Novel Potent and Selective Inhibitors of p90 Ribosomal S6 Kinase Reveal the Heterogeneity of
RSK Function in MAPK Driven Cancers

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Abstract:

The p90 Ribosomal S6 Kinase (RSK) family of serine/threonine kinases is expressed in a
variety of cancers and its substrate phosphorylation has been implicated in direct regulation of
cell survival, proliferation and cell polarity. This study characterizes and presents the most
selective and potent RSK inhibitors known to date, LJH685 and LJI308. Structural analysis confirms binding of LJH685 to the RSK2 N-terminal kinase ATP-binding site and reveals that the inhibitor adopts an unusual non-planar conformation that explains its excellent selectivity for RSK family kinases. LJH685 and LJI308 efficiently inhibit RSK activity in vitro and in cells. Furthermore, cellular inhibition of RSK and its phosphorylation of YB1 on Ser102 correlate closely with inhibition of cell growth, but only in an anchorage-independent growth setting, and in a subset of examined cell lines. Thus, RSK inhibition reveals dynamic functional responses among the inhibitor-sensitive cell lines, underscoring the heterogeneous nature of RSK dependence in cancer.

Implications: Two novel potent and selective RSK inhibitors will now allow a full assessment of RSK's potential as a therapeutic target for oncology.

Introduction:

The p90 Ribosomal S6 Kinase (RSK) comprises a family of four closely related proteins that are widely expressed in cancer cell lines and tissues and are activated in response to a number of growth factors and hormones. RSK substrate phosphorylation has been functionally linked to cell survival, proliferation and more recently to cell motility(1-4). RSK kinases have a unique structure containing two non-identical kinase domains, N-terminal and C-terminal, separated by a linker region. The currently favored model for RSK activation entails ERK phosphorylation of Thr573/577 (residue numbering for RSK1/RSK2 amino acid sequence) in RSK’s C-terminal kinase domain which ultimately leads to a phosphorylation-based docking site for PDK1 in the hydrophobic motif of RSK on Ser380/386. PDK1 phosphorylation of residue
Ser 221/227 then activates RSK’s N-terminal kinase domain, which in turn phosphorylates numerous nuclear and cytoplasmic proteins that account for the diverse cellular roles of RSK. Since the essential first step of RSK activation is its phosphorylation by ERK, RSK is positioned to be a critical effector of activated MAP kinase (MAPK) signaling that accompanies mutational activation of KRAS or BRAF in cancer. Indeed, substrates described for RSK have been used to predict proliferative and anti-apoptotic functions as well as contributions to invasion and motility, possibly associated with transition from an epithelial to mesenchymal phenotype (1, 2, 4, 5).

One of RSK’s physiological substrates with a particularly diverse set of downstream effects is Y-box binding protein 1 (YB1). YB1 regulates transcription and translation by binding to its recognition motifs in both DNA and RNA to regulate key cell processes such as proliferation, motility and stemness characteristics (6-11). Two kinases, RSK and AKT, have been studied as regulators of YB1 function through phosphorylation of YB1 on Serine 102 (12, 13). Previous studies have described somewhat opposing roles for phospho-YB1 as a modulator of both transcription and translation (8, 11, 14). Thus we hypothesize that YB1 has the potential to be strongly phosphorylated by RSK in the context of its activation downstream of mutated KRAS or BRAF, and thereby to serve as both a mediator of RSK’s functional effects and a marker for its activity.

Exploration of RSK and its substrate phosphorylation role in cancer has been previously hindered by the lack of optimal loss-of-function tools. Published inhibitors of RSK have helped gain some understanding of RSK’s role in cellular signaling. However, these molecules have limited use in dissecting RSK biology and in the assessment of RSK as a potential cancer target due to poor selectivity, or because they only target a subset of RSK family isoforms.
instance, the irreversible pyrrolopyrimidine RSK inhibitor (FMK) does not inhibit RSK3 since this isoform lacks a properly positioned active site cysteine needed for covalent binding (15). Another widely used RSK inhibitor, BI-D1870, potently inhibits all RSK isoforms in biochemical assays by binding to its N-terminal kinase domain, yet also has numerous off-target activities (16, 17).

In this study, we introduce two potent and highly selective inhibitors of the four RSK isoforms that overcome these issues. We then use these inhibitors to assess RSK’s potential as a therapeutic target in oncology by exploring the correlation between YB1 phosphorylation and RSK-inhibitor induced functional effects across a panel of MAP kinase activated cell lines.

**Experimental Procedures:**

**Materials**

All cell lines used in this study were obtained from ATCC (Manassas, VA), were authenticated by SNP6 profile, and were used for experiments within 30 passages of authentication. Cell propagation was in accordance with the ATCC recommended conditions. Cell media, fetal bovine serum, PBS and trypsin were supplied by CellGro (Mediatech, Inc., Manassas, VA) and Cambrex/BioWhittaker (Walkersville, MD). Soft Agar and TransIT-LT1 Transfection reagent were acquired from VWR International (Radnor, PA). Growth Factor Reduced Matrigel was purchased from Collaborative Biomedical Products (Bedford, MA). Primary antibodies for Western blotting were acquired from Cell Signaling Technology (Beverly, MA). The secondary anti-mouse and anti-rabbit antibodies conjugated with HRP were obtained from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England). Secondary antibodies conjugated to
fluorescent probes as well as rhodamine-phalloidin for immunofluorescence were purchased from Molecular Probes/ Invitrogen (Eugene, OR).

