for 15 minutes at 125°C in 10 mmol/L citrate buffer (pH 6.0) or 10 mmol/L Tris/EDTA (pH 8.0). Sections were blocked with peroxidase blocking reagent along with 15 μL/mL normal goat serum (Jackson ImmunoResearch) and then incubated with indicated primary antibodies overnight at 4°C. Secondary antibody was applied and detected by 3,3′-diaminobenzidine reaction. The sections were counterstained with Gill’s hematoxylin 3 (Fisher Scientific), dehydrated, cleared, and mounted with Permount (Fisher Scientific).

Analysis of human breast tumors
Approval was obtained from the Case Western Reserve University Cancer Institutional Review Board before initiating these studies. De-identified, paraffin-embedded tissues were collected from the University Hospitals Case Medical Center Department of Pathology Archives. Samples were sectioned and immunohistochemistry was performed as described for mouse tissues. All samples were blindly scored by 2 independent pathologists. Scores were assigned based on 2 parameters: percent of tumor that is positive (10% = 1, 20% = 2, 30% = 3, etc.) and staining intensity (1+, 2+, 3+). The final score was calculated as the product of the intensity and percentage scores with the highest value being 30 [3+ × 10 (100% stained)]. A section with a combined score ≥ 1 was deemed to have positive staining for p-Src or p-S6.

Statistical analysis
Differences in expression of p-Src and p-S6 among human breast tumors subtypes were compared using Fisher exact test. Animal data were analyzed with SAS v9.2. For comparison of tumor sizes, the log of the tumor sizes at each measured time point was used as the dependent variable. A generalized mixed model incorporating repeated measures over 4 days, 2 weeks, or 4 weeks of treatment was used to compare groups. The data were adjusted for baseline tumor size at treatment initiation. Each mouse was analyzed as a random effect. P values for body weight differences associated with treatment effects and final tumor weights were calculated using 2-tailed Student t tests. Poisson regression was used to ascertain differences in the number of metastases between the treatment groups. There was one extreme outlier in the MMTV-PyMT vehicle-treated group, which did not allow appropriate fitting of the model. Hence, the data were analyzed omitting this outlier. The direction of the findings was consistent with what would be expected if the outlier were incorporated, making this a conservative omission. Tumor regression and staining for p-Src and p-S6 among treatment groups were compared using Fisher exact test for both the MMTV-PyMT and MMTV-NeuT models. P values of less than 0.05 were considered statistically significant.

Results
Coactivation of Src and the mTOR pathway in human breast tumors
To assess the potential for Src and mTOR pathway interactions in breast cancer, we examined the extent of their coactivation in 60 human tumors representing the four receptor-defined subtypes. Immunostaining for activated Src (p-Src) and mTOR pathway activation (p-S6; Fig. 1A) revealed that nearly 50% of breast tumors simultaneously activate these two pathways, with HER2+ tumors having the highest frequency of coactivation (Fig. 1B). However, the absolute levels of p-Sr and p-S6 are not correlated (Supplementary Fig. S1), suggesting that, whereas these two proteins may converge on a common signaling pathway, they do not depend on one another for basal activation.

Dasatinib prevents rapamycin-induced feedback activation of AKT through inhibition of SFKs
We next asked whether SFKs may impact the well-described feedback activation of AKT that occurs with mTOR inhibitors such as rapamycin (33). We first confirmed this feedback activation in human breast cancer cell lines, representing luminal (MC7F), HER2+ (BT474), and TN (MDA-MB-231) subtypes. Escalating doses of rapamycin (1–100 nmol/L) resulted in increased phosphorylation of AKT (Supplementary Fig. S2A). This feedback activation was observed as early as 30 minutes in the BT474 cells, and peak phosphorylation occurred in less than 24 hours in all lines (Supplementary Fig. S2B). To examine the effect of SFK inhibition on rapamycin-induced feedback activation, cells were treated with dimethyl sulfoxide (DMSO), dasatinib, rapamycin, or the combination of dasatinib and rapamycin for 1, 24, and 48 hours (Fig. 2A and Supplementary Fig. S3). As expected, rapamycin increased the phosphorylation and activation of AKT. However, this feedback activation was eradicated by addition of dasatinib. Although rapamycin can induce Src activation in some cell lines (34), we found no consistent induction of Src activation upon
rapamycin treatment in the 3 breast cancer cell lines tested (Supplementary Fig. S2A and S2B). Of note, dasatinib inhibits phosphorylation of S6 in both BT474 and MDA-MB-231 cells (Fig. 2A), and the MCF7 cells in low serum conditions (data not shown), consistent with the previous observation that S6K is a Src substrate (18). Surprisingly, inhibition of AKT activity, with the combination of dasatinib and rapamycin, did not increase apoptosis to a greater extent than the most effective single agent drug for each cell line (Supplementary Fig. S4).

