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 regression than either single agent. Furthermore, the combination of dasatinib and rapamycin delays tumor recurrence following the cessation of treatment. In a model of 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.
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 due to 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 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.
Growing evidence suggests that the non-receptor tyrosine kinase Src, and other Src-family kinases (SFKs) 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 IGF-1R 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 mTORC-1 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, multikinase-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 epidermal growth factor receptor-2-positive (HER2+) and/or hormone receptor-positive (HR+) (19) and triple-negative (TN) breast cancer (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 PI3K/AKT/mTOR signaling may overcome the limited clinical efficacy when targeting either pathway alone.
In the current study, we show that Src and mTOR are both active in ~50% of human breast cancers representing all four 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 TN breast cancer. We further demonstrate a synergistic effect on tumor regression when combining dasatinib with rapamycin in two 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). Cells were maintained at 37°C with 5% CO₂. All lines were propagated in respective media: MCF7 (Dulbeco’s Modified Eagles Medium (DMEM)), BT474 (Hybri-Care Media (ATCC)), and MDA-MB-231 (RPMI-1640), supplemented with 10% FBS. BT474 also received 10ng/ml human insulin (Roche). Drug concentrations used for in vitro studies with dasatinib, rapapmycin (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, rsbweb.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-Erbb2)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 1mm$^3$ in size was implanted into the inguinal mammary fat pad of adult syngeneic FVB/N females (Genetically engineered Mouse Model (GEMM)-derived allografts or GDAs). Mice were palpated weekly for tumor formation and monitored for toxicity by assessing grooming and weight. Once tumor volumes reached ~300mm$^3$ 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 \((\text{length} \times \text{width}^2)/2\). For the MMTV-PyMT cohort, tumors from vehicle-treated mice were only measured at 4 and 15 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. SD, PR, and 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 three hours of the last treatment and placed in 4% paraformaldehyde in PBS, fixed for 4 hours at room temperature, and transferred to 1x PBS prior to 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 min at 125 °C in 10 mM citrate buffer (pH 6.0) or 10mM
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 prior to 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 two independent pathologists. Scores were assigned based on two 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+ x 10 (100% stained)]. A section with a combined score ≥ 1 was deemed to have positive staining for p-Src or p-S6.

**Statistics**

Differences in expression of p-Src and p-S6 among human breast tumors subtypes were compared using Fisher’s 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’s \( t \)-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's exact test for both the MMTV-PyMT and MMTV-NeuT models. \( P \)-values of less than 0.05 were considered statistically significant.
RESULTS

Co-activation 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 co-activation 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 co-activation (Fig. 1B). However, the absolute levels of p-Src and p-S6 are not correlated (Supplementary Fig. S1), suggesting that, while 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 (MCF7), HER2+ (BT474) and triple-negative (MDA-MB-231) subtypes. Escalating doses of rapamycin (1-100nM) 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 24hr 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. While rapamycin can induce Src activation in some cell lines (34), we found no consistent induction of Src activation upon rapamycin treatment in the three breast cancer cell lines tested (Supplementary Fig. S2A-B). 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 Src, and, like dasatinib, completely blocked the feedback-activation of AKT that occurs with rapamycin treatment (Fig. 2B). To further assess the impact of Src, MDA-MB-231 cells were generated that stably express wild-type Src (Src wt) or a dasatinib-resistant Src (Src DR) (Fig. 2C). Rapamycin still induced phosphorylation of AKT in the presence of either Src (Fig. 2D). However, while dasatinib blocked AKT activation in the Src (wt) cells, it was not able to block this induction in the Src (DR) cells. Thus, dasatinib prevents rapalog-feedback on AKT by inhibiting Src or another SFK.

