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Loss-of-function RNAi screens in breast cancer cells identify *AURKB*, *PLK1*, *PIK3R1*, *MAPK12*, *PRKD2*, and *PTK6* as sensitizing targets of rapamycin activity

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Abstract

The use of molecularly targeted drugs as single agents has shown limited utility in many tumor types, largely due to the complex and redundant nature of oncogenic signaling networks. Targeting of the PI3K/AKT/mTOR pathway through inhibition of mTOR in combination with aromatase inhibitors has seen success in particular sub-types of breast cancer and there is a need to identify additional synergistic combinations to maximize the clinical potential of mTOR inhibitors. We have used loss-of-function RNAi screens of the mTOR inhibitor rapamycin to identify sensitizers of mTOR inhibition. RNAi screens conducted in combination with rapamycin in multiple breast cancer cell lines identified six genes, *AURKB*, *PLK1*, *PIK3R1*, *MAPK12*, *PRKD2*, and *PTK6* that when silenced, each enhanced the sensitivity of multiple breast cancer lines to rapamycin. Using selective pharmacological agents we confirmed that inhibition of *AURKB* or *PLK1* synergizes with rapamycin. Compound-associated gene expression data suggested histone deacetylation (HDAC) inhibition as a strategy for reducing the expression of several of the rapamycin-sensitizing genes, and we tested and validated this using the HDAC inhibitor entinostat *in vitro* and *in vivo*. Our findings indicate new approaches for enhancing the efficacy of rapamycin including the use of combining its application with HDAC inhibition.

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Conflict of interest

None.

Keywords

Rapamycin; Breast cancer; RNAi screen; HDAC inhibition

Introduction

The concept of curative chemotherapy cancer first emerged in the 1960s with the use of combinations of cytotoxic drugs with differing mechanisms for the treatment of patients with Hodgkin's lymphoma or pediatric acute lymphoblastic leukemia (ALL) [1]. Combinations of cytotoxic drugs, and more recently, molecularly targeted agents in combination with cytotoxic drugs have become the mainstay of medical oncology [2]. The ability of combinations to circumvent tumor resistance is well established [2,3]. A spectrum of cooperative mechanisms between targeted agents are known, including examples of combinations enhancing response by vertically targeting the same pathway or horizontally targeting parallel pathways [2,3]. RNA interference (RNAi) screening affords an unbiased method for gene function discovery and is a powerful tool for aiding drug development, enabling target identification and validation. RNAi screening can be employed to identify proteins that alter the survival of tumor cells or modulate the activity of a drug of interest. Pharmacological inhibition of proteins identified by RNAi can be used to phenocopy these effects potentially enabling the identification of rational drug combinations [4]. Such combination treatments may improve therapeutic efficacy, overcome tumor resistance to a single chemotherapeutic agent, and lower individual drug toxicities. To identify proteins, that when silenced by RNAi to generate a loss-of-function (LOF) phenotype, enhance the activity of the mTOR inhibitor rapamycin we have conducted siRNA screens, focused on the human kinome, in multiple breast cancer cell lines, with the aim of identifying new drug combination strategies for this important class of drugs.

The mTOR pathway is deregulated in many human cancers, including breast cancer [5], and has been actively pursued as an antitumor target for some time. The best-characterized inhibitors of the serine/threonine protein kinase mTOR, are rapamycin (sirolimus) and its analogs (rapalogs), temsirolimus (CCI-779, Torisel, Wyeth) and everolimus (RAD001, Afinitor, Novartis) [6]. Rapamycin, a bacterial product with immunosuppressive properties, interacts with the intracellular receptor FKBP12 to bind mTOR, blocking activity of the mTOR complex 1 (mTORC1) [7]. The rapalogs exhibit antiproliferative activity in tumor cell lines and xenografts, and therapeutic responses have been seen in clinical trials in some cancer types particularly advanced renal cell carcinoma (RCC) [8,9]. However, in many cancers including breast cancer, rapamycin is cytostatic, causing G1 cell cycle arrest [10] and exhibits only limited single agent clinical benefit when used in advanced/metastatic breast cancer (reviewed in [11]). This limitation may, at least in part, be due to the activation of signaling feedback loops, including the activation of AKT, that paradoxically results in enhanced cell survival [12]. Recently though, based on data from the phase III BOLERO-2 clinical trial [13], the mTOR inhibitor everolimus, in combination with the aromatase inhibitor, exemestane, was approved by the United States Food and Drug Administration for the treatment of postmenopausal women with advanced hormone receptor-positive, HER2-negative breast cancer.

The success of everolimus in advanced hormone receptor-positive, HER2-negative breast cancer suggests that strategies could be developed that exploit the activity of this class of drug more broadly including to other sub-types of breast cancer. One approach to this would be to identify one or more proteins that when inhibited synergize with mTOR inhibition to decrease tumor growth. RNAi screening has the potential to guide the choice of drug combinations by identifying proteins that, when inhibited, modulate the activity of a drug of interest. In this study, we have used RNAi screens focused on the human kinome to identify kinases or kinase related proteins that when inhibited sensitizes breast cancer cells to rapamycin and demonstrate strategies for modulating the activity of these proteins through the application of both specific and broad pharmacological inhibitors.

