p70S6 kinase is a critical node that integrates HER-family and PI3 kinase signaling networks

Mark J. Axelrod a,b, Vicki Gordon a, Rolando E. Mendez b, Stephanie S. Leimgruber c, Mark R. Conaway d, Elizabeth R. Sharlow i, Mark J. Jameson b, Daniel G. Gioeli a, Michael J. Weber a,⁎

a Department of Microbiology, Immunology, and Cancer Biology, University of Virginia, Charlottesville, VA, USA
b Department of Otolaryngology—Head and Neck Surgery, University of Virginia, Charlottesville, VA, USA
c Department of Pharmacology, University of Virginia, Charlottesville, VA, USA
d Department of Public Health Sciences, University of Virginia, Charlottesville, VA, USA

1. Introduction

Resistance to targeted therapies often occurs because compensatory signaling blunts the short-term cytotoxic effects of target inhibition, without selection for resistant mutant cells. This mechanism of resistance can be intrinsic or adaptive, but in either case there is a perceived need to develop combinations of drugs that target not only the oncogenic driver, but also the compensatory response. We previously reported that inhibition of the MAP kinase (MAPK) pathway in prostate cancer xenografts induced upregulation not only of many components of the MAPK pathway, but also of other signaling pathways (e.g. Wnt, STAT); co-inhibition of these adaptive responses caused synergistic cytotoxicity [1]. Numerous subsequent reports support this concept. For example, in colorectal cancer cells, co-activation of MET and epidermal growth factor receptor (EGFR) resulted in synergistic proliferation due to the ability of both proteins to cause increased signaling through the MAPK and AKT pathways [2]. In non-small cell lung cancer (NSCLC),

Abbreviations: p70S6K, p70S6 kinase; PI3K, phosphoinositide-3-kinase; RPPA, Reverse Phase Protein Array; mTOR, mammalian target of rapamycin; MAPK, mitogen activated protein kinase; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; RTK, receptor tyrosine kinase; FOX, forhead box; T-PER, tissue protein extraction reagent; HNSCC, head and neck squamous cell carcinoma; PKA, protein kinase A; 4E-BP1, eukaryotic initiation factor 4E binding protein; elf4E, eukaryotic initiation factor 4E.

⁎ Corresponding author at: Department of Microbiology, Immunology and Cancer Biology, University of Virginia School of Medicine, 2-13 Jordan Hall, Charlottesville, VA 22908-0734, USA. Tel.: +1 434 924 5022 (office), +1 434 249 4391 (mobile); fax: +1 434 982 0689.

E-mail addresses: mj7a@virginia.edu (M.J. Axelrod), vg6z@virginia.edu (V. Gordon), rem5d@virginia.edu (R.E. Mendez), ss14q@virginia.edu (S.S. Leimgruber), mmct@virginia.edu (M.R. Conaway), em7g@virginia.edu (E.R. Sharlow), mj4e@virginia.edu (M.J. Jameson), dgg3f@virginia.edu (D.G. Gioeli), mjw@virginia.edu (M.J. Weber).
both MET and EGFR provide anti-apoptotic signaling, and thus simultaneous co-targeting of these receptors resulted in enhanced apoptosis compared to inhibition of only one target [3]. Compensatory signaling can also be induced by relief of negative feedback. For example, inhibition of MAPK signaling leads to upregulation of phosphoinoside-3-kinase (PI3K) and AKT signaling, and vice versa [4–6], thus leading to an interest in drug combinations that target both of these pathways. Another example is the upregulation of receptor tyrosine kinase (RTK) signaling in response to inhibition of PI3K [7], AKT [8] or BRAF [9]. AKT-mediated phosphorylation negatively regulates the forkhead box (FOX) protein family of transcription factors [10], leading to upregulation of a number of RTKs through a FOXO-dependent mechanism, thereby blunting the effects of drugs targeting PI3K or AKT. Similarly, BRAF inhibition leads to the dysregulation of FOXO3 activity, which in turn reduces the apoptosis generated through inhibition of mutant-BRAF driven melanomas by vemurafenib [11]. Co-inhibition of HER-family proteins along with AKT or BRAF resulted in enhanced anti-tumor benefit compared to either therapy individually [8].