**Kinase Inhibitors**

LJH685 and LJI308 were synthesized using methodology described in Supplemental Methods. All kinase inhibitors were synthesized in house and dissolved in DMSO 99.9% HPLC grade (Sigma Aldrich, Milwaukee, WI). The final dilution was performed in the treatment medium. Unless otherwise specified, cells were treated for 4 hours with inhibitors at the following concentrations which were selected to achieve complete inhibition based on testing a range of doses of each: 10µM LJH685 or LJI308, 6µM FMK-PA, 1.0µM PD0325901, 50µM I-9, 5µM MK2206, 3 µM KU-0063794, 250nM RAD001 or equivalent volume of DMSO for negative control samples.

**Inhibition of RSK1, RSK2, RSK3 activity**

Enzymatic activity of RSK isoforms 1, 2, and 3 (PV4049, PV4051, PV3846) was assessed using recombinant full-length RSK protein purchased from Invitrogen (Life Technologies, Grand Island, NY). RSK1 (1 nM), RSK2 (0.1 nM), or RSK3 (1 nM) was allowed to phosphorylate 200 nM peptide substrate (biotin-AGAGRSRHSSYPAGT-OH) in the presence of ATP at concentration equal to the Km for ATP for each enzyme (RSK1- 5 µM, RSK2- 20 µM, RSK3-10 µM) and appropriate dilutions of RSK inhibitors. Additional details are provided in Supplemental Methods.

**KinomeScan™**
Kinase selectivity profiling was carried out by KinomeScan (Ambit/DiscoveRx, Fremont, CA). Data are reported as 100 minus percent of control, where larger numbers indicate more complete binding.

**pYB1 Quantification**

A quantitative electrochemiluminescence (ECL) assay was developed to measure cellular levels of YB1 protein phosphorylated at Ser102. This assay was built using ECL reagents from MesoScale Discovery (MSD, Rockville, MD). Specifically, cell lysate generated using RIPA lysis buffer (Sigma, R0278-500ml) was added to unblocked 96-well high bind ECL plates and incubated overnight at 4°C on a plate shaker. The following day, plates were washed with 1x wash buffer followed by two-hour room-temperature incubation with 10% BSA diluted in 1x tris-wash buffer (50nm Tris pH 7.5, 0.15M NaCl, 0.02% Tween-20). Plates were then washed with 1x wash buffer followed by two-hour room temperature incubation with a phospho-specific Ser102 YB1 antibody (Cell Signaling Technologies; C34A2; 1.3ug/ml). Plates were washed again with 1x wash buffer and then incubated for two-hours at room temperature with a sulfo-tag goat anti-rabbit signaling antibody (MSD; 0.5 ug/ml). Plates underwent a final wash before addition of 1.5X MSD Read buffer and detection of the phospho-YB1 signal in a MSD Sector 6000 plate reader. We routinely ran this assay using a mouse BAF cell line engineered to express activated FGFR, but it is readily applicable to other cell lines with sufficient baseline phosphorylation of YB1. Although total YB1 levels were not affected by inhibitor treatment, we routinely normalized the phospho-YB1 signal to total YB1 signal for EC$_{50}$ calculation. Total YB1 was detected through the same protocol described for phospho-YB1, but utilized a total
YB1 antibody (Santa Cruz Biotechnology, SC-101198, (59-Q); 1µg/ml) with a sulfo-tag goat anti-mouse antibody (MSD; 0.5µg/ml) for detection.

**Assessment of Cell Growth**

Cell growth in anchorage-dependent and anchorage-independent assays was quantified using CellTiter Glo or Alamar Blue reagents according to supplier protocols. Details of the assays are provided in Supplemental Methods.

**Cell Cycle Analysis and Apoptosis Detection**

MDA-MB-231 and H358 cells were assessed for cell cycle distribution by PI staining (details in Supplemental Methods). For caspase 3 activation analysis, the cells were plated on minimal-attachment plates (Nano-culture plates, Scivax USA, Inc, Woburn, MA) and treated with indicated doses of LJH685 in presence of 1µM NucView 488 probe (Biotium Inc., Hayward, CA). The presence of cleaved fluorescent substrate indicative of active caspase 3 was detected at 3 hour intervals as signal emitted at 488nm using IncuCyte fluorescent microscopy Essen Bioscience (Ann Arbor, Michigan).