Materials and methods

Cell lines, antibodies, compounds, RNA, protein and cell cycle assays

MCF-7, MDA-MB-231 and MDA-MB-468 cells were obtained from ATCC (Manassas, VA); see Appendix S1 and Fig. S1A for further details. See Table S1 for the details of the antibodies, compounds, and qRT-PCR primer used in this study. Gene specific mRNA levels were measured by either qRT-PCR or by a branched DNA-based assay, and protein levels by Western blotting using standard methods (see Appendix S1). Cell cycle distribution, determined by DNA content, was measured by flow cytometry (see Appendix S1).

RNAi screen and analysis

RNAi screens were conducted using siRNAs corresponding to 931 genes arrayed from the Human Druggable Genome Version 2.0 library (Qiagen Inc., Germantown, MD). The siRNAs target 691 genes annotated as associated with kinase activity and 240 additional genes. See Table S2 for full details of genes targeted. For screening, four siRNAs per gene were arrayed in 384 well plates, one siRNA per well; see Appendix S1 for details of siRNA transfection. Paired screens were conducted in each cell line; 48 hours post siRNA transfection one screen received 0.1% (v/v) DMSO, while the other received 10 nM rapamycin in 0.1% (v/v) DMSO. Cell viability was assessed through quantification of ATP (CellTiter-Glo luminescent Reagent, Promega; Madison, WI) 72 hours after compound addition. Luminescent intensities were measured using a Victor plate reader (PerkinElmer; Waltham, MA). Untransfected cells and wells transfected with negative (AllStar siNegative; Qiagen) and positive (AllStar siCelldeath; Qiagen) control siRNAs were included on every plate. Quality control metrics for each screen are described in Fig. S1B and C. The data for each experimental siRNA were normalized using the plate median luminescence signal (excluding controls) and then z-score transformed; the z-score for each siRNA/rapamycin combination was compared with that obtained for the siRNA/vehicle only treated sample.

Two criteria were used for the identification of putative sensitizers of rapamycin activity with either or both criteria satisfied for two or more siRNAs per gene in two or more cell lines. Criteria 1 was fulfilled if RNAi in the presence of rapamycin induced a decrease in viability indicated by a z-score of -1.645 (corresponding to a $p < 0.05$) that represented at least a 10% decrease in viability over that seen in the absence of rapamycin. Criteria 2 was

fulfilled if RNAi in the presence of rapamycin generated a z-score of -1 that represented at least a 15% decrease in viability over that seen in the absence of rapamycin. Knowledge-based gene networks were generated using Ingenuity Pathway Analysis (IPA) tools (Ingenuity Systems®; Redwood City, CA). Follow up RNAi analysis of specific genes was conducted using siRNAs obtained from Qiagen or Ambion (Austin, TX). Sequences and qRT-PCR validation of silencing are detailed in Table S1D.

Drug combination studies and Connectivity Map analysis

Five hundred cells/well were plated in 384 well plates and cultured overnight; DMSO or drug was then added. After 72 hours of incubation, cell viability was assessed as described above. The effect of drug combinations was evaluated by the combination index (CI) method using the CombuSyn software (ComboSyn, Inc., Paramus, NJ). The calculation is based on the median effect model of Chou–Talalay [14]. Synergism, additive effect and antagonism are defined as $CI < 1$, $CI = 1$, and $CI > 1$, respectively.

The Connectivity Map Version 2 dataset was analyzed using the Oncomine analysis tool (<https://www.oncomine.org/resource/login.html>). Oncomine defines the top 5% over- and under-expressed genes for each individual inhibitor and concentration tested in the Connectivity Map as an individual gene set.

NanoPro Capillary Isoelectric Focusing Immunoassay

To profile the unphosphorylated, mono and dual-phosphorylated isoforms of ERK, a NanoPro Capillary Isoelectric Focusing immunoassay (CIFI) was used as described previously [15] and detailed in the Appendix S1.

In vivo MDA-MB-231-luciferase xenograft model

To test the efficacy of the rapamycin and entinostat combination *in vivo*, xenografts were generated using MDA-MB-231 cells expressing luciferase (kindly provided by Jeffrey Green, CCR, NCI) injected into the mammary fat pad of female SCID mice. See Appendix S1 for details. This animal study was approved and performed in accordance with the National Institutes of Health Intramural Animal Care and Use Program.

Statistical analysis

A Student t-test performed in Excel (two-tailed distribution and two-sample unequal variance) was used to determine statistical differences between groups. A value of $p < 0.05$ was considered significant.

