Combination of the mTOR inhibitor ridaforolimus and the anti-IGF1R monoclonal antibody dalotuzumab: preclinical characterization and phase I clinical trial

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ABSTRACT

Purpose: mTOR inhibition activates compensatory IGFR signaling. We evaluated the ridaforolimus (mTOR inhibitor) and dalotuzumab (anti-IGF1R antibody) combination.

Experimental design: In vitro and in vivo models, and a phase I study where advanced cancer patients received ridaforolimus (10-40 mg/day QD×5/week) and dalotuzumab (10 mg/kg/week or 7.5 mg/kg QOW) were explored.

Results: Preclinical studies demonstrated enhanced pathway inhibition with ridaforolimus and dalotuzumab. With 87 patients treated in the phase I study, main DLTs of the combination were primarily mTOR-related stomatitis and asthenia at doses of ridaforolimus lower than expected, suggesting blockade of compensatory pathways in normal tissues. Six confirmed partial responses were reported (3 breast cancer patients); 10/23 breast cancer patients and 6/11 ER+/high proliferative breast cancer patients showed antitumor activity.

Conclusions: Our study provides proof-of-concept that inhibiting the IGF1R compensatory response to mTOR inhibition is feasible with promising clinical activity in heavily pretreated advanced cancer, particularly in ER+/high proliferative breast cancer.

Statement of translational relevance:

There is a strong rationale for targeting the PI3K/AKT/mTOR pathway in advanced cancer patients, with potential to improve clinical outcomes in subsets of patients. Preclinical and early clinical results support the combination of ridaforolimus and dalotuzumab to abrogate feedback activation of AKT following mTOR inhibition. In particular, our findings reinforce the value of mTOR inhibition in the treatment of ER+ breast cancer.
INTRODUCTION

The phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway plays a critical role in cell growth, proliferation, and survival (1). Multiple receptor tyrosine kinases (eg, insulin-like growth factor-1 receptor [IGF1R] and human epidermal growth factor [HER] receptor family) activate this pathway via adapter proteins or binding of the p85-PI3K regulatory subunit (2). In breast cancer, aberrant activation of the PI3K pathway has been reported in approximately 50% of primary tumors and has been associated with the HER receptor family, estrogen receptor (ER), and IGF1R signaling pathways (3-6), and with Resistance to endocrine therapy and anti-HER2 therapy (7, 8).

Due to its central role in cancer development and progression, various therapeutic strategies have focused on blocking specific signaling molecules of PI3K pathway. Perhaps the most extensively tested druggable component of the pathway has been the kinase mTOR (comprised of mTORC1 and mTORC2) due to a better understanding of and discovery of its modulation by rapamycin and its analogs (9, 10).

However, rapamycin analogs have shown modest and variable antitumor activity as single-agent therapies for most tumor types, possibly due to the lack of identification of the most suitable patient population and/or optimal dose (10, 11). One mechanism of sub-optimal response to mTOR inhibitors is related to the activation of compensatory pathways. When mTORC1 is active, S6K1 directly phosphorylates the adapter protein of IGF1R insulin receptor substrate-1 (IRS1), leading to its degradation. A decrease in IRS1 levels reduces IGF1R signaling and activation of the PI3K/AKT pathway. Conversely, mTORC1 inhibition relieves this negative feedback loop, resulting in sustained IGF1R/IRS1 signaling and activation of AKT (12, 13).

Inhibition of mTOR in cancer cell lines and in patient tumor biopsies causes activation of AKT kinase, which is associated with induction of IRS1 but could be prevented by IGF1R inhibition (13, 14). Furthermore, activation of compensatory pathways has also been observed with other inhibitors of the PI3K/AKT/mTOR pathway, suggesting that is a general mechanism of response to inhibition of this pathway and that concomitant blockade of these compensatory responses may be required for optimal therapeutic efficacy (15, 16).
We have tested the hypothesis of preventing activation of IGF1R signaling by exploring the combination of the mTOR inhibitor ridaforolimus with the anti-IGF1R monoclonal antibody dalotuzumab in preclinical models and in a phase I study in patients with advanced solid tumors, including ER+ breast cancer patients.

METHODS

Preclinical assessment of combination effects between ridaforolimus and dalotuzumab

Cell lines

293FT producer cells were obtained from Invitrogen. Other cell lines were purchased from cell line banks American Type Culture Collection (ATCC), Japanese Collection of Research Bioresources Cell Bank (RIKEN), or Deutsche Sammlung von Mikroorganismen und Zellkulturen (DKMZ). The cells were grown under culture conditions recommended by the vendors. Cells were expanded and immediately frozen for experimentation. The authenticity of the cell lines were verified by short tandem repeat (STR) profiling analysis or similar methodologies by the banks. In addition, the mutation and gene expression levels from the targeted exome sequencing (TES) data were compared with the published mutation (COSMIC; Sanger data base) and gene expression data.

In vitro studies

A short hairpin RNA (shRNA) enhancer screen was performed in a colorectal cancer cell line. Details are available in the Supplementary Appendix.

