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14. ABSTRACT

The Ser/Thr protein kinase p90-kDa ribosomal S6 kinase (RSK) is an important downstream effector of mitogen-activated protein kinase but its roles in prostate cancer have not been previously examined. We have found that RSK regulates the growth of the human prostate cancer lines, LNCaP and PC-3, and that increased RSK levels stimulate the growth of a normal prostate cell line, RWPE-1. To further understand the role that RSK plays in prostate cancer we are developing prostate cell lines that overexpress RSK in an inducible manner and transgenic mice that overexpress RSK in the prostate. The potencies of RSK-specific inhibitors, which we are developing, are being evaluated in tissue culture and in PC-3 xenografts.

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Introduction

An increase in the activity of the mitogen-activated protein kinase (MAPK) has been correlated with the progression of prostate cancer to advanced disease in humans (1). The Ser/Thr protein kinase p90-kDa ribosomal S6 kinase (RSK) family are important downstream effectors of MAPK (2) but the roles they play in prostate cancer have not previously been examined (2). The overall objective of our research project is to evaluate the importance of RSK as a chemotherapeutic target in prostate cancer. In our previous report we found that RSK1 and RSK2 levels are higher in ~50% of human prostate tumors compared to normal prostate tissue (3). Furthermore, the RSK2 isoform stimulated expression of the prostate specific antigen (PSA), an important diagnostic marker for prostate cancer. Previously, we identified the first inhibitor of the RSK family, SL0101, by screening a collection of rare botanical extracts for their ability to inhibit RSK activity (4). Using this inhibitor we found that RSK regulates the growth of some human prostate cancer cell lines. Taken together, these observations suggest that RSK in a good drug target for prostate cancer.

Body

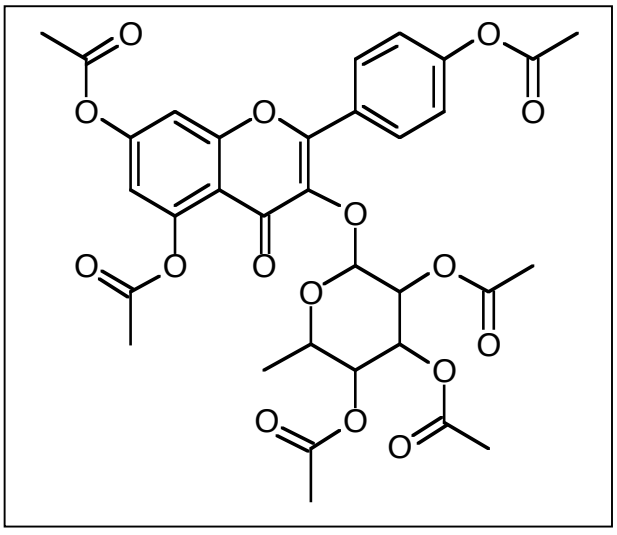
The research accomplishments associated with each Aim are described below:

Aim 1. Evaluate the efficacy of the RSK-specific inhibitor, SL0101, to inhibit proliferation of prostate cancer cells.

Task 1. Test ability of SL0101 to inhibit growth in culture and soft agar.

Previous progress on this Task was detailed in the 1st year report. In an ongoing collaboration with Dr. Hecht (Dept. Chemistry) we continue to synthesize and test SL0101 analogues to determine if we can identify a more potent RSK-specific inhibitor than SL0101. We have determined that enhancing the hydrophobic character of SL0101 increases its efficacy in intact cells (5). Therefore, Dr. Hecht synthesized a SL0101 analogue in which all available hydroxyl groups were acetylated, 6Ac-SL0101 (Fig. 1). In an *in vitro* kinase assay 6Ac-SL0101 was not as potent at inhibiting RSK activity as SL0101 or the analogue, 3Ac-SL0101 (Fig. 2). However, 6Ac-SL0101 was much more effective at inhibiting the growth of PC-3 cells than SL0101. The EC50 for inhibition of PC-3 proliferation was ~10 μM and ~50 μM for 6Ac-SL0101 and SL0101, respectively (Fig. 3). Thus 6Ac-SL0101 is ~5-fold more potent at inhibiting PC-3 growth compared to SL0101. 6Ac-SL0101 is defined as a pro-drug because it is only effective at inhibiting RSK activity in the intact cell. In the intact cell it is likely that the acetyl groups are removed by ubiquitous intracellular acetylases. The removal of the acetyl groups allow 6Ac-SL0101 to inhibit RSK activity. We hypothesize that 6Ac-SL0101 is a more potent RSK-inhibitor than SL0101 because its increased hydrophobic character facilitates its uptake into cells.