Adaptive responses to single-agent targeted therapy are extraordinarily complex [1,12] and differ substantially between cancer cell lines even within a single tumor and driver type [13]. Therefore, it is difficult to determine the optimal drug combination based on genotypic or molecular profiling of cells or tumors treated with a targeted agent. We and others have taken an empirical approach to identifying the adaptive responses that are most significant for maintaining the growth and survival of cells subjected to single-target inhibition, by screening combinations of targeted inhibitors for synergistic cytotoxicity [12–16]. Among the combinations we identified that have near-term potential clinical utility was co-inhibition of HER-family RTKs and PI3K/mammalian target of rapamycin (mTOR), which were synergistically cytotoxic where single-agent inhibition was ineffective. The potential utility of using drug combinations that inhibit these two target categories is widely recognized [4,7,17], which supports the validity of our approach.

Despite their potential benefits, there are numerous challenges associated with developing drug combinations. Synergistic cytotoxicity against tumors may be associated with enhanced toxicity for patients and an erosion of therapeutic benefit. This problem is exacerbated by the fact that each drug will have its own palette of off-target effects, and the combination may have adverse interactions. In addition, there are challenges associated with developing such combinations associated with issues of pharmacokinetics, drug interactions and intellectual property. Thus there is increasing interest in identifying points of convergence between signaling pathways that would yield targets whose inhibition would block two pathways that otherwise would be compensatory.

Signaling pathways are linked together in inter-dependent networks, communicating by feedback and feed-forward regulatory loops. These signaling networks have emergent properties associated with robust systems [18,19], raising the possibility that they contain critical nodes within the system, whose inhibition would lead to system collapse. Targeting such a critical node could enable the use of a single drug that might not only phenocopy the biological effects of dual-target inhibition, but also be effective in a broader range of biological settings.

In the current communication we identify p70S6 kinase (p70S6K) as being a critical node that links HER-family and PI3K pathway signaling, and is an effective target for single-agent therapy. We found that HER-family and PI3K/mTOR co-inhibition caused synergistic cytotoxicity, and utilized Reverse Phase Protein Arrays (RPPA) to identify p70S6K as being synergistically inhibited in response to these drug combinations. Expression of a constitutively active p70S6K construct protected the cells against apoptosis induced by combined HER-family and PI3K/mTOR inhibition. Direct inhibition of p70S6K using small molecule inhibitors phenocopied the growth inhibition and apoptosis caused by HER-family and PI3K/mTOR co-inhibition. Thus, p70S6K functions as a critical node in the signaling network that links HER-family and PI3K pathway signaling, and is an under-explored target for development of small molecule inhibitors that could function as single agents.

2. Methods and materials

2.1. Cell line and reagents

UMUC-6 bladder cancer cells were a gift from Dr. Dan Theodorescu (University of Colorado). Cal27 HNSCC cells were obtained from the American Type Culture Collection (ATCC). SCC61 HNSCC cells were a gift from Dr. Wendell Yarbrough (Vanderbilt University). UMUC-6 cells were maintained in MEM (Invitrogen) supplemented with 5% FBS (Gemini, Bio-products), 1 mM sodium pyruvate (Invitrogen) and 0.1 mM MEM non-essential amino acids (Invitrogen). Cal27 and SCC61 cells were maintained in DMEM/F-12 media (Invitrogen) supplemented with 5% FBS and 400 ng/mL hydrocortisone (Sigma-Aldrich).

Cells were grown in a humidified 37 °C incubator with 5% CO2. All cell lines were routinely tested and found to be free of mycoplasma contamination using MycoAlert (Lonza). Cell line identities were verified by STR analysis and comparison to published databases (University of Arizona). LY294002 was purchased from Calbiochem, Lapatinib from L.C. Laboratories, NVP-BEZ232 and BMS599626 from ChemieTek, PF04691502 and AT7867 from Selleckchem, and Ro31–8220 from Enzo Life Sciences. All of the primary antibodies used for Western blotting in this study were purchased from Cell Signaling Technologies with the exception of the total and phosphorylated ERK antibodies (Sigma) and the tubulin antibody (EMD Biosciences). The fluorescently labeled secondary antibodies used for Western blotting were purchased from Licor. The antibodies used for flow cytometry were as follows: the anti-RAT HA-tag antibody was purchased from Roche, the anti-rat FITC-conjugated secondary antibody from Invitrogen, the anti-rabbit PE-conjugated secondary antibody from Santa Cruz, and the cleaved Caspase 3 and phospho-S6 primary antibodies from Cell Signaling. 4',6-Diamidino-2-phenylindole (DAPI) was from Sigma-Aldrich.