These data suggest that Src potentiates PI3K/AKT signaling downstream of mTOR, however, dasatinib inhibits a number of additional kinases. To determine if another Src inhibitor has the same effects as dasatinib, MDA-MB-231 cells were similarly treated with saracatinib. This resulted in decreased phosphorylation of S6 in both BT474 and MDA-MB-231 cells (Fig. 2A), and the MCF7 cells in low serum conditions (data not shown), consistent with the previous observation that S6K is a Src substrate (18). Surprisingly, inhibition of AKT activity, with the combination of dasatinib and rapamycin, did not increase apoptosis to a greater extent than the most effective single agent drug for each cell line (Supplementary Fig. S4).

Combined dasatinib and rapamycin treatment induces tumor regression, inhibits metastasis, and delays recurrence of PyMT-induced mammary tumors

To investigate the efficacy of dual SFK and mTOR inhibition in vivo, MMTV-PyMT transgenic mice (35) were treated with vehicle, dasatinib, rapamycin, or combined dasatinib plus rapamycin for up to 30 days. No evidence of toxicity was observed (Supplementary Fig. S5A). We first evaluated the phosphorylation status of downstream signaling components of both pathways in the MMTV-PyMT tumors after only 3 hours of treatment. Similar to the in vitro analysis of breast cancer cell lines (Fig. 2), dasatinib not only blocks the increase in AKT phosphorylation observed with rapamycin, but also reduces p-AKT levels to below those of vehicle-treated control (Fig. 3A).

Using a 4-day treatment paradigm, many tumors completely regressed following combined drug treatment. In contrast, tumor response in mice treated with either agent alone included growth, stasis, and partial regression (Supplementary Fig. S6A). Indeed, representative magnetic resonance images demonstrated the disappearance of identifiable tumor mass in the combination-treated group (Fig. 3B). By day 15 of treatment, response rates of 25% and 63% (defined by RECIST criteria) were observed in dasatinib- and rapamycin-treated tumors, respectively (Fig. 3C); however, few achieved CR (dasatinib, 28%; rapamycin, 10%). The remaining mice in these two treatment groups displayed SD and PD, respectively. In contrast to the single agents, combining rapamycin with dasatinib induced PRs or CRs in all mice, with the majority of tumors (70%) regressing to below the limit of palpable detection (Fig. 3C and D and Supplementary Fig. S6B). Immunohistochemical analyses confirmed sustained inhibition of p-Src and p-S6 by dasatinib and rapamycin, respectively (Supplementary Fig. S7).