Figure 1 Schematic of the SL0101 analogue, 6Ac-SL0101.



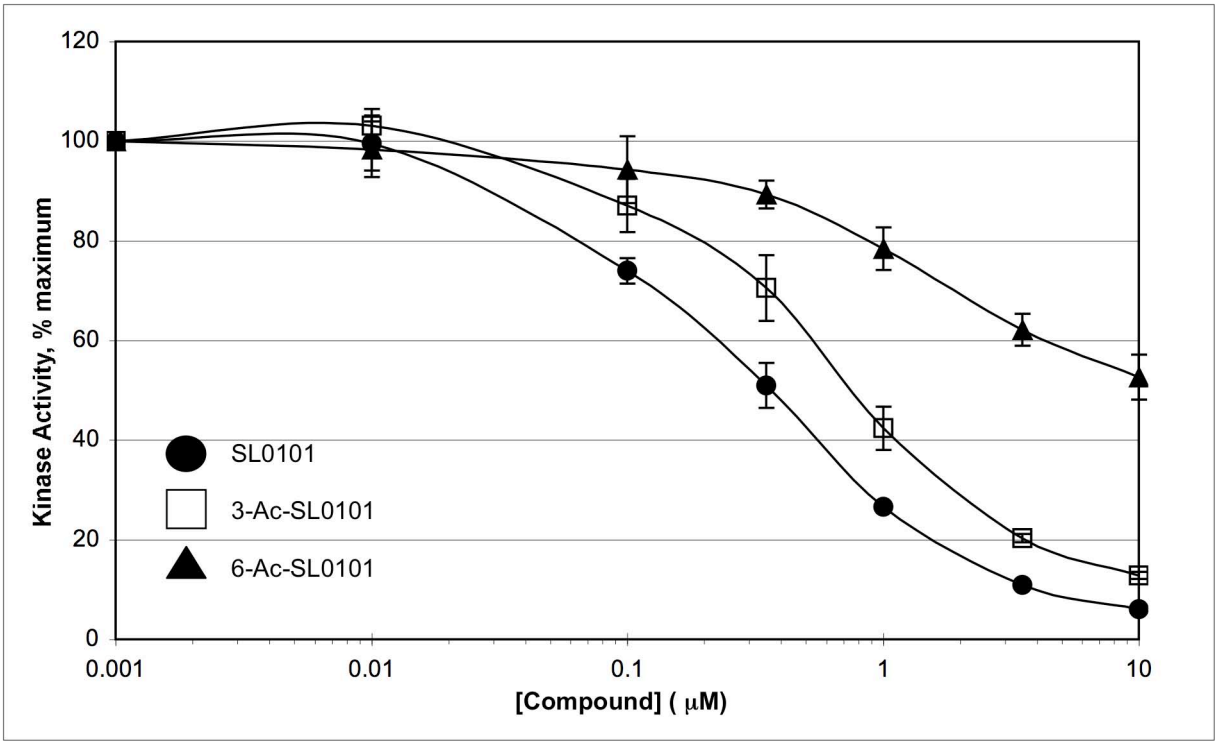


Figure 2 Effect of SL0101 and its analogues, 3-Ac-SL0101 and 6-AC-SL0101 on RSK activity in vitro. *The potency of the synthetic compounds in inhibiting RSK catalytic activity was measured as described in Smith et al. (4).*