2.2. Generation of stable UMUC-6-E389 cells

The plasmid containing the HA-p70S6K-E389-ΔCT construct was from Addgene (plasmid # 8993) [20] transferred into the pSLIK vector using Gateway recombination cloning according to the manufacturer’s protocol (Invitrogen) as previously described [3]. The pSLIK-HA-p70S6K-E389-ΔCT vector was transfected into 293-T cells along with the lentiviral packaging and envelope vectors pSPAX2 and pMDG by calcium phosphate transfection. Lentivirus was collected two days after transfection and filter sterilized through a 0.24 μm filter. UMUC-6 cells were transduced with lentivirus containing the pSLIK-E389 DNA or mock transduced. Both sets of cells were then exposed to 100μg/mL G418 until the mock-transduced plate was completely cleared. HA expression in the UMUC-6-E389 cells upon 2 μg/mL doxycycline treatment was assessed by flow cytometry.

2.3. Flow cytometry

Cells were plated for 24 h in phenol-red free RPMI-1640 and then treated as described in the text. Both floating and adherent cells were collected and pooled. The cells were fixed with paraformaldehyde and permeabilized with ice-cold methanol and stored in methanol at −20 °C until use. Prior to staining, the cells were pelleted in siliconized tubes and washed twice with PBS containing 1% BSA. The cells were then blocked using PBS containing 2% donkey serum, 1% BSA, 0.1% Triton X-100, and 0.05% Tween 20 for 10 min. Cells were then stained

3 C.C. Wang et al. Manuscript under review.
with the appropriate primary antibody or antibody mixture diluted in PBS with 1% BSA for 1 h. From this point forward, the cells were kept in the dark in order to preserve the fluorescence signal. Cells were then stained with the appropriate secondary antibody for 30 min. Finally, cells were stained with 1 μg/mL DAPI in PBS. The cells were stored at 4 °C overnight and then assayed using a FACScanLibur flow cytometer. Single stained controls were also assayed on the FACScanLibur to serve as compensation controls. Data analysis, including compensation, was performed using Flo-Jo flow cytometry analysis software.

2.4. Reverse phase protein array

RPPA analysis was performed by the RPPA Core Facility at MD Anderson. Cell lysates were prepared in accordance with their recommended protocols. Briefly, cells were treated with either DMSO, 0.5 μM lapatinib, 5 μM LY294002, or the combination for 24 h. Cell lysates were prepared in the same manner as for Western blotting (see below) with the exception that the lysis buffer used was a 1:1 mixture of 2x sample buffer and Tissue Protein Extraction Reagent (T-PER) (Pierce). Lysates were then processed according to the MD Anderson RPPA Core protocol.

2.5. Growth assays

Cells were plated in phenol-red free RPMI1640 + 0.5% FBS at a density of 3 × 10^3 cells/well in a volume of 80 μL for 24 h in 96-well plate format. The inhibitors and solvent were at a concentration 10× greater than the final experimental concentration. 10 μL of each drug and/or solvent was added to the appropriate wells. Cells were then incubated for 72 h; after which 10 μL of alamarBlue was added, cells were incubated for 4 h, and assayed using a fluorescence plate reader with a 540/25 nm excitation filter and a 620/40 nm emission filter.

2.6. Cell lysis and Western blotting

Cells were plated for 24 h in phenol-red free RPMI 1640 + 0.5% FBS and then treated as described in the text. Prior to cell lysis, each plate was treated with 1 μM pervanadate and 5 mM Calyculin-A for 1 min. The medium was aspirated off and the cells were washed for 30 s with ice-cold PBS containing pervanadate and Calyculin A. The PBS was removed and cells were lysed in a Triton-based lysis buffer (1% Triton X-100, 50 mMTris pH 7.5, 100 mMNaCl 50 mMNaF, and 5 mM EDTA) containing 1 μg/mL peptatin, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200 μM orthovanadate, 50 mM β-glycerophosphate, and 0.4 μM microcystin. Western blotting was carried out as previously described [21]. Immunoblots were analyzed using the Odyssey (LICOR Biosciences) imaging system.