**Results:**

**LJH685 and LJI308 are potent and specific RSK inhibitors**

To address the functional role of RSK, a novel series of inhibitors of the ATP binding domain of the N-terminal RSK kinase domain were developed. Two closely related difluorophenolpyridine representatives, LJH685 and LJI308, are shown in in Figure 1. LJH685 and LJI308 both inhibit RSK1, 2, and 3 biochemical activities with IC$_{50}$ of 0.004-0.013 µM (Figure 1A).
As a common shortcoming of many RSK inhibitors is their poor selectivity profile, we utilized KinomeScan™ kinase binding screening assays to assess the selectivity profiles of LJH685 and LJI308 binding to 96 kinases. LJH685 and LJI308 bound nearly 100% of RSK2 at 10µM concentration, but bound none of the other kinases to the same extent, suggesting good selectivity for RSK. Furthermore, when LJI308 was tested in an expanded panel of 442 kinases, it bound the targeted N-termini of remaining RSK isoforms, RSK1, RSK3 and RSK4 to similar extent (Table S1). Overall, only MEK4, S6K1, and HIPK1-3 bound either compound to the same degree. To determine whether the binding of LJI308 to MEK4, S6K1, and HIP kinases translates into inhibition of enzyme activity, we tested LJI308 in biochemical activity assays for these kinases. LJI308 inhibited S6K1 with an IC_{50} of 0.8 µM, representing a 200 fold lower inhibition than that of RSK2. LJI308 inhibited MEK4 less than 50% at 10 µM and HIP kinase1 less than 50% at 1 µM. This suggests that none of these kinases are inhibited with similar potency as the RSK isoforms, and that LJH685 and LJI308 are exceptionally selective inhibitors of RSK1, 2, 3, and 4.

BI-D1870-AE was tested against the same panel of 96 kinases and resulted in RSK2 binding to 99.9%, while it bound type II receptor for bone morphogenetic protein (BMPR) kinase, Polo-like kinases (PLK1 and PLK3) and several other kinases, including aurora kinase (AURKB) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (PI3CG) to a similar extent as the on-target effect on RSK2 (Figure 1B and Table S1). By this comparison, LJH685 and LJI308 have significantly improved specificity over BI-D1870-AE.

To confirm the binding site for LJH685 in RSK and to better understand its selectivity, we solved the crystal structure of LJH685 (Figure 2, PDB ID code 4NUS). LJH685 bound RSK2 in a non-planar conformation where the three aromatic rings form a propeller shaped
arrangement. Furthermore, the difluorophenol ring is binding to the gatekeeper area and is twisted 49 degrees from the plane of the pyridine ring. In most kinases, the area around the gatekeeper prefers substituents in the same plane as the hinge binding moiety. To accommodate the propeller arrangement, Phe212 of the DFG motif flips out in a conformation reminiscent of the classical type II, DFG-out orientation. However, Asp211 of the DFG blocks access to the hydrophobic pocket and interacts with the di-fluorophenyl ring and the catalytic Lys100. Thus, we propose that the non-planar shape of LJH685 and its conformational properties distinct from the currently available and commonly used inhibitor dihydropteridinone (BI-D1870) contribute to its significantly improved selectivity profile (manuscript in preparation). Furthermore, given the biochemical profile of structurally related molecules LJH685 and LJI308 and their mode of targeting RSK N-terminal kinase via interaction with the ATP binding site, these compounds are nearly identical with respect to RSK inhibition and are used interchangeably in further experiments.

**LJH685 potently and selectively inhibits RSK in cells.**

We tested the ability of these compounds to inhibit RSK in a cellular context. YB1’s description as a direct substrate for RSK made it an excellent candidate cellular marker for RSK activity. Experiments using EGF-stimulated COS7 cells as a model system for active MAP kinase signaling confirmed that in that context, YB1 is directly phosphorylated on Ser102 by RSK (Figure S1). We therefore used pSer102-YB1 as a cellular marker for RSK and asked whether its phosphorylation in MDA-MB-231 cancer cells that bear activating mutations within the MAPK signaling pathway could be modulated by RSK inhibitors. As shown in Figure 3A, LJH685 efficiently reduced phosphorylation of YB1 at sub-micromolar concentrations and caused nearly complete inhibition at higher concentrations. In comparison, the C-terminal RSK
inhibitor, FMK-PA, was similarly potent, but never achieved complete inhibition of YB1 phosphorylation even at high concentrations, possibly because it does not inhibit RSK3. In order to further quantitate RSK inhibition in cells, we developed an antibody-binding electrochemiluminescence-based (ECL) assay that quantifies cellular levels of YB1 phosphorylated at Ser102. LJH685 and LJI308 blocked YB1 Ser102 phosphorylation with similar potency (EC50=0.2-0.3 μM) and a reproducible maximum inhibition of >90% at 20 μM concentration, consistent with the qualitative results by Western blotting (Figures 3 & 4).

Because a number of other proteins have also been described as phosphorylated by RSK, we confirmed that phosphorylation of a second of these, the anti-apoptotic protein BAD at Ser112, is also inhibited by LJH685 at concentrations that correlate well with inhibition of pYB-1 (Fig S2).