In vivo xenograft studies

Immunodeficient female mice (HsdCpb:NMRI-Foxn1

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, Harlan Laboratories, Rossdorf, Germany) were implanted with tumor fragments from the lung adenocarcinoma LXFA629. The control group was dosed with dalotuzumab vehicle once per week (qwk) by intraperitoneal (IP) injection for 4 weeks (qwk×4). Ridaforolimus vehicle was dosed 5 times per week IP for 4 weeks. The experimental groups were dosed with dalotuzumab qwk (20 mg/kg, IP), ridaforolimus 5 times per week (1 mg/kg, IP), or the combination of dalotuzumab and
ridaforolimus at the same dose/schedules as the mono-therapy arms. Details are available in the **Supplementary Appendix**.

**Phase I clinical study of the combination of dalotuzumab and ridaforolimus in patients with solid tumors**

Patients aged 18 years or older with histologically confirmed advanced tumors unresponsive to standard therapy—or for whom standard therapy does not exist—were enrolled into the phase I, international, multicenter, open-label, single-arm, nonrandomized trial (ClinicalTrials.gov identifier: NCT00730379; http://clinicaltrials.gov/ct2/show/NCT00730379; Protocol 004). Eligible patients had Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 and adequate bone marrow, hepatic, and renal function. Patients with uncontrolled diabetes and uncontrolled hyperlipidemia at screening were excluded in the dose escalation portion. Diabetic patients requiring insulin were excluded from the dose escalation portion; however, well-controlled insulin-using diabetic patients were allowed in the dose expansion cohorts. The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. All patients gave informed consent, and approval was obtained from the ethics committees at each participating institution.

In the dose-escalation phase, the main objective was to determine the maximum tolerated dose (MTD) of the 2 drugs in combination. Enrollment in dose escalation followed a variation of the traditional “3 + 3” dose-escalation scheme, called the “rolling 6” trial design (17). Enrollment of a minimum of 3 patients per dose cohort was required, but enrollment of up to 6 patients per cohort was allowed (no intrapatient dose escalation was permitted). In dose expansion, at least 12 additional patients were to be enrolled at the recommended phase II dose (RP2D). Antitumor activity was analyzed in the dose-escalation and dose-expansion phases. A third part of the study was added as an amendment to seek preliminary evidence of antitumor activity in specific tumor types (advanced colorectal and NSCLC), and to gain additional safety and tolerability experience with the combination at the RP2D to better describe the adverse event (AE) profile. Secondary and tertiary objectives included pharmacodynamic (PD) and pharmacokinetic (PK) analyses, as well as
measurements of antitumor activity using various end points (objective response, metabolic response, tumor markers, 4-month progression-free survival [PFS] rate, and time on treatment).

Ridaforolimus was administered PO at a starting dose of 10 mg/day, once daily for 5 days (10 mg/day qd×5; dose level 1); ridaforolimus doses were escalated in sequential cohorts of patients in increments of 10 mg/day up to 40 mg/day (dose levels 2-4). Dalotuzumab was administered intravenously at 10 mg/kg/week for the first dose level; subsequent dose levels tested sequential escalating doses of ridaforolimus in combination with dalotuzumab at either 10 mg/kg/week (dose levels 2-4) or 7.5 mg/kg every other week (q14 days; dose levels 1.5-3.5). Ridaforolimus was given as monotherapy for the first week (days 1-5); this period was referred to as cycle 0. Cycle 1 began on the first day of dosing with the combination of ridaforolimus and dalotuzumab and lasted for 4 weeks (Supplementary Figure S1). The first 4 weeks of dosing were used as the toxicity evaluation period for the purposes of dose escalation. Dose-limiting toxicities (DLTs) were defined as any grade 4 neutropenia persisting for at least 5 days; grade 3 or 4 neutropenia associated with fever, antibiotics, or hospitalization for infection; grade 4 thrombocytopenia persisting for at least 5 days; grade 3 or greater hyperglycemia persisting for at least 5 days, grade 3 or greater diarrhea persisting for more than 24 hours, or grade 3 or greater nausea or vomiting (despite optimal medical management); and any other grade 3 or higher nonhematological toxicity—except for alopecia and transient electrolyte abnormalities—persisting in spite of treatment. Any toxicities that were at least possibly related to study drug that resulted in an inability to complete the DLT assessment period, interruption in dosing for more than 10 dosing days during the DLT assessment period, or a delay in the initiation of the next cycle for more than 10 dosing days were also considered DLTs. Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE version 3.0).

Safety and efficacy assessments

Patients were evaluated predose on day 1 of each week to assess ECOG performance status, vital signs, body weight, and AEs. Twelve-lead electrocardiogram measurements were taken at screening and predose for all cycles after cycle 1. Tumor assessment by Response Evaluation Criteria in Solid Tumors (RECIST v1.0) was
performed by computed tomography scan or magnetic resonance imaging predose and every 8 weeks thereafter for the remainder of the study. Measurable disease was not required for eligibility for efficacy analysis. $^{18}$F-Fluorodeoxyglucose-positron emission tomography ($^{18}$FDG-PET) was used to assess tumor metabolic rates during dose expansion within 14 days of starting therapy, and at the end of cycle 1, within 7 days before starting cycle 2. Metabolic response was defined in accordance with the European Organization for Research and Treatment of Cancer (EORTC) guidelines on $^{18}$FDG-PET imaging as a 20% decrease in tumor standardized uptake values (SUV) compared with baseline.