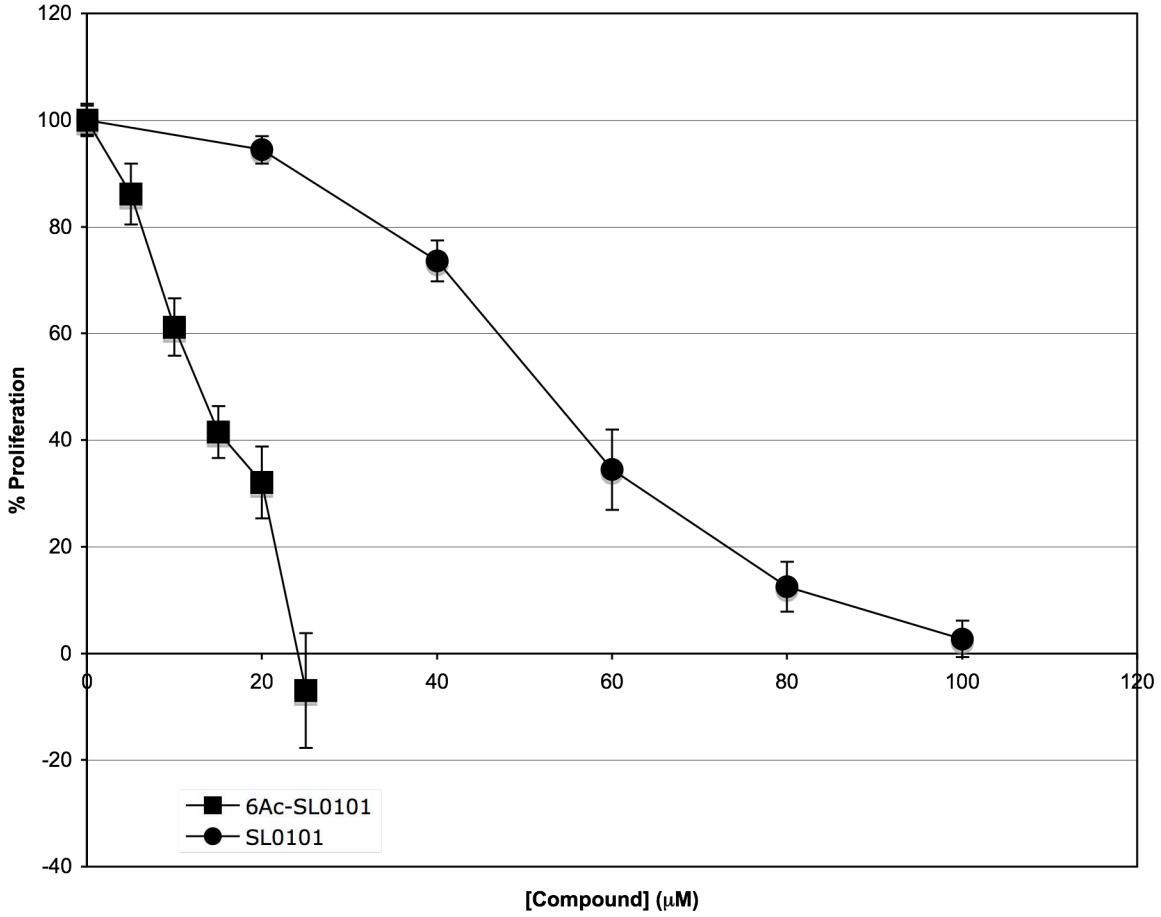


Figure 3 Effect of SL0101 and its analogue 6Ac-SL0101 on RSK activity in intact cells. *The potency of the synthetic compounds in inhibiting the proliferation of PC-3 cells was determined as described by Clark et al. (3).*

Task 2. Test ability of SL0101 to inhibit tumor formation in nude mice.