We next compared LJH685 and LJI308 with additional inhibitors of the MAP kinase signaling pathway and the parallel PI3 kinase signaling pathway for their ability to inhibit phosphorylation of YB1. In these experiments, we used MDA-MB-231 and NCI-H358 (H358) cells which have KRAS mutations that activate MAPK signaling and represent established models for late stage invasive triple negative breast cancer and non-small cell lung carcinoma, respectively. The cells were treated with 10μM LJH685, LJI308, or 6μM FMK-PA for 4 hours. In addition, inhibitors of MEK (PD0325901), ERK (I-9), AKT (MK2206) and two mTOR inhibitors targeting catalytic and allosteric domains of the molecule respectively (KU-0063794 and RAD001) were compared to LJH685 and LJI308 in this study system (15, 18-21). The doses were chosen based on previous data to give full target modulation by each compound (data not shown). As with LJH685 and LJI308, MAPK pathway inhibitors cause substantial inhibition of YB1 phosphorylation on Ser102 in both cell lines (Figure 3B, left panels), consistent with the role of these kinases in activating RSK (published and data not shown). Strikingly, although
previous publications implicate AKT in YB1 activation (12, 13), AKT inhibition did not affect YB1 phosphorylation, confirming the specificity of phospho-YB1 modulation by RSK in these cells (also see Figure S2). Surprisingly, in H358 cells inhibition of mTOR with either KU-0063794 or RAD001 caused partial inhibition of YB1 phosphorylation. This suggests that in this cell line, mTOR can influence YB1 phosphorylation, either directly or via crosstalk with RSK (Figure 3B, left panels).

Ribosomal S6 protein (S6RP) which is directly involved in modulation of protein synthesis (22), represents another protein sometimes described as a RSK substrate but well-established as phosphorylated by mTOR. Recent studies point to potential RSK involvement in direct S6RP enzymatic activation as well as indirect activation via phosphorylation of Raptor, a member of mTOR complex 1 (mTORC1) (23). Therefore, to examine this signaling convergence node, we assessed the levels of S6RP phosphorylated on Serine 235/236. As shown in Figure 3B left panels, the levels of S6RP phosphorylation were not convincingly modulated by LJH685 or LJI308, whereas both allosteric and catalytic mTOR inhibitors gave strong inhibition of S6RP phosphorylation (Figure 3B, left panels). Interestingly, in both cell lines the combination of the AKT inhibitor or either of the mTOR inhibitors with LJH685 caused an additive inhibition of S6RP phosphorylation (Figure 3B, right panels). These data suggest that RSK is a minor contributor and mTOR kinase a major contributor to phosphorylation of S6RP, but that YB1 is predominantly phosphorylated by RSK.

**LJH685 RSK inhibition correlates with antiproliferative effects in MAPK pathway dependent cancer cell lines only in anchorage-independent growth setting.**
We next examined the functional effects of LJH685 and, based on recent findings that implicate RSK in anchorage-independent growth modulation and cell transformation (24) assessed the effect of RSK inhibition in both attached and anchorage-independent cell culture settings. MDA-MB-231 and H358 cells were plated on conventional plastic tissue-culture plates as well as in soft agar and treated with increasing doses of LJH685. Cell proliferation was assessed using CellTiter Glo reagent and Alamar Blue stain for the two growth settings, respectively. YB1 phosphorylation was measured using the ECL assay in a parallel set of cells plated on plastic and identically treated with inhibitors as for the CellTiter Glo determination. As Figure 4A demonstrates, the growth of both cell lines in soft agar was efficiently inhibited using LJH685, with EC₅₀ values of 0.73 µM and 0.79 µM in MDA-MB-231 and H358, respectively. Surprisingly, the growth of either cell line in an attached setting was only affected by LJH685 at concentrations significantly above the doses at which RSK is fully inhibited, with an EC₅₀ in this setting of 48 µM and 66 µM in these cell lines, respectively. The inhibition of YB1 phosphorylation tracked closely with the cell growth inhibition in soft agar setting, with EC₅₀’s of 0.39 µM and 0.57 µM in each cell line, demonstrating that RSK inhibition was fully achieved, irrespective of the differential growth response. In a control experiment, we confirmed that the concentration at which LJH685 inhibits pYB1 in the anchorage-independent setting correlates well with the concentration at which it inhibits pYB1 on plastic (Fig. S2). The correlation of LJH685 EC₅₀ values for pYB1 & soft agar growth inhibition suggests that anchorage-independent growth in these cell lines is dependent on RSK kinase activity. In contrast, the inability of LJH685 to inhibit growth of the same cell lines on plastic indicates that in the attached growth setting, these cells do not depend on RSK function for proliferation.
To further examine the signaling events underlying the differential growth effects of RSK inhibition, we utilized ultra-low bind cell culture plates which maintain anchorage-independent cell growth without a scaffold or matrix and serve as a basic three-dimensional surrogate system. This system possesses the advantage over soft agar in that it allows for sufficient cell recovery and protein extraction for analysis, and cells in this setting were similarly sensitive to RSK inhibitor (data not shown). MDA-MB-231 cells plated in the anchorage-independent setting or on conventional plastic plates were treated with LJH685, mTOR inhibitor or vehicle control DMSO for 4 hours, and the total cell lysates were analyzed for presence of phosphorylated and total S6RP and YB1 proteins using Western Blot. As shown in Figure 4B, YB1 phosphorylation was efficiently inhibited by LJH685 and was unaffected by RAD001 in either growth setting, confirming that in this cell line YB1 remains under the control of RSK and is not affected by mTOR activity in either attached or anchorage-independent growth settings. In contrast, LJH685 efficiently reduced phosphorylation of S6RP in the anchorage-independent setting. This suggests that S6RP becomes a RSK dependent substrate in the anchorage-independent setting and may be a critical mediator of the growth inhibition in soft agar.