Pharmacodynamics assessments and biomarker evaluation

Pharmacodynamic effects of the combination of ridaforolimus and dalotuzumab in skin and tumor samples were determined in patients enrolled in the dose-expansion phase for whom paired specimens were available. Processing of the samples, immunohistochemistry, and statistical analysis were performed as described (18). Immunohistochemical analysis of skin and tumor biopsies was performed in formalin-fixed paraffin-embedded sections. Pharmacodynamic effects were also assessed in ER+ breast cancer patients. Based on the gene expression signature of a set of 97 genes that define the genomic grade index (GGI), ER+ breast cancer can be subdivided into highly proliferative poor prognosis (Luminal B [high GGI]) and low proliferative (Luminal A [low GGI]) categories.

To identify potential biomarkers that may help determine patient populations responsive to the combination of ridaforolimus and dalotuzumab, breast cancer cell lines and human breast tumor were used to evaluate correlations between mTOR inhibition and the RAS pathway signature, which is a transcription readout of RAS pathway activation (19). The RAS signature score was determined as the log(10) ratio relative to the mean of all samples in the respective experiment. To assess the expression of IGF1R signaling pathway components in human breast cancers, we evaluated gene expression profiling data sets from the Cancer Genome Atlas (TCGA).

RESULTS
Preclinical assessment of the combination of ridaforolimus and dalotuzumab

*IGF1R signaling mediates feedback activation of AKT upon mTOR inhibition*

Treatment with ridaforolimus resulted in inhibition of phosphorylation of downstream pathway components, p-S6 ribosomal protein, and p-4EBP, and in compensatory activation of AKT (*Figure 1A and B*). Cell lines exhibiting high levels of feedback induction by ridaforolimus were more sensitive to dalotuzumab (*Figure 1C*). Inhibition of IGF1R and IRS2, as well as components of mTORC2 complex, blocked the activation of AKT (*Figure 1D*), suggesting a causal link between the feedback activation of AKT following ridaforolimus exposure and IGF1R signaling.

*Enhancers of sensitivity to dalotuzumab.* The lentiviral shRNA enhancer screen targeting 480 distinct human kinases revealed that the strongest enhancers of dalotuzumab (top 3%) included several members of the PI3K pathway (PIK3CA, PDK1, AKT2, and FRAP1/mTOR) (*Figure 1E; Supplementary Table S1*). In addition, key members of the RAS-MAPK pathway, BRAF and MAP2K1, were also identified. To confirm the screening hits, we performed colony formation assays using HT29 cells. Seventeen of 31 shRNA hits inhibited HT29 cells growth in the absence of drug, preventing an assessment of dalotuzumab sensitization. Eleven out of 20 shRNAs screening hits resulted in 2-fold or greater enhancement of dalotuzumab mediated cell proliferation—shRNAs targeting both PI3KCA and mTOR (FRAP1) significantly enhanced dalotuzumab mediated colony growth inhibition (*Figure 1F*). When we tested a shRNA vector that efficiently silences lipid phosphatase PTEN, the primary negative regulator of PI3K signaling, we found that dalotuzumab-mediated growth inhibition was abrogated by the PTEN shRNA (*Figure 1F*), further confirming the role of PI3K pathway components (eg, mTOR and PI3KCA) in enhancing the antitumor activity of dalotuzumab.

*Combination therapy with ridaforolimus and dalotuzumab potentiates antitumor activity*

Western blot analysis showed that in the IGF1R-expressing H2122 NSCLC and MCF7 breast cancer cell line, ridaforolimus inhibited mTOR signaling (decreased levels of p-S6K) and activated AKT signaling (increased levels of p-AKT and its downstream targets, Foxo3A and PRAS40). Despite inhibiting mTOR signaling, AKT activation was not observed following treatment with ridaforolimus in H1703 cells, which express low levels of
IGF1R (Figure 2A, S2). These results suggest that active IGF1R signaling is associated with feedback activation of AKT following ridaforolimus treatment. Treatment of H2122 cells with dalotuzumab alone significantly inhibited IGF1R signaling by blocking ligand binding and promoting receptor internalization and degradation while combination treatment with ridaforolimus and dalotuzumab prevented AKT activation and blocked phosphorylation of Foxo3A and PRAS40, resulting in effective targeting of both upstream and downstream components of the PI3K pathway (Figure 2A). In MCF7 cells combination therapy blocked feedback activation of AKT and potentiated PI3K pathway inhibition (Supplementary Figure S2).

Dalotuzumab alone or in combination did not alter PI3K signaling in the low IGF1R H1703 cells (Figure 2A). The efficacy of combined treatment was further evaluated using an anchorage-independent growth assay. While single-agent treatment with dalotuzumab or ridaforolimus showed minimal to modest growth inhibition in the high IGF1R H2122 cell line, significant growth inhibition was observed in the combination treated group (Figure 2B). In contrast, combination therapy did not enhance growth inhibition in the low IGF1R H1703 cell line compared with monotherapy alone. Treatment with ridaforolimus resulted in G1 cell cycle arrest in H1703 cells, but not in H2122 cells. The addition of dalotuzumab to ridaforolimus had minimal effect in H1703 cells, while in H2122 cells the combination decreased the percentage of actively cycling cells and increased the proportion of dead or dying cells (Figure 2B). Combination therapy also induced apoptosis markers assessed by PARP cleavage in IGF1R-positive breast cancer cells (Supplementary Figure S2). These results were also observed in additional cell lines and are consistent with enhanced inhibition of PI3K signaling by combination therapy in high IGF1R expressing cancer cells.