Although dasatinib can inhibit the development of metastases in mouse models (36, 37), the data concerning the
effects of mTOR inhibition on metastasis are contradictory (38, 39). Therefore, we determined if the antimetastatic properties of dasatinib are maintained when combined with rapamycin. Lungs from PyMT tumor-bearing mice treated for 15 days with vehicle, or 30 days with dasatinib, rapamycin, or the combination of dasatinib and rapamycin were analyzed for differences in the number of pulmonary metastases. Lungs from vehicle-treated mice were observed after 15 days of treatment because the primary tumors grew too large to extend this cohort to the full 30-day treatment paradigm. Dasatinib significantly reduced the number of pulmonary metastases, consistent with the well-described...
Figure 3. Combined dasatinib and rapamycin inhibits activation of AKT and induces greater tumor regression of MMTV-PyMT tumors than either agent alone. A, dasatinib blocks rapamycin-induced activation of AKT (pAKT), in vivo. MMTV-PyMT mice bearing mammary tumors of ~300 mm³ were treated with vehicle, dasatinib, rapamycin, or the combination of dasatinib and rapamycin for 3 hours. Immunoblot analysis was performed on tumor lysates using antibodies against the indicated phosphorylated (p-) or total (t-) proteins and β-actin. B, magnetic resonance images of tumor-bearing MMTV-PyMT mice were obtained on day 1, just before treatment, and on day 4 of treatment with the indicated drug(s). Shown are representative coronal T2-weighted images with tumors outlined in yellow dashes. Arrowheads in the day 4 image from the combination-treated mouse indicate the location of tumors at day 1 that had completely regressed. Results are representative of three animals imaged per treatment group. C, waterfall plot of tumors from MMTV-PyMT mice treated as in A and B. Graphed is the percent change in individual tumor volume from baseline after 15 days of treatment. Dotted lines delineate PD, SD, PR, and CR based on RECIST criteria (32). P < 0.0001, vehicle (n = 21) versus dasatinib (n = 28); P < 0.0001, vehicle versus rapamycin (n = 23); P < 0.0005, dasatinib versus combination (n = 23), and P < 0.005, rapamycin versus combination. There was no difference in response between dasatinib- and rapamycin-treated groups (P = 0.50). D, combined results for the percent of tumors that completely regressed (below the limit of palpable detection) in all four treatment groups (including 4- and 30-day treatment cohorts) in the MMTV-PyMT tumor model (Supplementary Fig. S4). †, P < 0.001, dasatinib versus vehicle; ‡, P < 4.0E–11, combination versus vehicle; *, P < 0.0005, combination versus dasatinib; †, P < 3.0E–08, combination versus rapamycin; ‡, P < 0.05, rapamycin versus dasatinib. Rapamycin versus vehicle was not statistically different (P > 0.2).
role of SFKs in tumor progression and metastasis (40), and single-agent rapamycin treatment had minimal effect (Supplementary Fig. S8). Addition of rapamycin to dasatinib offered no benefit over dasatinib alone, nor did it negatively impact the efficacy of dasatinib in reducing metastases.

It has been proposed that dasatinib targets the "cancer-stem cell" or tumor-initiating cells responsible for metastasis and recurrence (41). Thus, we hypothesized that the synergistic effects seen with combination of rapamycin and dasatinib on primary tumor regression would translate into a delay in time to recurrence. Mice bearing MMTV-PyMT mammary tumors of ~300 mm³ were treated with vehicle, dasatinib, rapamycin, or the combined drugs for 2 weeks. At the end of treatment, tumors were measured and monitored for regrowth. Within 14 days after treatment cessation, most tumors from single agent-treated mice were larger than the initial tumor volume (Fig. 4), whereas tumors from combination-treated mice remained undetectable. By 28 days, 78% of tumors treated with the combined drugs had recurred, but only 33% were as large as the starting tumor. This considerable delay in tumor recurrence suggests an added benefit of the combined drugs for extending recurrence-free survival.

**Dasatinib synergizes with rapamycin to induce tumor regression in a HER2-induced mouse model of breast cancer**

The high degree of Src and mTOR pathway coactivation in HER2-amplified breast cancers (Fig. 1B) suggests that these tumors may be particularly responsive to combined therapy. Indeed, we found that only rapamycin + dasatinib treatment induced significant tumor regression in the MMTV-NeuT (42) transgenic mouse model of HER2⁺ breast cancer (Fig. 5A), wherein 100% of the combination-treated animals displayed PBs or CRs. In contrast, rapamycin alone resulted primarily in PD and SD whereas all mice in the dasatinib-treated group displayed PD, indistinguishable from the vehicle-treated controls (Fig. 5B). Thus, dasatinib was completely ineffective as a single agent, yet clearly potentiated the efficacy of rapamycin in HER2-induced tumors.