Results

Kinome focused loss-of-function RNAi screens in human breast cancer cell lines identify candidate sensitizing genes of rapamycin activity

To identify sensitizing genes of rapamycin activity in breast cancer cells, we conducted siRNA-based screens in three different breast cancer cell lines. We used one ER+/PR+ breast cancer line, MCF-7, as mTOR inhibition has shown clinical benefit in ER+ breast cancer [13] and two triple negative breast cancer (TRNBC) cell lines, MDA-MB-231 and

MDA-MB-468. Activation of the PI3K/AKT/mTOR pathway is frequently observed in TRNBC, but to date, the clinical application of mTOR inhibition as a strategy to treat this subtype of breast cancer has been less well studied [16]. We first confirmed that the mTOR inhibitor rapamycin induces a cytostatic effect on the growth of breast cancer cell lines (Fig. 1A). The two cell lines with mutations in components of the PI3K/AKT/mTOR pathway, MDA-MB-468 cells harbor a PTEN inactivating mutation and MCF7 cells harbor an activating mutation in PIK3CA, both exhibited concentration and time-dependent inhibition of growth in the presence of rapamycin. In contrast, MDA-MB-231 cells were highly resistant to exposure to rapamycin, exhibiting only a ~ 20% decrease in cell viability at the highest concentration of rapamycin (10,000 nM). To identify sensitizing genes of rapamycin we conducted paired siRNA based RNAi screens in each of the breast cancer cell lines, where 48 hours post siRNA transfection one screen received 0.1% (v/v) DMSO, while the other screen received 10 nM rapamycin in 0.1% (v/v) DMSO. The z-score-transformed data for each siRNA in the absence and presence of 10 nM rapamycin in each cell line are detailed in Table S2.

In contrast to RNAi screens conducted in the context of cytotoxic drugs where drug induced effects are typically greater than that induced by gene silencing, in this screen the effect of rapamycin alone on cell viability was less than the effects of silencing many of the genes targeted in the screen. This necessitated setting selection criteria that identified relatively small enhancements in activity when RNAi was combined with rapamycin. We used two criteria for the identification of putative sensitizers of rapamycin activity with either or both criteria needing to be reached for two or more siRNAs per gene in two or more cell lines. Criteria 1 was fulfilled if RNAi in the presence of rapamycin induced a significant decrease in viability that represented at least a 10% decrease in viability over that seen in the absence of rapamycin. Criteria 2 was fulfilled if RNAi in the presence of rapamycin generated a reduction in viability that represented at least a 15% decrease in viability over that seen in the absence of rapamycin. This analysis identified 16 kinase genes, one kinase regulatory subunit (*PIK3R1*) and two other genes (*AGXT* and *KIF1A*) as putative enhancers of rapamycin activity in two or more breast cancer cell lines; one further kinase gene *RPS6KA6* was also retained for further analysis though one siRNA induced only a 9% further decrease in cell viability in the presence of rapamycin in MDA-MB-231 cells (Fig. 1B). The distribution of the candidate rapamycin-sensitizing genes in the three breast cancer cell lines is shown in Fig. 2A. Only two genes, *AATK* and *MAPK12*, were identified as enhancing rapamycin sensitivity when silenced in all three-breast cancer cell lines. The greatest overlaps were seen for the two TRNBC cell lines (eight genes) and for MCF7 and MDA-MB-231 cells, also eight genes.

RNAi analysis confirms *AURKB*, *PLK1*, *PIK3R1*, *MAPK12*, *PRKD2*, and *PTK6* as sensitizers of rapamycin activity

To prioritize the candidate rapamycin-sensitizing genes for follow up analysis, we first confirmed their expression levels in the three breast cancer lines. The expression of all, but two of the candidate genes (*KIF1A* and *RPS6KA6*) was detected by qRT-PCR in all three lines (data not shown). *AURKB* and *PLK1* were highly expressed in all three of the breast cancer lines when compared with the reference normal breast tissue RNA (Fig. S1D).

Pathway analysis (Ingenuity) of the 20 rapamycin-sensitizing candidate genes generated a network that linked 12 of the candidate genes (Fig. 2B). As these 12 candidate genes were all expressed at quantifiable levels in all three-breast cancer lines and there was evidence of them acting within a shared functional space, we focused our follow up on these genes. For follow up analysis, we used one siRNA corresponding to each candidate gene that, with two exceptions, was the same as one of the siRNAs used in the screen (Qiagen) and a second siRNA from a different vendor with no sequence homology to the siRNAs used in the screen (Ambion). Silencing efficacy had been validated for most of these siRNAs by the vendors, but we also assessed the reduction in gene-specific mRNA levels for each siRNA in each cell line using qRT-PCR analysis (Table S1D) and Western blot analysis for the siRNAs corresponding to six of the genes (Fig. S2). We used the same procedure as for the siRNA screen for the follow up analysis of the 12 rapamycin-sensitizing candidate genes, but to enable statistical analysis each siRNA was the subject of four independent transfections in the absence or presence of 10 nM rapamycin.