Combination treatment was further evaluated in vivo using a primary human tumor–derived NSCLC xenograft model. Significant antitumor activity over single agents was observed (Figure 2C).

Biomarker analysis of response to ridaforolimus and dalotuzumab therapy

Sensitivity of breast cancer cell lines to mTORC1 inhibition significantly correlated with the RAS pathway signature (Figure 3A) (19). Notably, ER+ breast cancer cells expressed low RAS signature and were sensitive
to ridaforolimus treatment. A previous study found that IGF1R expression and activation was associated with response to anti-IGF1R monotherapy in a panel of breast cancer cell lines (20). We evaluated these biomarkers in 800 human breast tumors obtained from the Karolinska Institutet in Sweden. Two-dimensional hierarchical clustering of 5000 differentially expressed genes classified the tumors into 3 major subtypes: ER+, HER2+, and triple negative. The RAS gene expression signature was high in the triple negative subtype and low in the ER+ subtype (Figure 3B), consistent with the distribution of the RAS signature in the cell line panel. Analysis of human breast cancer gene expression profiling data from TCGA demonstrated that IGF1R, its ligand IGF1, and its adaptor protein IRS1 are significantly overexpressed ($p<1×10^{-10}$) in ER+ breast cancer (Figure 3C). Analysis of 2 independent breast cancer gene expression datasets suggests that within ER+ breast tumors, those that have high levels of proliferation have the highest levels of IGF1R (Figure 3C). Based on these biomarkers, we developed a hypothesis for defining the responder population to ridaforolimus and dalotuzumab combination therapy (Supplementary Figure S4). Containing all of the features proved to predict sensitivity to the combination at the preclinical level, the luminal B breast cancer subtype represents the ideal model for the development of the combined treatment with ridaforolimus and dalotuzumab. This is why the combination is being evaluated in patients with luminal B breast cancer, although we cannot exclude that other types of tumors with high levels of IGF1-R or IGF1 ligand, high PI3K signature and low RAS signature may similarly benefit from the combination.

**Phase I clinical evaluation of combined treatment with ridaforolimus and dalotuzumab**

Between July 11, 2008 and March 10, 2010, 87 patients were enrolled and treated in the dose-escalation and dose-expansion phases. All patients receiving at least one dose of study medication were included in the safety and antitumor activity analyses. The median age was 56 years (range 18-89), the median number of prior therapies was 3 (range 1-17), and the most frequent malignancies were breast cancer (n=23; excludes 2 patients with phylloides-type not included in breast cancer subtype efficacy analyses), colorectal cancer (n=19), NSCLC (n=16), and sarcoma (n=14; Supplementary Table S2). The most common breast cancer subtype was ER+ (n=18), of which 11 were ER+/high proliferative (Ki67 ≥15%). Sarcoma histology subtypes
included Ewing’s sarcoma (n=5), leiomyosarcoma (n=3), osteosarcoma (n=2), and chondrosarcoma, liposarcoma, synovial cell sarcoma, and desmoplastic round cell tumor (n=1 for each).

Dose-limiting toxicities and maximum tolerated dose

Thirty-seven patients were enrolled across 5 dose levels in the dose-escalation phase: ridaforolimus 10, 20, 30, and 40 mg/day qd×5 plus dalotuzumab at 10 mg/kg/week, and ridaforolimus 20 mg/day qd×5 plus dalotuzumab at 7.5 mg/kg q14 days. Thirty-two patients completed the first cycle of treatment (DLT observation period) and were therefore considered DLT-evaluable. Only one patient experienced a DLT (prolonged grade 2 stomatitis), which occurred at dose level 1 (ridaforolimus 10 mg/day qd×5/dalotuzumab 10 mg/kg/week). Dose level 4 (ridaforolimus 40 mg/day qd×5/dalotuzumab 10 mg/kg/week) was determined to be the preliminary MTD because none of the 6 evaluable patients experienced a DLT. Subsequently, dose expansion was initiated and 12 additional patients were enrolled at dose level 4. The combined 15 patients from dose-escalation and dose-expansion were evaluable for DLTs at dose level 4. Four patients experienced a DLT—one with grade 4 fatigue and 3 with grade 3 mucosal inflammation/stomatitis. Since the cumulative rate of observed DLTs at dose level 4 (4/15, 27%) exceeded the prespecified goal of an observed rate of DLTs of less than 25% at the RP2D, dose level 3 was opened for expansion with 12 additional patients (permitted by the protocol). Initially, 19 DLT-evaluable patients were treated at dose level 3 (dose escalation and expansion), and only one patient experienced a DLT of grade 4 mucosal inflammation (5%). Therefore, ridaforolimus 30 mg/day qd×5 and dalotuzumab 10 mg/kg/week was established as the RP2D. Considering all 41 DLT-evaluable patients treated at dose level 3 (all patients including those enrolled following protocol amendment), only 3 DLTs (grade 3 or 4 mucosal inflammation) were reported, for an overall cumulative DLT rate of 8% (see Supplementary Table S3 for a summary of all DLTs).