To explore the possibility that there may be functional effects of RSK inhibition in additional cell lines, we assessed the effects of LJH685 across a panel of 21 cell lines derived from five lineages representing models of lung, colon, pancreatic, breast and melanoma cancers. These cell lines were selected on the basis of MAPK cascade activating mutations, as well as the functional sensitivity of these cell lines to MEK inhibitors (data not shown). RAF and KRAS mutation status as well as cell line lineage are shown for each cell line (Table 1, column 1 through 3). As assessed by quantitative ECL p-YB1 assay described above, LJH685 inhibits YB1 phosphorylation across the cell lines with IC$_{50}$ ranging from 0.28 µM to over the limit of
assay detection, with 15 cell lines exhibiting IC₅₀ below 3.4 µM (Table 1, column 4). For the cell lines with the most potent inhibition of YB1 phosphorylation, the highest concentrations of LJH685 resulted in >80% maximum inhibition of YB1, establishing that YB1 phosphorylation in those cells was predominantly controlled by RSK. As with the MDA-MB-231 and H358 cell lines, LJH685 did not potently inhibit the growth of any of the tested cell lines in the attached setting (column 6). Surprisingly, only two cell lines in addition to MDA-MB-231 and H358, WM1799 and NCI-H2122, demonstrated a close correlation between phospho-YB1 inhibition and growth inhibition in soft agar (columns 4 and 5), suggesting that only these cell lines are also dependent on RSK for growth in this setting. Thus, our data demonstrates that the effect of RSK inhibition in cell lines with aberrantly activated MAPK signaling is highly growth setting specific and cannot be stratified by either lineage or specific mutation status. Additionally, although we confirm YB1 as a direct substrate for RSK in an expanded cell line set, we are unable to demonstrate a robust and consistent functional dependence on YB1 phosphorylation.

**RSK involvement in cell cycle regulation and apoptosis induction in MAPK driven cancer cell lines is anchorage-setting specific.**

While we demonstrate that RSK inhibition by LJH685 results in reduced numbers of cells for several cell lines in soft agar, it was not yet clear what aspect of cell growth in this setting is dependent on RSK enzymatic activity. Thus, we conducted an additional series of cell growth assays in an attempt to deconvolute the cell dependency on RSK in the soft agar anchorage-independent growth assay.

First, to understand whether RSK signaling is necessary only to support growth in the absence of attachment, or whether the lower cell density in soft agar contributes to the
differences in RSK inhibition responses, we used several other surrogate assays for anchorage-independent growth. H358 cells proved resistant to growing in a Matrigel matrix, but MDA-MB-231 cells could be grown in this matrix and exhibited 66% growth inhibition at 10 μM LJH685 with a concomitant morphology change in this setting (Figure S3). This finding suggested that these cells remain dependent on RSK in this alternate anchorage-independent assay. We next tested MDA-MB-231 and H358 cell lines for RSK dependency while growing on Scivax NanoCulture assay plates. Scivax plates enable a low level of cell attachment due to a nano-scale pattern printed on the bottom of assay plates that promotes three-dimensional cell growth. MDA-MB-231 and H358 cells cultured for 10 days on Scivax plates grew as loosely adherent clusters of cells (Figure 5A and Table S2). LJI308 was equally inhibitory to H358 growth in this setting as in soft agar, while MDA-MB-231 was less potently inhibited by LJI308 in this minimal-attachment setting.

Thirdly, we tested the effects of LJI308 on H358 and MDA-MB-231 cells in colony formation assays. This assay format tests the role of cell density in growth characteristics of cells by establishing sparse but attached growth conditions. As demonstrated in Figure 5B, H358 colony numbers were reduced more than 2-fold by a concentration of LJI308 that inhibits colony formation of these cells in soft agar. This result suggests that in addition to dependency on RSK for anchorage-independent growth, H358 cells are also dependent on RSK for growth on plastic when seeded at a low starting density. An alternative explanation is that RSK dependency in H358 cells is only observed after longer periods of incubation with LJH685 since the anchorage-independent and colony formation assays are performed with 10-days of compound incubation compared to three days of compound incubation in standard cell proliferation assays in the attached setting. MDA-MB-231 cells, on the other hand, appear to be less potently inhibited by
LJI308 for colony formation on plastic than for growth in soft agar just as they were somewhat less potently inhibited in the Scivax assay above, suggesting that these cells may rely on RSK activity primarily to survive anchorage independence. The difference in cell line sensitivity in colony formation and Scivax assays further underscores the cell-line specific nature of RSK dependence within the subset of cell lines showing sensitivity to LJH685 and LJI308.