The results of silencing six of the candidate genes (*BCR*, *NME2*, *PLK4*, *SRPK1*, *FRK*, or *AKT1*) on rapamycin sensitivity showed either siRNA or cell line specific effects that did not validate the screening data (Fig. S3). The follow up analysis of the other six genes, *AURKB*, *PLK1*, *PIK3R1*, *MAPK12*, *PRKD2*, or *PTK6*, however, did recapitulate the screening data (Fig. 2C). In the absence of rapamycin both siRNAs corresponding to *PLK1*, *PIK3R1*, or *PTK6* significantly reduced the viability of all three-breast cancer cell lines (Fig. 2Ci–iii, left side of each graph), but in most cases these siRNAs also enhanced the sensitivity of each cell line to rapamycin (Fig. 2Ci–iii, right side of each graph, and Fig. 2D). The siRNAs corresponding to *AURKB*, *MAPK12*, or *PRKD2* reduced the viability of at least two of the three breast cancer lines in the absence of rapamycin (Fig. 2Civ–vi, left side of each graph), but the silencing mediated by at least one siRNA per gene also enhanced the sensitivity of more than one line to rapamycin (Fig. 2Civ–vi, right side of each graph, and Fig. 2D). Overall, these results confirm that the LOF of *AURKB* (aurora kinase B), *MAPK12* (mitogen-activated protein kinase 12/p38 gamma), *PIK3R1* (phosphoinositide-3-kinase, regulatory subunit 1 (alpha)/p85), *PLK1* (polo-like kinase 1), *PTK6* (protein tyrosine kinase 6, *aka* BRK – breast tumor kinase), or *PRKD2* (protein kinase D2) sensitize multiple breast cancer cell lines to rapamycin activity.

Candidate rapamycin-sensitizing gene targets and mTOR

We next investigated whether the LOF of the six identified rapamycin-sensitizing genes enhance the activity of rapamycin by targeting the mTOR signaling directly or *via* parallel pathways. To do this, we first assessed if the silencing of any of the rapamycinsensitizing genes altered mTOR signaling by examining total and phosphorylated RSP6 and 4E-BP1 protein levels 72 hours post siRNA transfection of MDA-MB-231 cells. We observed no overt changes in 4E-BP1 protein levels following silencing of any of the six rapamycin-sensitizing genes (data not shown). We observed a slight decrease in the levels of both total and phosphorylated RSP6 following silencing of *PLK1* (Fig. 3A), but the silencing of the other rapamycin-sensitizing genes mediated no changes in either total or phosphorylated RSP6 levels (Fig. S4A). We also investigated the effects of silencing the two major components of the mTORC1 and two complexes on the six rapamycin-sensitizing genes to

determine if the inhibition of mTOR alters the expression of any of these genes. mTOR forms the catalytic subunit of two complexes: the mTORC1 complex that includes raptor and the mTORC2 complex that includes rictor. Rapamycin predominately inhibits the activity of mTORC1, though prolonged exposure to rapamycin may inhibit mTORC2 activity [17]. To determine whether mTORC1, mTORC2, or both, influence the expression of any of the rapamycin-sensitizing gene targets, we silenced *RPTOR* (Fig. 3B) or *RICTOR* (Fig. S4B) in MDA-MB-231 cells for 72 hours. Silencing of *RPTOR* mediated a decrease in the expression of AURKB, PLK1, and MAPK12 (Fig. 3B). The decrease in the expression of AURKB and PLK1 is consistent with effects of rapamycin on cell cycle regulated proteins. We observed no changes in the expression of PTK6, PIK3R1, or PRKD2 (data not shown). Loss of RICTOR mediated a small decrease in the levels of AURKB and PLK1 (Fig. S4B) but mediated no changes in the expression of the other four rapamycin-sensitizing genes (data not shown). Together, these data suggest that the LOF of the identified rapamycin-sensitizing genes are predominantly exerting their effects *via* parallel pathways and not as a consequence of targeting mTOR signaling.

Inhibitors of *AURKB* or *PLK1* phenocopy the effect of rapamycinsensitizers identified by RNAi

Our RNAi-based observations suggest that combining rapamycin with selective inhibitors of AURKB or PLK1 should mediate a synergistic effect on the growth of breast cancer cells, including TRNBC cells. ZM447439 is an ATP-competitive inhibitor of aurora kinases with selectivity for aurora B kinase [18]. Aurora B kinase function is essential for normal cell division and cancer cells treated with ZM447439 pass through S-phase, but fail to divide, exhibiting defects in chromosomal alignment and segregation [18], but apoptotic cell death is typically only seen at high doses (μM range) of ZM447439 [19,20]. Using low concentrations of rapamycin and concentrations of ZM447439 that as a single agent induced less than ~10% reduction in viability we observed a synergistic effect in all three breast cancer lines (Table 1). This synergistic effect was most pronounced in MDA-MB-468 cells. The polo-like kinase 1 serine/threonine kinase also plays an essential role in mitosis and cell cycle progression. BI2536, an ATP-competitive kinase PLK1 inhibitor [21] causes perturbation of the spindle assembly, resulting in mitotic arrest, disruption of cytokinesis, and apoptosis [22]. The combination of rapamycin and the PLK1 inhibitor, BI2536, was less synergistic than when combined with inhibition of AURKB, with MCF7 cells more susceptible to the rapamycin/BI2536 combination than the TRNBC cell lines (Table 1).