Safety

All treatment-related AEs reported in at least 10% of patients across all dose levels including the RP2D are listed in Table 1. Treatment-related AEs were mostly grade 1 to 2; grade 3 or worse AEs included hyperglycemia (12%), mucosal inflammation (9%), thrombocytopenia (9%), and asthenia (7%). Management of
hyperglycemia included the use of oral antidiabetic agents or insulin in 26 of 50 patients (46%), with 21
patients receiving an oral agent and 5 patients receiving insulin. No patients required treatment discontinuation
or dose reduction, but 6 patients required dose interruption due to hyperglycemic AEs; all restarted therapy
after a period of interruption (dose level 2, n=1; dose level 3, n=4; dose level 4, n=1). Treatment
discontinuation and dose reductions due to stomatitis were required in 3 (dose level 1, n=1; dose level 4, n=2)
and 9 patients (dose level 2, n=1; dose level 3, n=6; dose level 4, n=2), respectively. The median time to onset
of stomatitis and mucosal inflammation across all dose levels ranged from 9 to 15.5 days; at the RP2D (dose
level 3), the median time to onset was 9 days. Treatment-related AEs in dose level 3 (RP2D) were similar to all
dose levels with the exception of slightly higher levels of diarrhea and thrombocytopenia (Table 1). Patients
received a mean dose of ridaforolimus ranging from 77% to 89% of the expected total dose across dose
levels—notably, the mean total dose received (relative to planned) was the lowest in the dose level 4 cohort at
77%, reflecting a higher frequency of dose interruptions and dose reductions for the management of AEs.

Pharmacodynamic effects

Twelve tumor-matched biopsy specimens were available for analysis. Biopsies were obtained at baseline
(n=22), day 5 of ridaforolimus monotherapy (n=22), and day 19 after 2 doses of dalotuzumab (n=19)
(Supplementary Figure S1) and stained using hematoxylin and eosin, and for Ki67, p-S6K, and p-PRAS40.
Gene expression profiling was also performed in available matched tumor samples. A marked inhibition of p-
S6K was observed with ridaforolimus treatment that persisted with the addition of dalotuzumab (Figure 4A;
representative images are shown in Supplementary Figure S2), indicating early and sustained inhibition of
the mTOR signaling pathway. Phosphorylated PRAS40, an AKT substrate, was used as a surrogate marker to
determine the level of AKT activation. The p-PRAS40 staining had a high background that was unchanged
upon ridaforolimus or combination treatment with dalotuzumab; therefore, no conclusion could be made
regarding the ability of the combination treatment to block feedback activation of AKT in tumor biopsy samples.
However, we had previously shown that the dalotuzumab doses tested in this study efficiently blocked IG1R
signaling (18). Analysis of changes in GGI in ER+ breast cancer patients showed that by day 19 of combined
treatment, patients responding to therapy demonstrated a significant reduction in the expression of GGI
signature compared with nonresponders (no response by RECIST, PFS ≥6 months, metabolic response, or tumor markers decline) (Figure 4B). In contrast, single-agent treatment with ridaforolimus had a modest decrease in the GGI by day 5 that did not reach statistical significance (Figure 4C). Staining for Ki67 also showed a corresponding decrease in proliferation in patients that responded to combination therapy or single-agent ridaforolimus; however, the change compared with nonresponders was not significant (Supplementary Figure S3).

**Antitumor activity**

All 87 patients enrolled were evaluable for antitumor activity; 6 partial responses (PR) were observed (breast cancer, n=3; NSCLC, n=1; ovarian cancer n=1; salivary gland cancer, n=1) corresponding to an objective response rate (ORR) of 7% (95% confidence interval: 2.6, 14.4). The 3 breast cancer patients with PR were treated at dose level 2 (n=1) and dose level 4 (n=2), and the other 3 patients with a PR were treated at dose levels 1.5, 3, and 4 (n=1 each). An additional 40 (46%) patients achieved stable disease (SD), and 27 (31%) had progressive disease (PD). Of note, out of 23 patients with breast cancer, 3 (13%) patients—all with ER+/high proliferative tumors—achieved a PR; another 11 (48%) were reported to have SD (5 [46%] with ER+/high proliferative tumors) and 7 (30%) had PD (3 [27%] with ER+/high proliferative tumors). Among patients with ER+/high proliferative breast cancer, the ORR was 27% (3 of 11 patients) (Table 2; Supplementary Table S4 and Figure 4D and 4E). A total of 15 patients (17%; breast cancer, n=5; NSCLC, n=3; ovarian cancer, n=2; and 1 each with colon cancer, salivary gland cancer, endometrial cancer, sarcoma, rectal cancer carcinoid) had PFS >4 months. All breast cancer patients with PR, and an additional breast cancer patient with SD who also displayed a decline in tumor biomarker, had PFS >6 months. Time on treatment with ridaforolimus and dalotuzumab was longer than the most recent prior treatment in 23 of 81 (28%) evaluable patients and was at least doubled in 13 (15%) patients.

Seventeen patients had baseline and follow-up PET scans that could be analyzed for metabolic response. Based on changes in SUV_{mean}, 9 (53%) of 17 evaluable patients were classified as metabolic responders (breast cancer, n=5; ovarian cancer, n=2; melanoma, n=1; rectal cancer, n=1). Forty-two patients also had a
measurable serum tumor marker (eg, CA 15-3 for breast cancer) at baseline, and at least one post-treatment evaluation. Eleven (41%) of 27 patients (breast cancer, n=8; ovarian cancer, n=2; endometrial cancer, n=1) had a 25% to 97% decrease in serum tumor markers and were considered to have a biomarker response. Ten breast cancer patients had evidence of antitumor activity following treatment with the combination of ridaforolimus and dalotuzumab, based on objective response by RECIST, PFS greater than 6 months, PET response, and decrease of tumor markers during treatment.