2.7. Statistics and synergy

The primary method of analysis is 2-way ANOVA for randomized block designs, which were used to control for experiment-to-experiment variation [22]. Contrasts were used to make specific comparisons between groups, and where appropriate, data were transformed to the log scale to facilitate interpretations as fold changes. Synergy was assessed by comparing observed values and values that would be predicted by Bliss-independence [23,24] and by testing for interactions terms in the two-way ANOVA.

3. Results

3.1. Concurrent inhibition of HER-family and PI3K/mTOR signaling results in synergistic cytotoxicity mediated by apoptosis

We tested over 500 drug combinations for synergistic cytotoxic effects in 21 epithelial cancer cell lines representing three distinct cancer lineages [14]. One combination that we identified as synergistic using the Bliss model of additivity is the combination of a HER-family kinase inhibitor and a dual PI3K/mTOR inhibitor. This combination caused synergistic cytotoxicity in multiple cell lines and cancer lineages such as bladder (UMUC-6) and head and neck squamous cell carcinoma (HNSCC) (Cal27 and SCC61) (Fig. 1A–C). To be certain that the biological effects of these drugs were due to inhibition of the expected target enzyme and not a consequence of off-target effects, we determined that multiple pharmacophores with the same putative target elicited the same biological effects, as described previously [14]. BMS599626, lapatinib, and AG1478 were functionally equivalent (Fig. 1 and data not shown), thus validating the HER family as the functional target. Similarly, NVP-BEZ235 (BEZ235), PF04691502 and LY294002 could substitute for each other (Fig. 1 and data not shown), thus validating PI3K and mTOR as the functionally significant targets in these combination experiments.

AlamarBlue was used to assay for growth inhibition in our high-throughput screens, but since this agent monitors cell metabolism rather than cell death, we directly tested the effects of these drug combinations on apoptosis (Fig. 1 D–E). In all three cell lines, combination treatment resulted in a 2–4 fold increase in apoptosis compared to vehicle treated cells and demonstrated enhancement of the apoptotic effect compared to cells treated with either a HER-family kinase or PI3K/mTOR inhibitor alone.

3.2. p70S6K is a node of convergence between HER-family and PI3K pathway signaling

To determine a molecular basis for the synergistic cytotoxicity we observed upon treatment with HER-family kinase and PI3K/mTOR inhibitors, we performed RPPA analysis on UMUC-6 cells treated with lapatinib (HER-family kinase inhibitor), LY294002 (PI3K/mTOR inhibitor) and the combination. 209 epitopes, including 56 phosphoepitopes, were examined and the data were calculated as positive or negative fold changes compared to the vehicle treated control cells. The phospho-epitopes were then rank-ordered according to the fold-change decrease in phosphorylation upon combination treatment. The most robust changes in phosphorylation were observed at multiple epitopes of the ribosomal protein S6, a substrate of p70S6K (Fig. 2A). Subsequent Western blots confirmed the results of the RPPA in UMUC-6 cells (Fig. 2B). In addition, these Western blots demonstrated that the activating phosphorylations on p70S6K (T389 and T412/S421) were also inhibited by combination treatment with lapatinib and LY294002. To determine whether other combinations of HER-family kinase and PI3K/mTOR inhibitors that caused synergistic cytotoxicity in other cell lines had similar effects, we treated Cal27 and SCC61 cells with lapatinib plus PF-04691502 and BMS599626 plus PF-04691502, respectively. In both cases, S6 phosphorylation was decreased in the combination treated cells compared to either of the single-drug treated conditions (Fig. 2C–E).

3.3. p70S6K activity mediates the cytotoxic response upon treatment with HER-family and PI3K/mTOR inhibitors

To test the functional role inhibition of p70S6K plays in the cytotoxicity that occurs from inhibiting the HER family and PI3K pathways, we performed an epistasis analysis in which cells dually inhibited by BEZ235 and lapatinib were “rescued” from apoptosis by expression of a constitutively active p70S6K mutant. We generated stable UMUC-6 cells containing a doxycycline-inducible constitutively active p70S6K mutant, HA-p70-E389-ΔC. To be certain that the biological effects of these drugs were due to inhibition of the expected target enzyme and not a consequence of off-target effects, we determined that multiple pharmacophores with the same putative target elicited the same biological effects, as described previously [14]. BMS599626, lapatinib, and AG1478 were functionally equivalent (Fig. 1 and data not shown), thus validating PI3K and mTOR as the functionally significant targets in these combination experiments.