**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, mammary tumor-bearing, Polyoma Virus Middle-T antigen transgenic mice (MMTV-PyMT) (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 four 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 complete response (CR) (dasatinib, 28%; rapamycin, 10%). The remaining mice in these two treatment groups displayed stable or progressive disease (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-D and Supplementary Fig. S6B). Immunohistochemical analyses confirmed sustained inhibition of p-Src and p-S6 by dasatinib and rapamycin, respectively (Supplementary Fig. S7).
While dasatinib can inhibit the development of metastases in mouse models (36, 37), the data regarding the effects of mTOR inhibition on metastasis are contradictory (38, 39). Therefore, we determined if the anti-metastatic 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 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 ~300mm³ 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), while 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 co-activation 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 PRs or CRs. In contrast, rapamycin alone resulted primarily in PD and SD while 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 sub-type 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 triple-negative 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 co-
activation in human breast tumors has not been evaluated. Our analysis revealed a high
percentage of breast tumors with co-activation of both pathways (Fig. 1B). Studies aimed at
identifying molecular markers predictive of a “dasatinib-response” suggest that the triple-
negative, or basal-like subtype is most sensitive to this agent (43). Anbalagan et al. reported
that total Src and p-Src are increased in triple negative breast cancer (TNBC) compared to
ER+BC (21). Interestingly, we found that the hormone receptor negative/HER2+ subtype had
the highest percent of tumors with Src and mTOR pathway co-activation (Fig. 1B), suggesting
that these tumors may be particularly responsive to the combination of dasatinib and mTOR
inhibition.

A major obstacle to mTOR therapies is an increase in the active, phosphorylated form of
the pro-survival factor AKT. This is due to the loss of negative feedback regulation (see review
(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 (Fig. 2A and 3A), suggests essential crosstalk 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 credentialed, 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 pre-clinical 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 sub-types (19, 20). However, Src is required for mammary tumor initiation by HER2 (44). Recently, Karim et al., 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. These distinct outcomes are likely due to 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/vasculature 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). While 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 pre-clinical 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 reverts cancer associated fibroblasts towards 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 micro-environment.

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.
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.
REFERENCES


FIGURE LEGENDS

Figure 1. Src and mTOR pathway activation in human breast tumors. A, Representative photomicrographs (10x) showing phosphorylated-Src (p-Src) and phosphorylated-S6 (p-S6) immunostaining in a cohort of 60 human breast tumors. Tumors were grouped by receptor-defined subtype. ER+/-, estrogen receptor-positive/negative; PR+/-, progesterone receptor-positive/negative; HER2+/-, human epidermal growth factor receptor-2-positive/negative. B, Summative results from immunohistochemical analyses described in A. Values are shown for each receptor-defined subtype. Co-expression of p-Src and p-S6 occurs in a greater percentage of ER-/PR-/HER2+ tumors compared with ER-/PR-/HER2- tumors (Fisher’s exact test, P<0.05).

Figure 2. Src inhibition blocks rapamycin-induced feedback activation of AKT in breast cancer cell lines. A, Western blot analysis for phosphorylated-AKT (p-AKT), total AKT, p-Src, total Src, p-S6, total S6 and β-actin was performed on lysates from human breast cancer cell lines treated for 48 hours with vehicle (DMSO), dasatinib (Das), rapamycin (Rap), or the combination of dasatinib plus rapamycin (Combo). MCF7 and BT474 cells were treated with 1μM dasatinib and/or 1nM rapamycin. MDA-MB-231 cells were treated with 100nM dasatinib and/or 2.5μM rapamycin. Results are representative of at least three independent experiments performed in duplicate. Ratios of p-AKT to total AKT were determined by densitometry using ImageJ. B, Saracatinib (Sara) (5μM) inhibits rapamycin (100nM)-induced pAKT similar to dasatinib (100nM). MDA-MB-231 cell lysates were harvested at 36 hours of treatment and probed with indicated antibodies as in panel A. C, Stable overexpression of Src in MDA-MB-231 cells...
transduced with wild-type Src (wt) or dasatinib-resistant Src (DR). D, MDA-MB-231 cells stably overexpressing Src (wt) or Src (DR) were treated with the indicated drug(s) as in panel B. Dasatinib fails to block rapamycin induction of pAKT in Src (DR) cells but not Src (wt) cells.