Previously, we had established the maximum tolerated dosage and maximum tolerated total dosage for the SL0101 analogue, 3Ac-SL0101, using the National Cancer Institute's general instructions. As discussed in the previous report 3Ac-SL0101 is more potent at inhibiting RSK activity in the intact cell than SL0101 and therefore, is being used in the *in vivo* studies. Before, testing the efficacy of 3Ac-SL0101 to inhibit the growth of PC-3 xenografts in nude mice it is important to determine the pharmacodynamic and pharmacokinetic properties of the drug. These studies are ongoing. Pharmacodynamic studies determine the effects of the drug in the living organism. To ensure that the 3Ac-SL0101 was being taken up by tissues and tumor we had to identify a molecular marker to serve as a convenient readout for the ability of 3Ac-SL0101 to inhibit RSK activity *in vivo*. Therefore, we investigated whether the phosphorylation of eukaryotic elongation factor 2 (eEF2) could be used as molecular marker. eEF2 mediates the translocation step in mRNA translation. eEF2 activity is regulated by phosphorylation and it is inactivated by a highly specific kinase, EF2 kinase (EF2K). RSK phosphorylates and inactivates EF2K in response to mitogenic stimulation, which leads to a decrease in phosphorylation of eEF2 (6). Thus under conditions such as serum deprivation, which leads to low RSK activity, eEF2 is phosphorylated by the active EF2K. However, stimulation of RSK activity by mitogens results in reduced phosphorylation of eEF2 due to inactivation of EF2K by RSK. Therefore, the phosphorylation state of eEF2 during mitogenic stimulation is an indicator of RSK activity. Treatment of PC-3 cells with the

mitogens, serum or phorbol dibutyrate (PDB), inactivated EF2K as determined by the reduced levels of phosphorylated eEF2 (Figs. 4 and 5). However, pre-incubation of the cells with 3Ac-SL0101 eliminated serum-induced eEF2K inactivation and the levels of phosphorylated eEF2 increased in a dose-dependent manner (Fig. 4). Thus the phosphorylation status of eEF2 is a valid molecular marker for RSK activity.

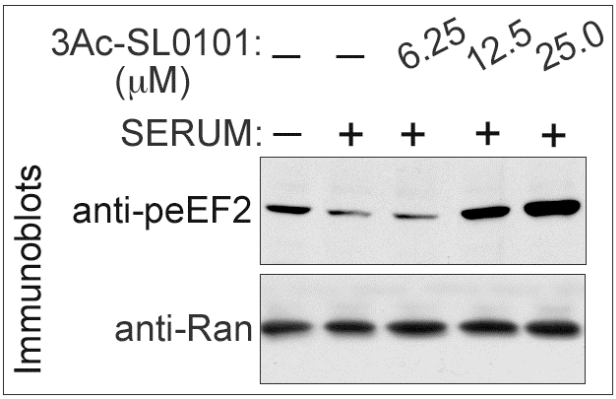


Figure 4 Dose-dependent inhibition of RSK activity by 3Ac-SL0101 in intact cells. *Serum-deprived PC-3 cells were pre-treated with vehicle or varying doses of 3Ac-SL0101 for 4 hrs. The cells were then treated with 10% serum for 20 mins prior to lysis. Protein concentration of lysates was measured and lysates were electrophoresed, transferred and immunoblotted. Equal loading of lysate is shown by the anti-Ran immunoblot.*

The next step in establishing the efficacy of 3Ac-SL0101 was to determine if we could inhibit RSK activity in PC-3 xenografts. Male SCID mice (7-8 weeks old) were subcutaneously injected with $5X10^6$ PC-3 cells/100 μl and tumors were allowed to develop until they were palpable, ~ 2 weeks. The animals were then injected with vehicle or 3Ac-SL0101 (100 mg/kg) intravenously through the tail vein. Animals were euthanized at various times up to 2 hrs after injection and the xenograft, blood and organs were isolated and analyzed. Typical results are shown in Fig. 5. As a control PC-3 cells grown in tissue culture and treated with PDB in the absence and presence of 3Ac-SL0101 were also immunoblotted on the same gel as the tumor sample. Treatment with 3Ac-SL0101 did not substantially alter the phosphorylation of eEF2 in the tumor. These results suggested that the compound was not effectively reaching the tumor.