AlamarBlue was used to assay for growth inhibition in our high-throughput screens, but since this agent monitors cell metabolism rather than cell death, we directly tested the effects of these drug combinations on apoptosis (Fig. 1 D–E). In all three cell lines, combination treatment resulted in a 2–4 fold increase in apoptosis compared to vehicle treated cells and demonstrated enhancement of the apoptotic effect compared to cells treated with either a HER-family kinase or PI3K/mTOR inhibitor alone.
UMUC-6-E389 control cells not treated with doxycycline, approximately half the vehicle treated cells stained positive for pS6. Treatment with lapatinib and BEZ235 in the absence of doxycycline reduced the percentage of pS6+ cells to 5% and 15% respectively. Treatment with the combination resulted in less than 1% of the cells staining positive for pS6. In UMUC-6-E389 cells treated with doxycycline, induction of the constitutively active p70 E389 construct resulted in an increased level of S6 phosphorylation in vehicle treated cells that stained positive for HA. Expression of this construct also provided significant protection against loss of S6 phosphorylation in both single drug treated cells and in cells treated with both lapatinib and BEZ235 that stained positive for HA (Fig. 3 A&C). These data indicate that the cells expressing the p70 E389 construct retain p70S6K activity in the presence of lapatinib and BEZ235.

Next, we looked at the effects of the p70 E389 construct on survival. Treatment with 2.5 μM lapatinib increased the percentage of doxycycline-naive cells that stained positive for cleaved caspase 3 by 2.5-fold. Although 750 nM BEZ235 had no effect on apoptosis as a single drug, the combination of lapatinib and BEZ resulted in a 3.7-fold increase in apoptosis versus control treated cells. The addition of doxycycline and expression of p70 E389 resulted in significant decreases in apoptosis in all treatment conditions in the population of cells expressing the HA-tagged construct. In cells treated with the drug combination, expression of p70 E389 resulted in a 23% reduction of apoptosis, from 27.6% to 4.6%, versus cells that were not treated with doxycycline, a level of apoptosis even lower than that seen in the untreated controls (Fig. 3 B&D). UMUC-6 E389 cells that were not treated with doxycycline behaved similarly to parental UMUC-6 cells in both the absence and presence of doxycycline (Supplemental Figure S1). Taken together, these data demonstrate that expression of a constitutively active p70S6K construct can rescue cells from the apoptotic effects of combined PI3K/mTOR and HER family kinase inhibition suggesting that p70S6K is a critical node within the PI3K and HER family signaling network, and its activity is sufficient to maintain cell survival.

---

**Fig. 1.** Combinatorial drug screening revealed synergy between HER-family and PI3K/mTOR inhibitors in multiple cell lines. The Bliss model of additivity was used to predict additive levels of cytotoxicity in combination treated cells. A. UMUC-6 bladder cancer cells were treated with 250 nM, 500 nM, and 1 μM lapatinib (HER-family inhibitor) in combination with 50 nM, 100 nM, and 250 nM NVP-BEZ235 (PI3K/mTOR inhibitor). B. Cal27 HNSCC cells were treated with 78 nM, 156 nM, and 313 nM lapatinib in combination with 16 nM, 32 nM, and 63 nM PF04691502 (PI3K/mTOR inhibitor). C. SCC61 HNSCC cells were treated with 2.5 μM, 5 μM and 10 μM BMS599626 (HER-family inhibitor) in combination with 16 nM, 32 nM, and 63 nM PF04691502. All data points represent the average of three independent experiments. The vertical error bars represent the standard error of the mean of the Bliss predicted additive cytotoxicity calculations. The horizontal error bars represent the standard error of the mean of the experimental cytotoxicity values. D. UMUC-6 (500 nM lapatinib, 100 nM BEZ235), Cal27 (78 nM lapatinib, 63 nM PF04691502), and SCC61 (2.5 μM BMS599626, 63 nM PF04691502) were treated for 72 h assessed for apoptosis by staining for caspase 3 cleavage. E. Quantification of fold change in apoptosis compared to control cells for three independent experiments in each cell line. Bars represent the mean fold change in apoptosis and error bars represent the standard error of the mean.
3.4. Direct inhibition of p70S6K recapitulates the effects of upstream HER family kinase and PI3K/mTOR inhibition