HDAC inhibition suppresses multiple sensitizers of rapamycin activity

As specific inhibitors of most of the rapamycin-sensitizers identified by RNAi are unavailable we sought an alternative approach to their pharmacological inhibition by conducting an analysis of the Connectivity Map dataset of compound-associated gene expression available for MCF7 cells [23]. Using our initial list of 12-candidate rapamycin-sensitizer genes we identified 15 compounds that reduced expression of three or more of these genes (see Table S3). Of particular interest was the down-regulation of the expression of *PIK3R1*, *PTK6*, and *PLK1* by the histone deacetylase inhibitor (HDACi) trichostatin A (TSA) (Table S3). The development of HDAC inhibitors has become an important therapeutic strategy in cancer as a means of reversing altered gene expression including

activation of silenced tumor suppressors. However, global expression analysis indicates that HDAC inhibition also represses expression of many genes [24,25]. Previous studies have shown that the HDACi LBH589 degrades aurora A and B kinases in renal cancer cells [26] and TSA reduces the levels of PLK1 protein in HeLa cells [27]. The combination of rapamycin and HDAC inhibition has been studied in models of number of cancers [28–31] and currently the combination of temsirolimus and vorinostat is undergoing phase 1 trial in patients with metastatic prostate cancer (ClinicalTrials.gov Identifier: NCT01174199), however, the combination of mTOR inhibition and HDAC inhibition has not been extensively studied in models of breast cancer. TSA has not been developed for therapeutic use, so we assessed two HDAC inhibitors under clinical development, suberoylanilide hydroxamic acid (SAHA) and entinostat (MS-275), in combination with rapamycin. SAHA enhanced rapamycin activity particularly in MCF7 cells, but entinostat showed a more consistent synergistic effect in all three of the breast cancer cell lines tested (Table 1).

Entinostat is a synthetic benzamide derivative that preferentially inhibits Class I HDACs. Treatment of the three breast cancer cell lines with 100 nM entinostat for 48 hours reduced transcript levels of most of the six validated rapamycin-sensitizing genes (Fig. 4A). Importantly, as seen for other HDAC inhibitors, entinostat decreased the levels of AURKB and PLK1 (Fig. 4B). There was also a dose-dependent decrease in PTK6, MAPK12, and PIK3R1 protein levels in MDA-MB-231 cells treated with entinostat, but we saw no consistent effects on PRKD2 protein expression following entinostat treatment. Further, the specific inhibition of mTOR via RNAi enhanced entinostat activity in MDA-MB-231 (Fig. 4Ci), inducing a similar enhancement in the reduction of viability as seen when rapamycin (10 nM) and entinostat (100 nM) were combined (Fig. 4Cii).

Downstream effects of the combination of rapamycin and entinostat on AKT and AKT signaling

Next, we investigated downstream effects of the rapamycin/entinostat combination in the TRNBC line MDA-MB-231. Consistent with previous studies [12] rapamycin treatment increased phosphoAKT^{Ser473} level (Fig. 5A). In contrast, entinostat did not induce AKT phosphorylation, and blocked rapamycin-induced AKT phosphorylation (Fig. 5A). Also as expected, rapamycin inhibited S6 ribosomal protein phosphorylation (pRPS6) (Fig. 5A). Entinostat also decreased pRPS6 and there was no observable pRPS6 in cells treated with the rapamycin/entinostat combination (Fig. 5A). The rapamycin/entinostat combination induced G₀/G₁ cell cycle arrest (Fig. 5B) and also enhanced the cleavage of PARP and caspase-3 (Fig. 5C). ERK pathway is another pro-survival pathway that may contribute to resistance to rapalogs [32]. A previous study reported that the HDAC inhibitors sodium butyrate and SAHA block ERK phosphorylation without affecting the total levels of ERK protein [33]. Consistent with these previous findings, we observed reduced levels of ERK phosphorylation in MDA-MB-231 cells treated with the rapamycin/entinostat combination (Fig. 5D). Western blot analysis cannot resolve monophospho- and diphospho-isoforms of ERK1 or ERK2 (monophosphorylated ERK lacks kinase activity) and so to profile these isoforms we used a capillary isoelectric focusing immunoassay. This assay detected decreased levels of the dual-phosphorylated isoforms of ERK1 and ERK2 following treatment with the rapamycin/entinostat combination (Fig. 5E).

The combination of rapamycin and entinostat inhibits the *in vivo* tumor growth of MDA-MB-231 breast cancer cells

Finally, we evaluated the potential of the rapamycin/entinostat combination to inhibit tumor growth *in vivo*. The rapamycin/entinostat combination significantly inhibited MDA-MB-231-luc xenograft growth (Fig. 6A and B). Rapamycin initially induced tumor growth inhibition, but after 2 weeks tumor growth increased (Fig. 6B). Overall, no toxic effects were observed in any of the treatment groups and no significant weight loss was seen (Fig. S5A); consistent with clinical data the rapamycin-treated group and the rapamycin/entinostat combination group had elevated levels of cholesterol (Fig. S5B).