**DISCUSSION**

Inhibition of mTOR induces feedback activation of AKT, which may potentially counteract antitumor effects of mTOR inhibitors (12, 13). Here we have confirmed that mTORC1 inhibition alone led to activation of AKT, both in cell lines and in a primary human tumor derived xenograft model. We observed that the sensitivity to dalotuzumab correlated with the level of induced AKT activation, which could therefore become a potential biomarker of sensitivity to the combination. In addition, knockdown of IGF1R and its signaling pathway also blocked AKT activation, providing further evidence for the role of the IGF1R pathway in this setting. Conversely, an RNAi enhancer screen indicated that silencing components of the PI3K pathway, notably mTOR, resulted in enhanced sensitivity to the IGF1R inhibitor dalotuzumab. Additionally, shRNAs targeting PI3KCA and mTOR (FRAP1) enhanced inhibition of colony growth by dalotuzumab. Taken together, our preclinical studies strongly supported the rationale for targeting both mTOR and IGF1R signaling pathways by combined ridaforolimus and dalotuzumab.

The results from our phase I study demonstrate that the combination of ridaforolimus and dalotuzumab is feasible, with encouraging activity in breast cancer patients with ER+/high proliferative tumors. The observed AEs were generally mild to moderate in severity and manageable, allowing patients to remain on treatment for long periods. However, as noted below, the grade of oral mucositis was higher than the one observed with single-agent ridaforolimus, suggesting that the inhibition of compensatory pathways may also be at play in tissues sensitive to mTOR inhibition. There was no evident pharmacologic interaction between study drugs, and drug levels were comparable with expected levels (based on single-agent trial reports). Based on our
results, we determined that the RP2D of ridaforolimus to combine with dalotuzumab 10 mg/kg/week for further clinical development is 30 mg qd×5. The RP2D for ridaforolimus is lower than the dose that has been evaluated for therapy as a single agent; this is due to the higher rates of stomatitis and mucositis observed with the combination, which is probably an indication that the combination also inhibits the feedback loop in normal tissues. Stomatitis/mucositis are well-known class effects of mTOR inhibitors and have been previously reported with similar incidence and grade with single-agent ridaforolimus (21) or other rapamycin analogs (22). In the present study, stomatitis/mucositis were manageable with local treatment and temporary dose interruption in most cases. Although concerns regarding metabolic toxicity have been raised with both mTOR (23) and IGF1R inhibitors (24), all the episodes of hyperglycemia and hypercholesterolemia reported with ridaforolimus and dalotuzumab occurred outside of the DLT period and did not interfere with dosing.

While ridaforolimus has not been formally tested as a single agent in breast cancer, in the initial phase I trial of single-agent oral ridaforolimus, one of 8 treated patients with metastatic breast cancer reported SD for about 5 months (21). Single-agent therapy with other mTOR inhibitors has shown modest activity in breast cancer patients (25, 26), while no signal of antitumor activity was observed with dalotuzumab in a recently reported phase I study that included 17 breast cancer patients (18). In our phase I study, clinical evidence of antitumor activity was observed in several patients; the combination of ridaforolimus and dalotuzumab produced an overall ORR of 7% and a 4-month SD rate of 17%. Notably, the most prominent results were observed in patients with breast cancer (ORR of 13% and a SD of 48%); especially among patients with ER+/high proliferation breast cancer who achieved an ORR of 27% (3 out of 11 patients), which was 20% higher than the overall population. With the limits of a phase I study, the clinical data with ridaforolimus and dalotuzumab support the anti-tumor activity seen in pre-clinical models, and provides evidence that the combination is safe and active in patients with advanced cancer, and appears to be particularly promising in patients with breast cancer.

Until recently, there has been little overall success in identifying biological markers that may identify a population responsive to either mTOR or IGF1R inhibitors. In this study, most breast cancer patients deriving
benefit from the combination had ER+/high proliferative tumors with 15% or more Ki67 positive staining. A Ki67 labeling index (LI) of greater than 13.25% is typically used to distinguish luminal B from luminal A tumors (27). Dysregulation of IGF1R or PI3K pathways has been associated with luminal B tumors; it has been recently reported that luminal B tumors have hyperactive PI3K signaling and are more likely to express IGF gene signature as compared with luminal A breast cancer (3, 4). The high-proliferative luminal B subtype is associated with poorer prognosis (6), and long-term outcomes following adjuvant tamoxifen with a 10-year disease-free survival (DFS) of ~50% (28). In addition, luminal B breast tumors are poorly responsive to primary chemotherapy, with pathological complete response rates that are worse than those of other molecular subtypes (29).