To determine whether p70S6K could function as a single therapeutic target with efficacy that emulates the combination therapy, we utilized a small molecule inhibitor of p70S6K, AT7867. AT7867 has been shown to be an effective inhibitor of cancer cell growth both in vitro and in vivo [25]. Treatment of all three cell lines with AT7867 for 72 h caused a dose-dependent decrease in growth with an average IC50 of approximately 4 μM (Fig. 4A). This decrease corresponded to a similar dose dependent decrease in the level of ribosomal protein S6 phosphorylation upon treatment with similar concentrations of AT7867 (Fig. 4B). These data are consistent with inhibition of p70S6K activity resulting in growth inhibition. The inhibition of growth and S6 phosphorylation upon treatment with AT7867 were similar to those observed upon treatment with the HER family kinase and PI3K/mTOR inhibitor combinations. To determine whether inhibition of p70S6K activity produced effects on apoptosis comparable to the combination of HER family kinase and PI3K/mTOR inhibitors (Fig. 1), we treated cells with increasing concentrations of AT7867 for 72 h and performed flow cytometry to assess the levels of apoptosis in these cells. AT7867 caused a dose-dependent increase in apoptosis in all three cell lines tested (Fig. 4C–D). These results were similar to the biological effects caused by the combination treatment (compare Figs. 1 and 4).

AT7867 has been demonstrated to inhibit the kinase activity of proteins other than p70S6K at the concentrations that caused cytotoxicity and apoptosis, such as other AGC kinases including protein kinase A (PKA) and Akt [25]. Therefore, we tested another structurally distinct compound, Ro31-8220, that has been shown to inhibit p70S6K activity. Although Ro31-8220 is primarily used as a protein kinase C (PKC) inhibitor, it inhibits p70S6K at a similar potency [26]. Importantly, the published range of Ro31-8220 and AT7867 targets is non-overlapping with the exception of p70S6K (Supplemental Table 1). Treatment with 2.5 μM Ro31-8220 resulted in a 72% increase in cytotoxicity compared to vehicle control cells in Cal27 cells (Fig. 4E). Treatment with the same concentration of Ro31-8220 caused a 95% reduction in S6 phosphorylation at serine 235/236 and an 84% reduction at serine 240/244, indicating that Ro31-8220 is a potent inhibitor of p70S6K activity at this concentration (Fig. 4F). Taken together, the effects of AT7867 and Ro31-8220 indicate that inhibition of p70S6K results in significant cytotoxicity and apoptosis that is comparable to the effects of combination treatment using HER family and PI3K/mTOR inhibitors.

4. Discussion

We have identified p70S6K as a critical node of convergence between the HER family and PI3K signaling modules, and shown that targeting this enzyme with small molecule inhibitors can provide an alternative to drug combinations that target both pathways. Although the
Fig. 3. Constitutive activation of p70S6K protects cells from combination-induced apoptosis. UMUC-6 cells were transduced by lentivirus containing a construct encoding a doxycycline-inducible HA-tagged C-terminus truncated copy of p70S6K with a threonine to glutamate mutation at position E389 (UMUC-6-E389). This construct has been shown to display constitutively kinase activity, even in the presence of inhibitors of upstream activators. Clones stably expressing this construct were selected by exposure to G418. Due to the 24 hour vs 72 hour incubation lengths, the concentration of lapatinib (5-fold) and BEZ235 (7.5-fold) used was higher than in Fig. 1 accounting for the differential apoptotic responses. A–B. UMUC-6 parental cells and UMUC-6-E389 were treated with either water or 2 μM doxycycline for 48 h and then for 24 h with either DMSO control, 2.5 μM lapatinib, 750 nM BEZ235, or the drug combination. The cells were stained with antibodies against the HA tag, cleaved caspase 3, and/or phosphorylated S6. In addition, the DNA in the cells was stained with DAPI. The cells were then analyzed by flow cytometry on a FACSCalibur. The levels of pS6 (A) and cleaved caspase 3 (B) in the HA positive population of the doxycycline-treated UMUC-6-E389 cells were compared to the populations of doxycycline (-) UMUC-6-E389. C–D. Quantifications of the flow cytometry data. The bars represent the mean of either two (pS6) or four (Cl. Casp. 3) experiments. Error bars represent the standard error for each experiment. The * indicates a p-value < .05 and the ** indicates a p-value < .01.