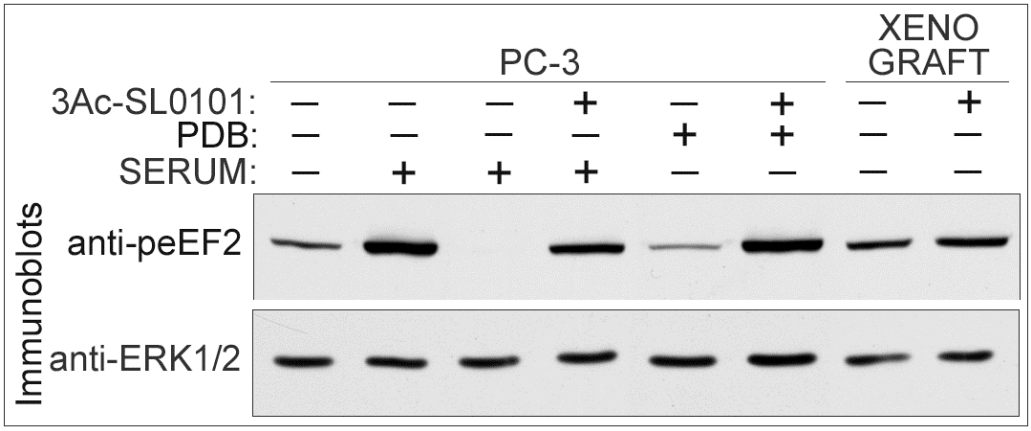


Figure 5 3Ac-SL0101 inhibits RSK activity in tissue culture and in vivo. *Serum-deprived PC-3 cells were pre-treated with vehicle or 50 μM 3Ac-SL0101 for 4 hrs. The cells were then treated with 10% serum or 500 nM PDB for 20 min prior to lysis. SCID mice containing PC-3 xenografts were treated with vehicle or 3Ac-SL0101 by tail vein injection. After 2 hrs the mice were euthanized and the tumor isolated and lysed. Protein concentration of lysates was measure and lysates were electrophoresed, transferred and immunoblotted. Equal loading of lysate is shown by the anti-ERK1/2 immunoblot.*

To investigate why we did not observe an effect of 3Ac-SL0101 on the tumor *in vivo* we initiated pharmacokinetic studies. We are currently analyzing the absorption, distribution, metabolism and excretion of

3Ac-SL0101 *in vivo*. In order to facilitate these studies we have isolated various tissues and blood from mice treated with 3Ac-SL0101 for various lengths of time. These samples will be extracted with methanol. The dried extracts will be sent to the Michigan State University Genomics Technology Support Facility for high resolution mass spectrometry using fast atom bombardment. This assay is very sensitive and will be able to detect μg quantities of 3Ac-SL0101 or any of its metabolites that are present in the organs or blood. To facilitate these studies we have already had the Michigan Facility analyze 3Ac-SL0101. In addition to these studies we tested whether we could use the phosphorylation status of eEF2 in the blood samples as a method to easily monitor the efficacy of 3Ac-SL0101 *in vivo*. As can be seen in Figure 6 we were able to observe an increase in phosphorylation of eEF2 in the blood after a 15 min treatment with 3Ac-SL0101. Thus by analyzing the tissue and blood distribution of 3Ac-SL0101 by mass spectroscopy and monitoring the *in vivo* efficacy of 3Ac-SL0101 in the blood we will be able to establish the pharmacokinetic properties of 3Ac-SL0101. These studies will allow us to adjust the dosing schedule of 3Ac-SL0101 in order to perform the efficacy studies. Additionally, these studies will provide insight into how we can improve the pharmacokinetic properties of 3Ac-SL0101.

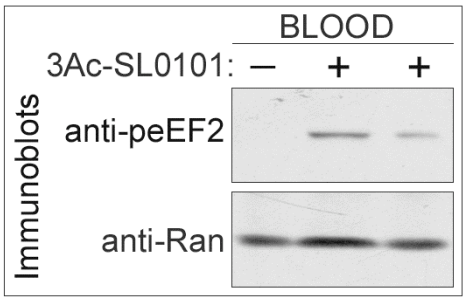


Figure 6 Detection of RSK inhibition by 3Ac-SL0101 in blood samples. *SCID mice were injected with vehicle or 3Ac-SL0101 and after 15 mins were euthanized. The blood from the animals was isolated, lysed, electrophoresed, transferred and immunoblotted. Equal loading of lysate is shown by the anti-Ran immunoblot.*

Aim 2. Test whether enhanced RSK1 and RSK2 activity increases prostate cell growth.