RSK has been previously described as mediating activation of several substrates such as Elk1, c-Fos and p27, which either directly regulate cell cycle kinases or drive expression of cell cycle regulator proteins (25-27). Thus, we examined the effect of RSK inhibition on cell cycle progression and apoptosis induction in either attached or unattached MDA-MB-231 and H358 cells. For both cell lines, there was some decrease in percentage of cells in S and G2 phases as a result of plating with no attachment on low-bind plates, but neither cell line showed strong effects of RSK inhibition on distribution within the phases of cell cycle (Figure S4). However, as shown in Figure 5C in both MDA-MB-231 and H358 cell lines, RSK inhibition in the anchorage-independent growth conditions led to a doubling of cell numbers in the sub-G1 phase of the cell cycle suggesting that this concentration of LJH685 could be inducing apoptosis. To confirm this, we tracked caspase 3 activity by non-cytotoxic NucView488 fluorescent probe in both cell lines while growing in the Scivax minimal-attachment plates in the presence of increasing doses of LJH685. As shown in Figure 5D, caspase 3 increased with time in both MDA-MB-231 and in H358 cells treated with 10 μM RSK inhibitor. These results confirm that in some cell lines such as MDA-MB-231 and H358, RSK contributes to survival in the anchorage-independent setting despite other differences in the nature of dependency on RSK.

Discussion:
Aberrant activation of the MAPK & PI3K signaling pathways is a hallmark of many cancer types. Convergent signaling nodes between these pathways could present new targeting points to achieve an effective therapeutic outcome. RSK represents such a point of convergence by incorporating sequential activation steps from both ERK and PDK1 and by phosphorylating numerous downstream substrates associated with cell proliferation, motility and cell polarity (1, 2, 4, 5). However, our understanding of this critical signaling node has been previously hindered by the lack of potent and specific tools that enable a deeper understanding of RSK’s role in cancer signaling networks. In this study we introduce two N-terminal RSK kinase inhibitors, LJH685 and LJI308, that are highly selective for all RSK isoforms. The selectivity of these inhibitors can possibly be explained by the compound’s unusual propeller conformation that can be accepted by RSK’s N-terminal kinase domain. The potency, selectivity, and effectiveness of LJH685 and LJI308 at inhibiting RSK activity in cells makes these molecules excellent new tools to better understand the role of RSK in MAP kinase pathway signaling and function.

We used LJH685 and LJI308 in functional cell viability assays in a panel of cell lines that bear activating mutations in the MAPK signaling cascade and are sensitive to MEK inhibitors. Surprisingly, LJH685 and LJI308 only inhibited the growth of a narrow sub-group of these cells, and only when these cells were grown in an anchorage-independent setting or at low cell densities in colony formation assays. Notably, while phosphorylation of YB1 at Ser102 was well-inhibited by LJH685 and LJI308, this did not closely correlate with widespread functional consequences. These results were surprising given recent studies that have linked YB1 to important physiological roles in cancer progression, including the expression of fibroblast growth factor receptor (FGFR), proliferating cell nuclear antigen (PCNA), MNK kinase and matrix metalloproteinase 2 (MMP2), among others (11, 12, 14, 28, 29). Many of these studies
investigate the importance of YB1 protein without specifically testing the role of Ser102 phosphorylation. While previous publications have indicated that YB1 is critical for these widespread roles, our results with LJH685 and LJI308 suggest that the importance of phosphorylation at Ser102 may be more nuanced.

Another intriguing possibility is that additional RSK substrates or the cell growth context may influence the functional consequence of inhibiting phosphorylation of YB1. For instance we find that in the subgroup of sensitive cell lines, phosphorylation of S6RP, a component of the 40S ribosome which plays an integral role in protein translation, is mediated by RSK preferentially in an anchorage-independent setting and correlates with the loss of cell viability under these growth conditions. Furthermore, we find that RSK and mTOR both contribute to phosphorylation of S6RP even in cells grown under attached conditions, pointing to convergence of signaling inputs. This result is consistent with the recent finding by Carriere et al. (23) that RSK is a key activator of mTORC1 complex protein RAPTOR. While we find YB1 phosphorylation to be predominantly controlled by RSK under both anchorage-dependent and anchorage-independent growth conditions, RSK substrates such as S6RP may be redundantly phosphorylated by other kinases in an anchorage-dependent context but exclusively regulated by RSK in the anchorage-independent setting.