Mutational activation of the PI3K pathway, whether through loss of PTEN expression or gain-of-function PIK3CA mutations, has been demonstrated to act as a mediator of resistance to anti-HER-family therapies both in vitro and in vivo [17]. Two of the three cell lines that demonstrated sensitivity to this combination in our studies contain activating PIK3CA mutations (SCC-61-E542K [27] and UMUC-6-E545K [14]. However, a third cell line that was sensitive to this inhibitor combination, Cal27, does not contain PIK3CA mutations at any of the canonical locations for mutations in exons 1, 4, 5, 6, 7, 9, and 20 [27]. This cell line also expresses full length, non-mutated PTEN [14,28]. Additionally, another cell line containing an activating PIK3CA mutation, the bladder cancer cell line 253-J, did not show sensitivity to the drug combination [14]. Therefore, it does not appear that mutational activation of the PI3K signaling pathway at the level of PIK3CA or PTEN is sufficient or necessary for sensitivity to co-inhibition of HER-family and PI3K/mTOR signaling. This underscores the point that analysis of genetic alternations may not be adequate for guiding the construction of combination therapies in all cases.

Since the mutational status of the PI3K signaling pathway does not predict sensitivity to the combination, we sought to identify a biomarker or predicted response to HER family and PI3K/mTOR co-inhibition. Utilizing RPPA technology, we discovered that phosphorylation of the ribosomal protein S6 is strongly inhibited at multiple residues upon combination drug treatment, and that none of the other protein phosphorylations exhibited a synergistic change in phosphorylation comparable to the synergistic change in cytotoxicity. S6 phosphorylation has been described as a marker for malignant progression in both bladder [29] and squamous cell [30,31] cancers. In addition, decreases in S6 phosphorylation have been correlated with anti-tumor efficacy of targeted drugs in pre-clinical studies [32]. S6 phosphorylation is also...
being tested as both a predictor of clinical response (ex. NCT00827359) and a pharmacodynamic marker of drug efficacy [33].

Since S6 phosphorylation is a validated biomarker, we tested whether the other responsive cell lines displayed a similar pattern of S6 phosphorylation in response to HER-family and PI3K/mTOR inhibitor treatment. S6 phosphorylation was significantly decreased by combination treatment in both Cal27 and SCC61 to a similar extent as in UMUC-6. These data indicate that inhibition of S6 phosphorylation does correlate to responsiveness to the HER-family and PI3K/mTOR inhibitor combination. Interestingly, the changes in S6 phosphorylation in response to treatment with these inhibitors utilized as single agents varied between cell lines. In UMUC-6 and SCC61, the two cell lines with activating PIK3CA mutations, treatment with a HER-family inhibitor had only a modest effect on S6 phosphorylation. In contrast, HER-family inhibition in Cal27 cells resulted in an over 50% decrease in S6 phosphorylation. Inhibition of PI3K/mTOR produced similarly variable results. In UMUC-6 cells, treatment with LY249002 resulted in a robust decrease in S6 phosphorylation, especially at Serine 235/236. In contrast, both HNSCC cell lines displayed only modest inhibition of S6 phosphorylation upon PI3K/mTOR inhibition. These differences can most likely be accounted for by differences in the upstream signaling states of the three cell lines. This may indicate that the combination of HER-family and PI3K/mTOR inhibition, combined with use of S6 phosphorylation as a biomarker, may be useful in a broad variety of genetic backgrounds. Importantly, these findings also suggest that p70S6K could be a therapeutic target in a wide variety of cancers that differ in upstream mechanisms of activation of the MAP and PI3K pathways.

It is generally acknowledged that p70S6K plays a role in regulating important cellular functions. However, the exact nature of that role is the subject of some dispute. It was initially believed that the main function of p70S6K was to regulate the translation of 5′ terminal oligopyrimidine tract (5′TOP) mRNAs through phosphorylation of S6. Ruvinsky et al. [34] showed that mutation of the serine sites of
p70S6K-mediated phosphorylation on S6 to non-phosphorylatable alanine residues did not have an effect on 5′TOP mRNA translation in mouse embryonic fibroblasts. Therefore, the importance of p70S6K signaling may lie in translation-independent cell functions such as regulation of proliferation [35], neurological function [36], and metabolism [37]. Our RPPA and Western blot data showed that a synergistic decrease in S6 phosphorylation correlated with synergistic apoptosis, a biological process regulated by p70S6K [38]. These data cannot, however, tell us whether p70S6K serves as a regulatory node by integrating signals from HER-family and PI3K/mTOR signaling to regulate this biology. Therefore, we tested the role of p70S6K using both pharmacological and genetic techniques. The data generated by both approaches, particularly by use of the constitutively active E389-p70S6K construct to protect UMUC-6 cells from apoptosis, demonstrate that p70S6K does in fact play a critical role in mediating the synergy caused by co-inhibition of upstream signaling. Use of direct inhibitors of p70S6K enzymatic activity to recapitulate the biological effects of inhibition of HER-family and p70S6K signaling demonstrates the druggability of this node, making it an attractive target for therapeutic intervention.