**Discussion**

Breast cancer is a heterogeneous disease that has benefited from the development of subtype-specific–targeted treatment paradigms. Single-agent hormone therapy and RTK-targeted therapies have shown significant efficacy, increasing both disease-free and overall survival. However, de novo and acquired resistance is a common barrier to the use of receptor-targeted therapies. Furthermore, patients with TN disease are left with limited treatment options other than systemic chemotherapy. The extensive redundancy of RTK signaling, coupled with an increased appreciation of the potential for growth factor–driven resistance to kinase inhibitors (2), requires the identification of a novel approach to target cancer cells. We have focused on the combinatorial blockade of two critical downstream effectors frequently dysregulated in most breast cancers. To date, Src and mTOR pathway coactivation in human breast tumors has not been evaluated. Our analysis revealed a high percentage of breast tumors with coactivation of both pathways (Fig. 1B). Studies aimed at identifying molecular markers predictive of a "dasatinib-response" suggest that the TN, or basal-like subtype is most sensitive to this agent (43). Anbalagan and colleagues reported that total Src and p-Src are increased in TNBC compared with ER+BC (21). Interestingly, we found that the hormone receptor negative/HER2⁻ subtype had the highest percent of tumors with Src and mTOR pathway coactivation (Fig. 1B), suggesting that these tumors may be particularly responsive to the combination of dasatinib and mTOR inhibition.

A major obstacle to mTOR-targeted therapies is an increase in the active, phosphorylated form of the prosurvival factor AKT. This is because of the loss of negative feedback regulation (see review; ref. 1 and Fig. 6). The ability of dasatinib to prevent this feedback activation of AKT in breast cancer cell lines, as well as PyMT tumors (Figs. 2A and 3A), suggests essential crossstalk between SFKs and the mTOR pathway. Indeed, we have shown that dual inhibition of SFKs and mTOR signaling leads to significant tumor regression in two highly creden
tialed, RTK-driven mouse models of mammary tumorigenesis, further indicating the importance of the intersection between the mTOR and SFK pathways. Multiple phase II and III clinical trials are currently evaluating either Src or mTOR inhibitors as single agents, or combined with conventional cytotoxic chemotherapies, as well as targeted therapies, for the treatment of breast cancer and other malignancies. Recently, the first phase I trial for the combination of rapamycin plus dasatinib (for the treatment of refractory neuroblastoma) was initiated (ClinicalTrials.gov) based on several in vitro studies showing a synergistic effect on cell-cycle arrest and apoptosis. However, this is the first preclinical in vivo evidence demonstrating a
rationale for the combinatorial inhibition of these two pathways in multiple subtypes of breast cancer.

We found that dasatinib alone is not sufficient to induce complete tumor regression in the majority of PyMT tumors (Fig. 3D), and is completely ineffective in the model of HER2+ disease (Fig. 5B). Our results mirror clinical studies where dasatinib, as a single agent, is ineffective in multiple breast cancer subtypes (19, 20). However, Src is required for mammary tumor initiation by HER2 (44). Recently, Karim and colleagues reported that dasatinib delays tumor onset in a mouse model of HER2+ disease that also has chronically active AKT through PTEN loss, but tumor growth was not inhibited (45). Together, these data indicate that Src/SFKs may be necessary for HER2-induced tumor initiation but not sustained growth. Conversely, in the studies reported herein, addition of dasatinib to rapamycin causes extensive tumor regression.

Figure 5. Effects of Src and mTOR inhibitors on HER2/neu-induced mammary tumor growth, in vivo. A, mice bearing primary MMTV-NeuT mammary tumors of ~300 mm3 were treated and analyzed as in Fig. 3. P < 0.0001, vehicle (n = 7) versus combination (n = 10); P = 0.0001, vehicle versus rapamycin (n = 9); P < 0.0001, dasatinib (n = 9) versus rapamycin; P = 0.0001, rapamycin versus combination and P < 0.0001, dasatinib versus combination. There was no difference in treatment effect between dasatinib- and vehicle-treated groups (P = 0.73). B, effect of combining dasatinib and rapamycin treatment on overall response (based on RECIST criteria) in MMTV-NeuT cohort. 1, Addition of dasatinib to rapamycin shifts outcome from mostly PD/SD to all PR/CR. (P < 0.0005).