We assessed potential biomarkers that would correlate with response to ridaforolimus and dalotuzumab. Breast cancer cell line sensitivity to ridaforolimus was highly correlated with activation of the RAS pathway, a finding that is consistent with resistance to other PI3K pathway inhibitors via activation of the parallel MAPK pathway. Activation of the RAS pathway has also been correlated with resistance to AKT inhibition (19). Conversely, sensitivity to anti-IGF1R therapy has been associated with expression levels of IGF1R and activation of the IGF1R pathway (20). The estrogen receptor has been shown to regulate the expression of IGF1R (30). Moreover, primary or acquired resistance to endocrine therapy, a major cause of mortality in ER+ breast cancer patients, is associated with reactivation of IGF1R signaling (31). These results suggest that the IGF1R pathway may play an important role in the progression of ER+ breast cancer. Our analysis of the RAS signature in human breast cancer tumors showed high levels expressed in triple negative breast cancer and low levels in the ER+ subtype. Gene expression profiling based on the TCGA data set revealed overexpression of IGF1R, its ligand IGF1, and the adaptor protein IRS1 in ER+ breast cancer. Although indirect, these findings suggest that breast cancer patients with the luminal B subtype expressing high levels of IGF1R pathway and low levels of RAS pathway activation would be responsive to ridaforolimus and dalotuzumab combination therapy (Supplementary Figure S4).
Results from the Breast Cancer Trials of Oral Everolimus-2 (BOLERO-2) trial demonstrated significant increases in PFS and ORR with everolimus (10 mg qd) and the aromatase inhibitor exemestane (25 mg qd) compared with exemestane alone, strongly supporting the value of mTOR inhibition in hormone receptor–positive breast cancer patients (32). Our findings reinforce the value of mTOR inhibition in the treatment of ER+ breast cancer and support the additional value of dalotuzumab to abrogate the feedback activation of AKT by mTOR inhibitors. Based on the results of this phase I study, several studies are exploring the combination of ridaforolimus and dalotuzumab in breast cancer and other cancers. A phase II trial to compare the combination of ridaforolimus and dalotuzumab with endocrine therapy in advanced luminal B breast cancer patients has been recently completed (NCT01234857).

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REFERENCES


## TABLES

**Table 1.** Summary of treatment-related adverse events occurring in at least 10% of patients across all dose levels or at dose level 3 (recommended phase 2 dose)

<table>
<thead>
<tr>
<th>Type of toxicity</th>
<th>All dose levels (N=87)</th>
<th>Dose level 3 (N=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All grades</td>
<td>Grade &gt;3</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Patients with ≥1 AE</td>
<td>83 (95)</td>
<td>40 (46)</td>
</tr>
<tr>
<td>Hyperglycemia</td>
<td>49 (56)</td>
<td>10 (12)</td>
</tr>
<tr>
<td>Mucosal inflammation</td>
<td>40 (46)</td>
<td>8 (9)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>36 (41)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Asthenia</td>
<td>34 (39)</td>
<td>6 (7)</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>26 (30)</td>
<td>0</td>
</tr>
<tr>
<td>Stomatitis</td>
<td>25 (29)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Decreased appetite</td>
<td>22 (25)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>20 (23)</td>
<td>4 (5)</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>20 (23)</td>
<td>8 (9)</td>
</tr>
<tr>
<td>Nausea</td>
<td>18 (21)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>17 (20)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Rash</td>
<td>12 (14)</td>
<td>0</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>10 (12)</td>
<td>0</td>
</tr>
<tr>
<td>Anemia</td>
<td>10 (12)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>9 (10)</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>

Only the highest reported grade of a given AE is counted for the individual subject.

AE, adverse event
Table 2. Summary of clinical efficacy in breast cancer patients

<table>
<thead>
<tr>
<th>Breast cancer type</th>
<th>n</th>
<th>RECIST (PR)</th>
<th>PFS ≥ 6 months</th>
<th>18FDG-PET response</th>
<th>Tumor marker decline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>23</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Triple negative breast cancer</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HER2+</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>ER+</td>
<td>18</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>High proliferation (Ki67 ≥15%)</td>
<td>11</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Low proliferation (Ki67&lt;15%)</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown proliferation</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Overall, 10 of 23 (43%) breast cancer patients and 6 of 11 (54%) patients with ER+/high proliferative breast cancer showed some evidence of antitumor activity (either response by RECIST, PFS ≥6 months, metabolic response, or tumor markers decline).

RECIST, response evaluation criteria in solid tumors; PR, partial response; PFS, progression-free survival; 18FDG-PET, [18F]Fluorodeoxyglucose-positron emission tomography; HER2, human epidermal growth receptor 2; ER, estrogen receptor.
FIGURE LEGENDS

Figure 1. Combination therapy with mTOR and IGF1R inhibitors block feedback activation of AKT. Diversity in feedback activation of AKT by ridaforolimus was observed in a panel of breast cancer cell lines (A). Cell lines exhibiting feedback activation of AKT following ridaforolimus treatment showed sensitivity to anti-IGF1R therapy (B). Knock down of IGF1R and its signaling pathway components blocked feedback activation of AKT (C). A lentiviral shRNA screen identified mTOR as an enhancer of the activity of dalotuzumab in tumor cell lines, and AKT activation by mTOR was confirmed to be mediated via IGF1R signaling pathway. (D) Representative results from a shRNA screen targeting various PI3K and MAPK kinases in HT-29 CRC cells. mTOR, mammalian target of rapamycin; IGF1R, insulinlike growth factor 1 receptor; shRNA, short hairpin RNA; PI3K, phosphatidylinositol 3-kinase; CRC, colorectal cancer; PTEN, phosphatase and tensin homolog