Task 1. Produce stable RWPE-1 and LNCaP lines that overexpress RSK1 and RSK2.

Previously, we had reported that we had optimized the transfection method, Nucleofection, for use with the normal prostate line, RWPE-1, which are very difficult to transfect using standard procedures. It is not necessary to use Nucleofection with the prostate cancer line, LNCaP, because we are able to achieve >70% transfection efficiency using Lipofectamine 2000. In the Tet-on system the expression of the gene of interest is regulated by doxycycline in a concentration-dependent manner. However, the transactivator that is commercially available (Clontech) has a number of drawbacks that severely limit its use *in vivo*. Therefore, we obtained a modified Tet-on system from Hillen and colleagues, in which the transactivator has been optimized and has been successfully used *in vivo* (7). We are currently screening to identify those clones that produce at least a ten-fold activation in gene expression in the presence of doxycycline and that have a very low background in the absence of doxycycline. The screening process requires a great deal of effort. In order to identify clones that stabley express the transactivator it is necessary to transfect each clone with a construct that encodes a gene under the control of a Tet-sensitive promoter. The transfected clones are then treated with or without doxycycline and the effects on gene expression are determined. To facilitate the screening process we are using the reporter gene, luciferase, to identify clones of interest.

In initial studies we have observed that enhanced RSK2 levels significantly increase the proliferation of RWPE-1 cells compared to the vector (Fig. 7). These results are very exciting because we have previously determined that RSK2 levels are increased in human prostate cancers compared to normal prostate tissue (3). These results

suggest that RSK2 may act as an oncogene in prostate cells and support the continued evaluation of RSK as a drug target for prostate cancer.

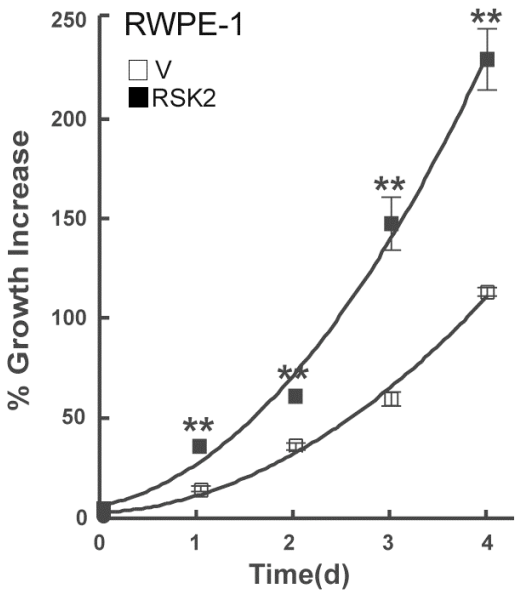


Figure 7 RSK2 stimulates the proliferation of the normal prostate line, RWPE-1. *RWPE-1 cells were transfected with a construct encoding RSK2 or vector control. At various times cell viability was measured as described by Clark et al. (2).*

Aim 3: Determine whether increased RSK1 or RSK2 activity induces prostate cancer in a transgenic mouse model.

Task 1: Generate transgenic vectors.

To facilitate overexpression of RSK1 and RSK2 in the prostate we obtained the ARR_2PB promoter from Robert Matusik (University of Vanderbilt) (8). This composite probasin (PB) promoter gives high levels of prostate-specific gene expression. The MkbpAII vector (Jeff Rosen, Baylor College of Medicine) was modified for use as a prostate-specific transgenic vector. The MMTV was removed and the ARR_2PB was subcloned into the BssHII and BamH1 site to generate the vector $ARR_2PBKbpA$ (Fig. X). This vector contains a 640 bp of the rabbit beta-globin gene containing 18 bp of exon II, 572 bp of intron 2, and 50 bp of exon II 5' to the unique EcoR1 cloning site. Having an intron 5' of the gene of interest has been found to increase expression of the gene of interest. Additionally, 3' from the gene of interest the bovine growth hormone poly A signal is inserted to ensure proper processing of the mRNA.