Follow-up analysis of the protein from tumor tissues (following 5 weeks of treatment) showed entinostat treatment alone and with rapamycin decreased AURKB and PLK1 protein levels (Fig. 6C). The rapamycin/entinostat combination specifically decreased the level of p-ERK (Fig. 6C). Rapamycin alone increased the level of phosphoAKT^{Ser473}, but when combined with entinostat, the phosphorylation of AKT was completely inhibited (Fig. 6C). In contrast to our *in vitro* results, we did observe decreased expression of PRKD2 (PKD2) following treatment with rapamycin or entinostat *in vivo*, that was further enhanced to undetectable levels in tumors from animals treated with both rapamycin and entinostat. PIK3R1 expression was reduced in tumors from entinostat and rapamycin and entinostat treated animals, while the expression of MAPK12 was only reduced in the tumors from rapamycin and entinostat treated animals. PTK6 expression was reduced in tumors from rapamycin treated mice, but not in tumors subjected to other treatments (Fig. 6C).

Discussion

The recent approval of everolimus, in combination with the aromatase inhibitor letrozole, for the treatment of ER positive breast cancer indicates the potential for enhancing the clinical benefit of rapalogs to target the mTOR pathway when used in conjunction with a second agent [34]. In this study we used an RNAi screening strategy, focused on the human kinome, to identify genes that when inhibited enhance the sensitivity of multiple breast cancer lines to rapamycin. Our RNAi screens initially identified 20 candidate rapamycin-sensitizing genes. We conducted follow up analysis of 12 candidate genes, chosen on the basis of gene network analysis, and validated six, *AURKB*, *PLK1*, *MAPK12*, *PIK3RI*, *PTK6* and *PRKD2*, as rapamycin-sensitizing genes in at least two different breast cancer cell lines. A spectrum of cooperative mechanisms between targeted agents are known, including examples of combinations enhancing response by vertically targeting the same pathway or horizontally targeting parallel pathways [2,3]. Analysis of the effects of silencing each of these genes on established down-stream targets of mTOR and analysis of the silencing RPTOR or RICTOR suggests that the loss-of-function of these proteins is most likely acting to sensitize breast cancer cells through disruption of parallel signaling pathways, as opposed to enhancing vertical inhibition of the PI3K/mTOR pathway.

The identification of *MAPK12*, *PIK3RI*, and *PTK6* as sensitizers of rapamycin is of particular interest. Increased expression of *PTK6* has been observed in breast cancer [35,36]. *PTK6* inhibitors are under development [37], and it will be interesting to determine if any of these synergize with rapamycin. There is also increasing interest in the role of *PIK3R1* in

tumorigenesis, as recent deep-sequencing efforts have identified the presence of recurrent mutations in this gene in several cancer types, in particular endometrial cancer [38], and a significant proportion of breast cancers [39]. *PIK3R1* encodes p85 α , the regulatory subunit of phosphoinositide 3-kinase, and as such is an important modulator of PI3K/AKT, exerting both positive and negative effects on PI3K signaling and thus cell proliferation and survival (reviewed in [40]). The best-characterized function of p85 α is its role in stabilizing the catalytic subunit of PI3K, p110, but it also interacts with additional proteins and a number of recent studies have described direct interactions with PTEN [40]. In our study, all three-breast cancer cell lines, irrespective of PI3K/AKT activation state exhibited decreased viability following the silencing of *PIK3R1*, suggesting a pro-survival function for p85 α in this context. Further work will now be required to determine whether the sensitization of rapamycin following LOF of *PIK3R1* relates to a single functional role of p85 α or represents a cumulative effect due to its other functional roles.

MAPK12 encodes p38 gamma (p38 γ), one of the four isoforms of the p38 MAP-kinase family (reviewed in [41]). p38 α is expressed ubiquitously while p38 γ expression is principally expressed in skeletal muscle. However, we observed expression of *MAPK12* in all three-breast cancer cell lines (data not shown) and several studies have suggested a role for p38 γ in breast cancer progression and metastasis [42–44]. Interestingly, p38 γ was recently identified using a similar RNAi screening strategy as used here as a sensitizer of a PARP inhibitor [45].