A study by She et al. [39] demonstrated that knockdown of eukaryotic initiation factor 4E binding protein (4E-BP1), but not p70S6K, recapitulated the growth inhibitory effects of co-inhibition of AKT and MEK in breast cancer cell lines harboring both activating PIK3CA and Ras mutations. When phosphorylated, 4E-BP1 binds to and inactivates eukaryotic initiation factor 4E (eIF4E), which plays an important role in mediating cell growth and proliferation through regulation of 5′-cap dependent mRNA translation [40]. Thus, the She study identified 4E-BP1 as a critical node of convergence between Ras and AKT signaling. However, we did not detect a significant decrease of 4E-BP1 phosphorylation upon combination treatment with HER-family and PI3K/mTOR inhibitors. Also in contrast to that study, our RPPA and epistasis data indicate that p70S6K, not 4E-BP1, is the critical node of convergence between the two pathways that were inhibited in this study. It is possible that the differences observed between the two studies are due to the dissimilar genetic backgrounds of the cell lines used. The She study focused on cell lines in which activating mutations of both the Ras/MEK/ERK and PI3K/AKT pathways coexisted. Conversely, none of the cell lines in our current study contain mutations activating the Ras/MEK/ERK pathway. De novo and acquired resistance mediated through up-regulation of PI3K pathway signaling has been reported in patients treated with HER-family inhibitors such as lapatinib [41] thus leading to attempts to utilize combinations of inhibitors of Her-family tyrosine kinases and PI3K/AKT pathways coexisted. Conversely, none of the cell lines in our current study contain mutations activating the Ras/MEK/ERK pathway.

De novo and acquired resistance mediated through up-regulation of PI3K pathway signaling has been reported in patients treated with HER-family inhibitors such as lapatinib [41] thus leading to attempts to utilize combinations of inhibitors of Her-family tyrosine kinases and PI3K/AKT pathways coexisted. Conversely, none of the cell lines in our current study contain mutations activating the Ras/MEK/ERK pathway.

5. Conclusions

Combinations of targeted cancer therapies are viewed as necessary to block adaptive resistance mechanisms. However, significant issues including enhanced toxicities and the potential requirement of drug company cooperation are impediments to the implementation of combination drug therapy. Identification of critical nodes in cell signaling networks is an attractive alternative to inhibiting multiple targets. Using phosphoproteomic, empirical and epistasis experiments we show that p70S6K is a critical node integrating PI3K and HER family signaling. Moreover, direct inhibition of p70S6K kinase phenocopies co-inhibition of HER family and PI3 kinase. These data indicate that direct targeting of critical nodes may be a viable strategy to overcome the limitations of combinatorial drug therapies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2014.03.013.

Authors’ Contributions

Mark Axelrod—Conception and design, development of methodology, acquisition of data, analysis and interpretation, writing/review of manuscript

Vicki Gordon—acquisition of data

Rolando Mendez—acquisition of data

Stephanie Leimgruber—acquisition of data

Mark Conaway—development of methodology, analysis and interpretation

Elizabeth Sharlow—development of methodology, writing/review

Mark Jameson—development of methodology, study supervision

Dan Gioeli—conception and design, development of methodology, analysis and interpretation, writing/review of manuscript, study supervision

Michael Weber—conception and design, development of methodology, analysis and interpretation, writing/review of manuscript, study supervision

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The study was supported by NIH grant K08-DE019477 (Jameson), NIH grant P30-CA044579 (Weber), a grant from the Melanoma Research Alliance (Weber), a grant from the James and Rebecca Craig Foundation (Weber), and a UVA Pilot Project Grant (Jameson/Gioeli) funded by the UVA Cancer Center and the UVA Department of Otolaryngology—Head and Neck Surgery.

References