Figure 2. Cotreatment with dalotuzumab and ridaforolimus enhances antitumor activity in IGF1R expressing tumors. (A) Western blot analysis of PI3K pathway components in cancer cells treated with dalotuzumab, ridaforolimus, or the combination. In IGF1R-expressing cancer cell lines (H2122), combination treatment suppresses the activation of AKT demonstrated by decreased phosphorylation of AKT and downstream targets FOXO3A and PRAS40. (B) Combination therapy with ridaforolimus and dalotuzumab results in increased cell death in anchorage independent growth inhibition assays in IGF1R expressing cells (H2122). No such combination benefit was observed in cancer cells expressing low levels of IGF1R (H1703). (C) In vivo, combination therapy with ridaforolimus and dalotuzumab significantly inhibits growth over mono-therapies. Tumor growth of primary human tumor–derived lung adenocarcinoma xenograft model LXFA629 in control (vehicle) or experimental agent treated mice (n=10/group) were plotted. Combination therapy with ridaforolimus and dalotuzumab significantly enhanced tumor growth inhibition compared to vehicle treated (P<0.0001) or single-agent treated groups (P<0.05). PI3K, phosphatidylinositol 3-kinase; IGF1R, insulin-like growth factor 1 receptor; NSCLC, non-small cell lung cancer

Figure 3. RAS pathway activation and sensitivity to mTOR inhibition in ER+ breast cancer cell lines. (A) RAS signature is associated with sensitivity to ridaforolimus in a panel of breast cancer cell lines. (B) ER+ tumors...
have low levels of RAS pathway activation—unsupervised 2D clustering of the 5000 most variable genes segregate breast tumors into ER+, HER2+, or triple negative (ER−, PR−, HER2−) subtypes. RAS pathway activation is low in the ER+ tumors. (C) IGF1R signaling pathway components are overexpressed in ER+ breast tumors. Differential expression of selected genes in the IGF1R receptor signaling pathway was compared between ER+ (n=228) and ER− (n=87) breast tumors profiled by TCGA. The fold overexpression and P value for differential expression are indicated. mTOR, mammalian target of rapamycin; ER+, estrogen receptor–positive; PR−, progesterone receptor–negative; IGF1R, insulinlike growth factor 1 receptor; TCGA, the Cancer Genome Atlas

Figure 4. Effect of ridaforolimus and dalotuzumab treatment on tumor markers. (A) Ridaforolimus and dalotuzumab treatment results in a significant decrease in the GGI at day 19 compared with day 0 in patients who responded to the combination therapy. (B) Patients responding to treatment with ridaforolimus alone had less pronounced changes in GGI by day 5 compared with day 0. (C) Example of tumor response (partial response; RECIST) in a breast cancer patient with liver metastases treated at dose level 4 (ridaforolimus 40 mg/day and dalotuzumab 10 mg/kg/week). (D) PET-CT evaluation of a breast cancer patient after one cycle of treatment at dose level 4: CT scans revealed a 28% decrease in the sum of the longer diameters of the target lesions, which corresponded to 48% decrease in \(^{18}\)FDG uptake. Arrows indicate target lesions of interest. mTOR, mammalian target of rapamycin; p-S6RP, phosphorylated S6 ribosomal protein; GGI, genomic grade index; RECIST, Response Evaluation Criteria in Solid Tumors; PET-CT, Positron emission tomography-computed tomography; NR, nonresponders; R, responders
Figure 1.

A) Relative fold change of P-AKT T308, S6RP S235/6, and P-4E BP T65.

B) P-AKT activation versus sensitivity to dalotuzumab.

C) Relative change in P-AKT for different proteins.

D) Scatter plot showing the correlation between negative log P-value and the change in P-AKT.

Significance
Dalotuzumab/no drug

Relative fold change

P = 0.010

Sensitivity to dalotuzumab

Negative Log P value

FRAP1, mTOR, PDK3, PK3CA, MAP3K5, NEK8, AKT2, BRAF, PI3KCA, MAP2C1

Research.

Figure 2.

A

 Dalotuzumab
 Ridaforolimus

 IGFIRβ
 P-S6K (T389)
 S6K
 P-AKT (S473)
 AKT
 P-Foxo3A (S253)
 P-PRAS40 (S246)
 Actin

 H1703 H2122

 B

 H2122

 Relative colony area

 Ridaforolimus (log nM)

 H1703

 Relative colony area

 Ridaforolimus (log nM)

 C

 Tumor volume (mm$^3$)

 Days of treatment

 Control
 Ridaforolimus
 Dalotuzumab
 Ridaforolimus/ dalotuzumab

 64%
 70%
 98%
Figure 3.

A

B

C

<table>
<thead>
<tr>
<th>P value</th>
<th>Fold change</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.52E-20</td>
<td>2.90</td>
<td>IGF1R</td>
</tr>
<tr>
<td>1.55E-16</td>
<td>2.43</td>
<td>IRS1</td>
</tr>
<tr>
<td>2.64E-10</td>
<td>2.21</td>
<td>IGFBPS</td>
</tr>
<tr>
<td>6.88E-4</td>
<td>1.52</td>
<td>IGF1</td>
</tr>
<tr>
<td>0.013</td>
<td>1.23</td>
<td>IGF2</td>
</tr>
<tr>
<td>0.034</td>
<td>1.11</td>
<td>IRS2</td>
</tr>
<tr>
<td>1.000</td>
<td>-1.27</td>
<td>IGFBP3</td>
</tr>
</tbody>
</table>
Combination of the mTOR inhibitor ridaforolimus and the anti-IGF1R monoclonal antibody dalotuzumab: preclinical characterization and phase I clinical trial

Serena DiCosimo, Sriram Sathyanarayanan, Johanna C Bendell, et al.

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