RNAi screens represent an unbiased approach to the identification of putative molecular targets, but the lack of specific small compound inhibitors that can be used for follow up analysis is likely to remain a barrier to the exploitation of such data. We phenocopied the effects of silencing *PLK1* or *AURKB* through application of small compound inhibitors, but as no specific chemical inhibitors are available for the other sensitizer proteins we sought to identify another pharmacological approach to inhibiting their activity. By analyzing publically available drug associated gene expression data we identified HDAC inhibition as a possible strategy and showed that the HDACi entinostat suppressed the expression of our validated rapamycin-sensitizer genes and was synergistic with rapamycin both *in vitro* and *in vivo*. Previously, entinostat has been shown to induce apoptosis in ERBB2 expressing breast cancer cells as a consequence of down-regulating ERBB3, while TRNBC cell lines including MDA-MB-231 and MDA-MB-468 exhibited less sensitivity to entinostat [46]. Our *in vivo* data using the MDA-MB-231 cell line are consistent with these data in that we saw minimal effect when entinostat was administered alone, but in combination with rapamycin we saw a significant reduction in tumor growth. The synergistic effect of combining rapamycin with HDAC inhibition has been reported in several tumor models [28–31] and we have now extended this to breast cancer cells. HDAC inhibition as an approach for the treatment of breast cancer has been pursued as both a single agent and in combination with other drugs [47] and entinostat is currently the subject of a number of clinical trials in breast cancer (in combination with exemestane, Clinical Trials Identifier: NCT02115282; in combination with Fulvestrant: NCT02115594, and in combination with lapatinib, or lapatinib and trastuzumab: NCT01434303). Our study suggests that its

combination with rapamycin or its analogs may also be worth pursuing as a therapeutic combination in breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix: Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.canlet.2014.08.043.

Abbreviations

RNAi	RNA interference
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
mTOR	mechanistic target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
LOF	loss-of-function
TRNBC	triple negative breast cancer
RSP6	ribosomal S6 kinase
RCC	Renal cell carcinoma

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Fig. 1.

Kinome focused loss-of-function RNAi screens in human breast cancer cell lines identify sensitizing genes of rapamycin activity. (A) The human breast cancer cell lines MDA-MB-231, MDA-MB-468 and MCF7 were treated with DMSO or increasing doses of rapamycin. Each data point represents the mean $\pm$ standard deviation (SD) of at least six wells. (B) The z-score of cell viability in the absence (DMSO) or presence of rapamycin (10 nM) for those siRNAs corresponding to the putative rapamycin sensitizing genes that fulfilled either criteria 1, a z-score -1.645 ($p < 0.05$) in the presence of rapamycin that

was a 10% decrease over that seen in the absence of rapamycin, or criteria 2, a z-score -1.00 in the presence of rapamycin that was a 15% decrease over that seen in the absence of rapamycin, for two or more siRNAs, in two or more cell lines.

Fig. 2.

RNAi analysis confirms six candidate-sensitizing genes of rapamycin activity. (A) Initially identified candidate-sensitizing genes of rapamycin activity in each of the three breast cancer cell lines studied. (B) Network analysis of candidate-sensitizing genes of rapamycin activity functionally linked 12 genes. (C) Confirmation of the sensitization of breast cancer cells to rapamycin activity following silencing of (i) *AURKB*, (ii) *PLK1*, (iii) *PIK3R1*, (iv) *MAPK12*, (v) *PRKD2*, or (vi) *PTK6*. Cells were transfected with siRNAs obtained from two vendors: A = Ambion and Q = Qiagen. Data are shown as the relative viability of siNeg

transfected cells (no rapamycin) calculated from four transfections per siRNA. Statistical analysis of data (Student t-test) in the absence of rapamycin (DMSO) is shown on the left hand side of each graph and in the presence of rapamycin is shown on the right hand side of each graph, *p 0.05, **p 0.01, ***p 0.001. (D) Comparison of the viability of rapamycin treated cells in the absence and presence of gene silencing in: (i) MDA-MB-231, (ii) MDA-MB-468, and (iii) MCF7 cell lines. Data are shown as the relative viability of rapamycin treated siNeg transfected cells versus rapamycin treated silenced cells calculated from four transfections for each siRNA. The statistical analysis (Student t-test) of rapamycin treated siNeg transfected versus rapamycin treated silenced cells is indicated for each siRNA, *p 0.05, **p 0.01, ***p 0.001.

Fig. 3. Identified rapamycin-sensitizing gene targets do not directly influence mTOR signaling. (A) Western blot analysis showing the expression of total and phosphorylated RSP6 following PLK1 loss-of-function in MDA-MB-231 cells (72 hours post siRNA transfection). (B) Western blot analysis of the expression of PLK1, AURKB, and MAPK12 72 hours post silencing of *RPTOR*.

Fig. 4.

The HDAC inhibitor entinostat decreases expression of rapamycin-sensitizing genes of rapamycin activity. (A) The expression of *AURKB*, *PLK1*, *PIK3R1*, *MAPK12*, *PRKD2*, and *PTK6* shown following drug exposure, DMSO or entinostat (100 nM) for 48 hours. Gene expression was assessed using gene specific qRT-PCR(β -actin normalized); data are shown as the median value of three qRT-PCR reactions. (B) Western blot analysis, using the antibodies indicated, of protein from MDA-MB-231 cells treated with the concentrations of entinostat shown for 72 hours; tubulin expression was included as a loading control; AcH3

refers to acetylated histone H3, and H3 to histone H3. (C) (i) MDA-MB-231 cells were transfected with either a control siRNA (siNeg) or *MTOR* siRNAs. Forty-eight hours post-siRNA transfection DMSO or increasing concentrations of entinostat were added. Cell viability was measured after further 72 hours of incubation. (ii) Cell viability of MDA-MB-231 cells treated with either siRNA or drug alone or combinations of siRNA and drug, or combination of drug. Each bar represents the mean $\pm$ SD of six wells after a total of 120 hours.