Interestingly, even within the subgroup of cell lines sensitive to LJH685, we detect differences in their dependency on RSK for survival, attesting to the high variability of RSK involvement in cellular survival mechanisms. Our study suggests that a role for RSK signaling in anchorage-independent survival persists in certain cancer contexts, but cannot be generalized to apply wherever there is mutational activation of the MAP kinase signaling cascade. The
effects of signaling pathway crosstalk and incomplete functional dependence on a single protein phosphorylation may mask the subtle roles of RSK’s substrate phosphorylation.

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Figure Legends

Figure 1. LJH685 and LJ1308 are potent and specific RSK inhibitors. (A) Chemical structure of LJH685 and LJ1308. IC₅₀ for RSK inhibition and % of target binding at 10µM inhibitor are shown below. NT=not tested (B) Visualization of KinomeScanTM kinase competition binding data for LJH685 vs BI-D1870-AE binding to 96 kinases at 10 µM concentration. Larger circles indicate higher-percentage binding.

Figure 2. Crystal structure of LJH685 bound to RSK2 confirms binding to the ATP site with pyridine nitrogen as the hinge binder. The inhibitor demonstrates non-planar conformation where the three aromatic rings retain a propeller-shaped arrangement. The co-ordinates and structure factors for RSK2 in complex with LJH685 have been deposited to the rcsb (http://www.rcsb.org) with the PDB ID code 4NUS.

Figure 3. LJH685 modulates YB1 phosphorylation but affects phosphorylation of S6RP only in combination with mTOR inhibitors. MDA-MB-231 (A, B) and H358 (B) cells were treated with the indicated kinase inhibitors for 4 hours either alone or in combinations. Cell lysates were analyzed by Western blot with indicated antibodies.

Figure 4. Inhibition of RSK in cells correlates with decreased cell number and broad phosphorylation effects only in an anchorage-independent setting. (A) Growth of MDA-MB-231 and H358 cells was assessed in anchorage-dependent setting using CellTiter Glo assay (blue diamonds) and in anchorage-independent setting using Alamar Blue staining (red squares) with increasing concentrations of LJH685 in each growth environment. Percent of control cell number (y-axis) was compared to percent of control cellular Ser102 YB1 phosphorylation (red triangles) measured using quantitative ECL assay. EC₅₀ for each assay is shown below the
curve. (B) MDA-MB-231 cells were grown in uncoated plastic or ultra-low bind plates and treated with the indicated kinase inhibitors for 3 hours. Cell lysates were analyzed by Western blot with indicated antibodies.

Figure 5. RSK inhibition affects MDA-MB-231 and H358 colony formation, growth with minimum attachment, and viability. (A, B) MDA-MB-231 or H358 cell lines were grown with the indicated concentrations of LJI308 as colonies on minimal-attachment Scivax plates (A) or as colonies on conventional tissue-culture plates (B). Cell cycle distribution and viability were determined after treatment with 10 µM LJH685 on attached or anchorage-independent conditions for 5 days (C) or after treatment with increasing LJH685 doses during growth in Scivax plates and assessed for caspase 3 activation (D). *, $P \leq 0.005$ at 10 µM LJH685 dose compared to DMSO treated control. Data depicted is for period after stabilization of specific caspase 3 signal.
Table 1. Cellular EC$_{50}$ of LJH685 modulation of YB-1 protein phosphorylation levels at Ser 102, as well as cell growth on plastic and in soft agar in a panel of cell lines with mutationally activated MAPK pathway.

<table>
<thead>
<tr>
<th>Cancer Type/Cell Line</th>
<th>Mutation Status</th>
<th>pYB1</th>
<th>Soft agar</th>
<th>Attached</th>
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<tbody>
<tr>
<td><strong>Lung</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calu6</td>
<td>Q61K</td>
<td>9.95 (58)</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>NCI-H2122</td>
<td>G12C</td>
<td>0.63 (87)</td>
<td>61, &gt;33</td>
<td>26</td>
</tr>
<tr>
<td>NCI-H358</td>
<td>G12C</td>
<td>0.57 (91)</td>
<td>0.79</td>
<td>66</td>
</tr>
<tr>
<td>NCI-H727</td>
<td>G12V</td>
<td>0.64 (81)</td>
<td>30</td>
<td>&gt;100</td>
</tr>
<tr>
<td>NCI-H2087</td>
<td>wt (N-Ras Q61K)</td>
<td>0.28 (82)</td>
<td>1.1</td>
<td>64</td>
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<td><strong>Colon</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SW620</td>
<td>G12V</td>
<td>3.23 (74)</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SW480</td>
<td>G12V</td>
<td>2.22 (61)</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HT29</td>
<td>V600E</td>
<td>&gt;20 (43)</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td><strong>Pancreatic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capan-2</td>
<td>G12V</td>
<td>0.9 (78)</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>MiaPaCa2</td>
<td>G12C</td>
<td>3.35 (64)</td>
<td>62, &gt;33</td>
<td>&gt;100</td>
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<tr>
<td>SW1990</td>
<td>G12D</td>
<td>1.6 (69)</td>
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<tr>
<td>Panc02.03</td>
<td>G12A/G12D</td>
<td>&gt;20 (31)</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td><strong>Breast</strong></td>
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<tr>
<td>MDA-MB-231</td>
<td>G464V</td>
<td>0.39 (93)</td>
<td>0.73</td>
<td>48</td>
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<tr>
<td><strong>Melanoma</strong></td>
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<tr>
<td>A375</td>
<td>V600E</td>
<td>0.38 (96)</td>
<td>&gt;100</td>
<td>52</td>
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<tr>
<td>G361</td>
<td>V600E</td>
<td>0.49 (88)</td>
<td>38</td>
<td>70</td>
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<tr>
<td>Colo205</td>
<td>V600E</td>
<td>&gt;20 (48)</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>V600E</td>
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<td>WM983B</td>
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<td>WM266-4</td>
<td>V600D/V600E</td>
<td>0.37 (88)</td>
<td>22, &gt;33</td>
<td>&gt;100</td>
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</table>
Figure 1A.