Efficacy of Dual SFK and mTOR Inhibition in Breast Cancer

Figure 6. Model of PI3K/AKT/mTOR signaling and the effects of targeting mTOR and SFKs. A, the PI3K/AKT/mTOR pathway is tightly regulated by multiple feedback loops (dashed lines) to control growth factor signaling. B, aberrant RTK signaling in tumor cells can override this regulation and addition of mTOR inhibitors (C) can further increase activation of AKT. This occurs through multiple mechanisms, including derepression of the negative feedback loops, whereby S6K induces degradation of IRS-1/2 (uncoupling RTK signaling to PI3K) and S6K inhibits the mTORC2 complex. D, we report that rapalog-induced activation of AKT can be blocked with the dual Src/c-Abl inhibitor, dasatinib; however, the specific SFK(s) and directed downstream targets are unknown. As both Src kinase and mTOR pathway activity are found to be increased in breast cancer, these findings suggest pathway convergence that promotes tumor viability. This novel convergence reveals a potential vulnerability and supports the use of combinatorial agents that act downstream of RTKs.
These distinct outcomes are likely because of the fact that mTOR inhibition has additional consequences other than simply activating AKT, including loss of protein synthesis. Thus, rapamycin-treated tumors are already compromised and the blockade of AKT survival signaling by dasatinib potentiates the effects of rapamycin. In vitro, alterations in proliferation and/or apoptosis between single-agent and combination treatment were not significant (data not shown and Supplementary Fig. S4), suggesting the in vivo synthetic lethality is likely the result of combined extrinsic effects on the tumor microenvironment/vascularity as well as signaling within the tumor. Indeed, endothelial AKT signaling has been proposed as the rate-limiting event for rapamycin inhibition of PyMT mammary tumor progression (46). We and others have shown that, in several different mouse models of breast cancer, rapamycin induces extensive coagulating necrosis, which may be attributed to inhibition of angiogenesis (29, 47–49). Although this is sufficient to induce cytostasis, it is not effective at eliminating the tumor. In addition to the tumor cell-autonomous effects of dasatinib, there is also evidence for extrinsic effects of this drug on the tumor vasculature and stroma. In preclinical models of multiple myeloma, dasatinib inhibits tumor vessel growth, which correlates with combined targeting of platelet-derived growth factor receptor β (PDGFRβ) and vascular endothelial growth factor receptor (VEGFR)/c-Src signaling pathways (50). In lung carcinoma, dasatinib partially reverses cancer-associated fibroblasts toward a phenotypically normal state, and this is associated with reduced tumor cell proliferation (51). We hypothesize that the combinatorial effect of both drugs observed in vivo results from the ability of dasatinib to eliminate the remaining tumor cells that are compromised, but not killed, by the effects of rapamycin, through effects on the tumor and the microenvironment.

In summary, we have identified a novel therapeutic approach for multiple subtypes of breast cancer that circumvents receptor targeting. The ability of dasatinib to significantly augment the effects of mTOR inhibition in vivo identifies an intrinsic vulnerability in breast cancer cells. Although it is well established that mTOR inhibitors induce rebound activation of AKT, no studies have placed Src within this feedback pathway. Our findings indicate that one or several SFKs converge on the PI3K/AKT/mTOR pathway to regulate tumor viability. Identifying which SFK(s) modulate reactivation of AKT in response to rapamycin will lend further insight into the mechanism(s) of feedback regulation. The combined use of dasatinib and mTOR inhibitors has not yet been evaluated in patients for breast cancer and the results presented herein provide a rationale for clinical assessment of the safety and efficacy of this approach.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support
This work was supported by the NIH (CA090398 to R.A. Keri and P30 CA043703 to the Case Comprehensive Cancer Center Histology and FACS Core Facilities).

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Received December 19, 2013; revised May 15, 2014; accepted June 8, 2014; published OnlineFirst July 14, 2014.

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