Fig. 5.

Effects of combining rapamycin and entinostat on AKT phosphorylation, cell cycle and apoptosis, and ERK phosphorylation in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with rapamycin or entinostat or both and protein harvested at the stated time points for Western blot analysis of pAKT, total AKT and pRPS6. (B) The combination of rapamycin and entinostat enhances arrest in the G0/G1 phase of the cell cycle and (C) induces apoptosis as indicated by cleavage of caspase-3 and PARP after 72 and 96 hours of drug treatment. (D) ERK phosphorylation was reduced in cells treated with the rapamycin/

entinostat combination. (E) A capillary isoelectric focusing immunoassay (CIFI) shows that the diphosphorylated isoforms of ERK1 and ERK2 were decreased in MDA-MB-231 cells treated with the rapamycin/entinostat combination. Panel (i) shows a representative immunoassay for the drug treatments indicated 72 hours after initiation of treatment, panels (ii) and (iii) show the quantitative data for ERK1 and ERK2 levels assessed by CIFI 24, 48 and 72 hours after initiation with the drug treatments indicated.

Fig. 6.

The combination of rapamycin and entinostat inhibits the growth of MDA-MB-231 xenografts *in vivo*. Rapamycin and/or entinostat were administered five times per week by intraperitoneal injection and oral gavage respectively into mice carrying xenografts generated using MDA-MB-231-luc cells (11 or 12 mice per treatment group). MDA-MB-231-luc xenograft growth was monitored weekly. (A) Representative luminescent images taken 4 weeks following initiation of drug administration. (B) Quantification of the luminescent signal detected from MDA-MB-231-luc xenografts in animals in each treatment

group over time. Data are shown as the mean and standard deviation of the photon flux (10^9) measured using a XenogenIVIS 100 system (VivoVision, Caliper Life Science) and analyzed using Living Image 4 software (Caliper Life Science). Week 0 indicates the average signal for each group of animals pre-treatment; animals were randomized to each treatment group. The number of animals in each treatment group after 5 weeks of drug treatment is indicated. Statistical analysis (Student t-test) was conducted on the luminescent signal (photon flux) measured after 4 and 5 weeks of treatment; at 4 weeks, controls (n = 7) versus rapamycin plus entinostat treated animals (n = 11), p = 0.04, at 5 weeks controls (n = 7) versus rapamycin plus entinostat treated animals (n = 11) p = 0.003, *p < 0.05, **p < 0.01. (C) Western blot analysis with indicated antibodies on protein extracted from tumor tissues; data from two different animals (labeled 1 and 2) from each treatment group are shown. The analysis of the expression of PLK1, AURKB, PRKD2, PIK3R1, and MAPK12 shows β -ACTIN expression as a loading control. The analysis of PTK6 expression shows TUBULIN expression as a loading control.

Table 1

Synergism of AURKB1, PLK1 and HDAC inhibitors with rapamycin.

Cell line	Rapamycin		ZM447439		B12536		SAHA		Entinostat	
	nM	CI	nM	CI	nM	CI	nM	CI	nM	CI
MDA-MB-231	1	100	0.19	1	6.79	100	1.97	100	0.19	
	1	500	0.22	10	0.67	500	0.47	500	0.74	
	10	100	0.17	1	15.64	100	58.5	100	0.19	
MDA-MB-468	10	500	0.72	10	0.64	500	0.54	500	0.72	
	1	100	0.03	1	0.19	100	0.41	100	0.55	
	1	500	0.0001	10	0.6	500	0.63	500	0.91	
MCF7	10	100	0.001	1	2.24	100	18.57	100	0.4	
	10	500	0.004	10	1.56	500	5.21	500	0.79	
	1	100	0.01	1	0.09	100	0.07	100	0.15	
	1	500	0.05	10	1.16	500	0.25	500	0.48	
	10	100	0.49	1	0.17	100	0.09	100	0.15	
	10	500	0.09	10	0.56	500	0.16	500	0.45	

Breast cancer cell lines were exposed to the AURKB inhibitor ZM44739 (100 or 500 nM), or the PLK1 inhibitor, B12536 (1 or 10 nM), or the HDAC inhibitors, SAHA (100 or 500 nM), or entinostat (100 or 500 nM), in the absence of rapamycin or the presence of either 1 or 10 nM rapamycin. Cell viability (CellTiter-Glo, Promega) was assessed following 72 hours of drug treatment and these data were used to determine the combination indexes (CI) for each drug combination. A CI of <1 indicates synergism, a CI = 1 indicates an additive effect and a CI > 1 indicates antagonism.