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC_{50} (μM)</th>
<th>% target binding at 10 μM</th>
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</thead>
<tbody>
<tr>
<td>RSK 1</td>
<td>0.006</td>
<td>NT</td>
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<tr>
<td>RSK 2</td>
<td>0.005</td>
<td>99.95</td>
</tr>
<tr>
<td>RSK 3</td>
<td>0.004</td>
<td>NT</td>
</tr>
<tr>
<td>RSK 4</td>
<td>NT</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC_{50} (μM)</th>
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<tr>
<td>RSK 1</td>
<td>0.006</td>
<td>100</td>
</tr>
<tr>
<td>RSK 2</td>
<td>0.004</td>
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<tr>
<td>RSK 3</td>
<td>0.013</td>
<td>99.95</td>
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<tr>
<td>RSK 4</td>
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</table>

Figure 1B.
Figure 2.
Figure 3.

A) µM Inhibitor

LJH685

<table>
<thead>
<tr>
<th>Phospho-YB1</th>
<th>RAS-GAP</th>
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<tbody>
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FMK-PA

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<th>RAS-GAP</th>
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</table>

B) MDA-MB-231

Inhibitor Targets: N-RSK ERK MEK C-RSK AKT mTOR + LJH685

<table>
<thead>
<tr>
<th>p-YB1</th>
<th>YB1</th>
<th>p-S6RP</th>
<th>S6RP</th>
<th>RAS-GAP</th>
</tr>
</thead>
<tbody>
<tr>
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</table>

H358

<table>
<thead>
<tr>
<th>N-RSK</th>
<th>ERK</th>
<th>MEK C-RSK</th>
<th>AKT</th>
<th>mTOR</th>
<th>+ LJH685</th>
</tr>
</thead>
<tbody>
<tr>
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<table>
<thead>
<tr>
<th>p-YB1</th>
<th>YB1</th>
<th>p-S6RP</th>
<th>S6RP</th>
<th>RAS-GAP</th>
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</thead>
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Figure 4.

A

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<tr>
<th></th>
<th>CTG</th>
<th>SAA</th>
<th>pYB1</th>
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<tbody>
<tr>
<td>EC\textsubscript{50}</td>
<td>48 µM</td>
<td>0.73 µM</td>
<td>0.39 µM</td>
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B

<table>
<thead>
<tr>
<th></th>
<th>Attached</th>
<th>Anchorage Independent</th>
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<tbody>
<tr>
<td>p-YB1</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>YB1</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>p-S6RP</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>S6RP</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>RAS-GAP</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>
Figure 5

A. % of Media Alone

- H358
- MDA-MB-231

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>LJ308 0.1 μM</th>
<th>LJ308 1 μM</th>
<th>LJ308 10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>H358</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MDA-MB-231</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

B. Absorbance (RLU)

- H358
- MDA-MB-231

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>LJ308 1 μM</th>
<th>LJ308 10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>H358</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
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</tbody>
</table>

C. Attached vs. Anchorage Independent

- H358
- MDA-MB-231

<table>
<thead>
<tr>
<th></th>
<th>Attached</th>
<th>Anchorage Independent</th>
</tr>
</thead>
<tbody>
<tr>
<td>H358</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td></td>
<td></td>
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</tbody>
</table>

D. Active Caspase 3

- MDA-MB-231

<table>
<thead>
<tr>
<th>DMSO</th>
<th>0.1 μM LJ685</th>
<th>1 μM LJ685</th>
<th>10 μM LJ685</th>
</tr>
</thead>
<tbody>
<tr>
<td>H358</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference
Molecular Cancer Research

Novel Potent and Selective Inhibitors of p90 Ribosomal S6 Kinase Reveal the Heterogeneity of RSK Function in MAPK Driven Cancers

Ida Aronchik, Brent A. Appleton, Stephen E. Basham, et al.

Mol Cancer Res Published OnlineFirst February 19, 2014.

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