**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 ~300mm³ 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 prior to 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 have completely regressed. Results are representative of 3 animals imaged per treatment group. C, Waterfall plot of tumors from MMTV-PyMT treated as in panels A and B. Graphed is the percent change in individual tumor volume from baseline after 15 days of treatment. Dotted lines delineate progressive disease (PD), stable disease (SD), partial response (PR) and complete response (CR), based on RECIST criteria (32). P<0.0001, vehicle (n=21) *versus* dasatinib (n=28); P<0.0001, vehicle *versus* rapamycin (n=20); P<0.0005, dasatinib *versus* combination (n=23) and P<.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 day 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).

**Figure 4. Addition of dasatinib to rapamycin delays tumor recurrence in the MMTV-PyMT mice.** MMTV-PyMT mice bearing mammary tumors of ~300mm³ were treated with vehicle, dasatinib (dashed lines), rapamycin (gray lines) or the both drugs (black lines) for 2 weeks. Each line represents a distinct tumor. As expected, tumors from vehicle-treated mice continued to grow and these mice were removed from the experiment at the end of 2 weeks (data not shown). Drug(s) were withdrawn at 2 weeks and mice were palpated bi-weekly for tumor regrowth. The addition of dasatinib to rapamycin delays recurrence by greater than 2 weeks (14 days vs. 28 days) compared with single agent-treated mice.

**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 ~300mm³ 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. *Addition of dasatinib to rapamycin shifts outcome from mostly PD/SD to all
PR/CR. (P<0.0005). Progressive Disease (PD), Stable Disease (SD), Partial Response (PR), Complete Response (CR).

**Figure 6. Model of PI3K/AKT/mTOR signaling and the effects of targeting mTOR and Src family kinases (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 de-repression 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. Abbreviations: RTKs, receptor tyrosine kinases; mTORC1, mammalian target of rapamycin complex 1; S6K, ribosomal S6 kinase; IRS-1/2, insulin receptor substrate 1/2 (IRS-1/2); mTORC2, mammalian target of rapamycin complex 2.
Figure 1

A

Breast cancer sub-type analysis of Src and mTOR activation in human tumors

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Figure 2

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B

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D

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Figure 3

A

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B

Day One

Day Four

C

MMTV-PyMT Tumors (15 days)

% Change in tumor volume from baseline

D

Complete Regression (% of Tumors) PyMT

<table>
<thead>
<tr>
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<th>Dasatinib</th>
<th>Rapamycin</th>
<th>Combination</th>
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<td>0/33 (0%)</td>
<td>14/45 (31%)*</td>
<td>3/34 (8%)†</td>
<td>34/49 (70%)**,#,†</td>
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</tbody>
</table>
Figure 4

The figure shows the tumor volume (fold change) over days post treatment for three different treatments: Dasatinib (n=10), Rapamyicin (n=9), and Combination (n=9). The x-axis represents days post treatment ranging from 0 to 28 days, and the y-axis represents tumor volume (fold change) ranging from 0 to 6. The treatment period is indicated as 2 weeks.
Figure 5

A

MMTV-NeuT Tumors (15 days)

% Change in tumor volume from baseline

-100 -80 -60 -40 -20 0 20 40 60 80 100 2000 3000 4000 5000

Vehicle Dasatinib Rapamycin Combination

B

Response (% of each treatment group) NeuT

<table>
<thead>
<tr>
<th></th>
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<th>Rapamycin</th>
<th>Combination</th>
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</tbody>
</table>
Figure 6

A. Intact mTOR Signaling Normal Cell

B. Constitutive Signaling Tumor Cell

C. Rapamycin-Treated Tumor Cell

D. Rapamycin + Dasatinib Treated Tumor Cell

RTKs → IRS-1/2 → P3K → AKT → mTORC1, mTORC2 → S6K

RTKs → SFKs → P3K → AKT → mTORC1, mTORC2 → S6K

RTKs → SFKs → P3K → AKT → mTORC1, mTORC2 → S6K

RTKs → SFKs → P3K → AKT → mTORC1, mTORC2 → S6K

Rapamycin + dasatinib
Combined SFK/mTOR inhibition prevents rapamycin-induced feedback activation of AKT and elicits efficient tumor regression.

Jennifer L. Yori, Kristen L. Lozada, Darcie D. Seachrist, et al.

Cancer Res  Published OnlineFirst July 14, 2014.

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