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

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ABSTRACT

Therapies targeting oncogenic drivers rapidly induce compensatory adaptive responses that blunt drug effectiveness, contributing to therapeutic resistance. Adaptive responses are characteristic of robust cell signaling networks, and thus there is increasing interest in drug combinations that co-target the driver and the adaptive response. An alternative approach to co-inhibiting oncogenic and adaptive targets is to identify a critical node where the activities of these targets converge. Nodes of convergence between signaling modules represent potential therapeutic vulnerabilities because their inhibition could result in the collapse of the network, leading to enhanced cytotoxicity. In this report we demonstrate that p70S6 kinase (p70S6K) can function as a critical node linking HER-family and phosphoinositide-3-kinase (PI3K) pathway signaling. We used high-throughput combinatorial drug screening to identify adaptive survival responses to targeted therapies, and found that HER-family and PI3K represented compensatory signaling pathways. Co-targeting these pathways with drug combinations caused synergistic cytotoxicity in cases where inhibition of neither target was effective as a monotherapy. We utilized Reverse Phase Protein Arrays and determined that phosphorylation of ribosomal protein S6 was synergistically down-regulated upon HER-family and PI3K/mammalian target of rapamycin (mTOR) co-inhibition. Expression of constitutively active p70S6K protected against apoptosis induced by combined HER-family and PI3K/mTOR inhibition. Direct inhibition of p70S6K with small molecule inhibitors phenocopied HER-family and PI3K/mTOR co-inhibition. These data implicate p70S6K as a critical node in the HER-family/PI3K signaling network. The ability of direct inhibitors of p70S6K to phenocopy co-inhibition of two upstream signaling targets indicates that identification and targeting of critical nodes can overcome adaptive resistance to targeted therapies.

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, forkhead box; T-PER, tissue protein extraction reagent; HNSCC, head and neck squamous cell carcinoma; PKA, protein kinase A; 5′TOP, 5′ terminal oligopyrimidine tract; 4E-BP1, eukaryotic initiation factor 4E binding protein; eIF4E, eukaryotic initiation factor 4E.

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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 FOXD3 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[1] [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[1]. 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[2] [13–16]. 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% CO_2. 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-BEZ235 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 phopho-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 100 μg/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

[1] D.G. Roller et al. Manuscript in preparation.
[2] M.J. Axelrod et al. Manuscript submitted.
[3] C.C. Wang et al. Manuscript under review.