To facilitate the detection of the exogenously introduced RSK2 we inserted the haemagglutin epitope tag (HA) in frame at the 5' of RSK1 and RSK2. The HA-tagged RSK2 was digested from pKH3RSK2 using Xba I and Xma I and subcloned into $ARR_2PBKbpA$ with an EcoR I/Xba I linker. The HA-tagged RSK1 was digested from pKH3RSK2 using Xba I and EcoR1 and subcloned into $ARR_2PBKbpA$ with an EcoR I /Xba I linker. The inserts were sequenced by the University of Virginia Biomolecular Research Facility. To demonstrate that the transgenic vector was functional we transfected LNCaP cells with the transgenic vector encoding HA-RSK2. HA-RSK2 was expressed only in response to the synthetic androgen, R1881 (Fig. 8). Furthermore, the levels of HA-RSK2 were equivalent to those obtained with a vector that constitutively expresses high levels of RSK2. These studies validated the transgenic vector for use in the production of the RSK1 and RSK2 transgenic animals.

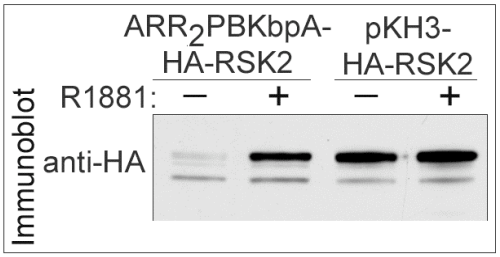


Figure 8 Inducible expression of RSK2 using the prostate-specific transgenic vector $ARR_2PBKbpA$. *LNCaP cells were transfected with either a construct that inducibly expresses ($ARR_2PBKbpA$-HA-RSK2) or constitutively expresses HA-RSK2 (pKH3-HA-RSK2). The cells were treated with 10 nM R1881 for 24 hrs, lysed, electrophoresed and immunoblotted.*

Task 2: Produce and characterize transgenic animals.

The University of Virginia Transgenic Facility is generating the RSK1 and RSK2 transgenic animals. Progress in this Aim was delayed due to difficulties in generating the transgenic vectors as well as the additional drug development studies that were needed in Aim 1.

Key Research Accomplishments

- Discovered a new synthetic analogue of SL0101, 6Ac-SL0101, which is more potent at inhibiting the growth of the prostate cancer line, PC-3, than SL0101.
- Identified a molecular marker to facilitate pharmacodynamic studies of SL0101 and its analogues *in vivo*.
- Developed a blood assay to simplify the pharmacokinetic studies of 3Ac-SL0101 *in vivo*.
- Initiated screening of RWPE-1 and LNCaP lines for optimum induction of Tet-responsive gene expression.
- Discovered that RSK2 may be oncogenic in prostate cells.
- Produced a transgenic vector that will result in high levels of prostate-specific gene expression.
- Generated RSK1 and RSK2 transgenic vectors.

Reportable Outcome

Manuscripts: Clark, D.E., Errington, T.M., Smith, J.A., Frierson, Jr., H.J., Weber, M.J. and Lannigan, D.A., (2005) The Ser/Thr protein kinase, RSK, is an important regulator of prostate cancer cell proliferation. Cancer Res. 65:1027-1034.

Smith, J.A., Maloney, D.J., Hecht, S. Lannigan, D.A. (In preparation) Structure/Activity relationship studies of SL0101 and its derivatives with RSK.

Grant Submissions: NIH R01 "RSK2 Regulation of Cancer Cell Growth"

Conclusions

We have found that increased RSK2 enhances the growth of the normal prostate line, RWPE-1. These results are physiologically significant given that RSK2 levels are increased in ~ 50% of prostate cancers compared to normal prostate tissue. We have developed analogues with improved efficacy in inhibiting the growth of the advanced prostate cancer line, PC-3, compared to SL0101. We are in the process of generating cell lines and transgenic mice that will be a valuable resource to the prostate cancer research community.

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Appendices

None