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### Title and Subtitle

**Overcoming Resistance to Inhibitors of the Akt Protein Kinase by Modulation of the Pim Kinase Pathway**

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### Abstract

Small molecules targeted at specific signal transduction pathways hold great promise for creating a new approach to prostate cancer treatment. In this proposal, the applicant research team demonstrates that resistance to small molecule AKT protein kinase inhibitors is mediated by the ability of the Pim protein kinases to in part stimulate increases in receptor tyrosine kinases (RTKs). In particular the Pim protein kinases are shown to induce resistance to c-Met inhibition. The c-Met tyrosine kinase is an activator of AKT. Results demonstrate that Pim can phosphorylate eIF4B and control IRES mediated translation of these RTKs. Additional experiments show that inhibition of AKT/PI3K kills through the induction of ROS, and the Pim protein kinase through the induction of Nrf2 blocks the action of these agents. Animal experiments demonstrate that a combination of Pim and AKT inhibitor decreases the growth of subcutaneous grafts of human prostate cancer. The knowledge gained through these studies will be essential to the development of combined chemotherapeutic strategies to treat prostate cancer.

### Subject Terms

Prostate cancer; AKT protein kinase; PI3 kinase; Pim protein kinase; FOXO transcription factors; Protein kinase inhibitor; c-MET tyrosine kinase; hepatocyte growth factor; Receptor tyrosine kinases; GSK690693
# Table of Contents

1. Introduction........................................................................................................ 4
2. Keywords............................................................................................................. 4
3. Accomplishments............................................................................................. 4
4. Impact................................................................................................................ 15
5. Changes / Problems.......................................................................................... 16
6. Products............................................................................................................. 16
7. Participants & Other Collaborating Organizations.......................................... 17
8. Special Reporting Requirements...................................................................... 17
9. Appendices......................................................................................................... 17
1. Introduction
Over 90% of prostate cancer patients have abnormalities in the AKT signaling pathway. These abnormalities are driven by mutations in the PTEN and AKT proteins as well as deletions and inversions of these proteins. Small molecule drugs have been developed to block this pathway with the hope of inhibiting tumor growth. Although activation of the AKT pathway is a cancer driver for prostate carcinoma, inhibiting this protein kinase pathway is not sufficient to block tumor growth. Research from multiple laboratories has demonstrated that one reason is that AKT inhibition leads to increases in receptor tyrosine kinases that have the capacity to stimulate tumor growth. Data from our laboratory has demonstrated that the Pim protein kinase plays a role in this biology stimulating receptor tyrosine kinase increases. This grant proposal has explored the resistance to small molecule AKT protein kinase inhibitors mediated by the Pim-1 protein kinase, and examined in detail whether unique Pim protein kinase inhibitors that synergize with AKT inhibitors to block prostate cancer growth overcome this resistance. The knowledge gained through the studies is essential for the development of this combined chemotherapeutic strategy targeting both Pim and AKT protein kinases.

2. Keywords
Prostate cancer
AKT protein kinase
PI3 Kinase
Pim protein kinase
FOXO transcription factors
Protein Kinase Inhibitors
c-MET tyrosine kinase
hepatocyte growth factor
Receptor tyrosine kinases
GSK690693

3. Accomplishments
Major goals of the project
The goals of the project were to attempt to understand how AKT and Pim kinases interact to drive the growth of prostate cancer. In part this dual action occurs through the regulation of receptor tyrosine kinases. It would appear that this regulation is through the control of translation. At the start of this project the mechanism of action Pim protein kinase was not well defined so an essential element in this research was to discover its mechanism in part through understanding the targets it phosphorylates. The Specific Aims of the project were to:

1) Decipher the mechanisms by which AKT inhibition elevates the cellular levels of Pim-1 protein kinase.
2) Examine the mechanism(s) by which Pim-1 protein kinase controls the expression of cell surface RTKs.
3) Study, using mouse models, whether combination therapy using AKT and Pim inhibitors blocks prostate cancer growth.

Accomplishments under these goals

Year 1-
In year 1 we have completed work on Task 1-4 outlined for year one. This analysis led to an impressive and novel understanding of how Pim-1 regulates the level of receptor tyrosine kinases and thus the growth of prostate cancer. We have discovered that Pim-1 regulates the phosphorylation of eIF4B and being doing so is able to control the translation of the MET receptor tyrosine kinase in prostate cancer. This regulates the activity of the MET/HGF axis and potentially can affect the ability of these cells to spread and metastasize.

Task 1, Year 1. Task 1 was to carry out experiments to examine whether inhibition of AKT with small molecule inhibitors increased the levels of Pim-1 mRNA. To determine the mechanism by which AKT inhibitors increase Pim-1 levels, PC3-LN4 cells were treated with either GSK690693 or MK2206 overnight and mRNA was isolated. As shown by qRT-PCR, both agents increase the levels of Pim-1 mRNA GSK690693 inhibits all three isoforms of AKT 1,2 and 3 while MK2206 inhibits only AKT 1,2. These data suggest that inhibition of the AKT 1,2 isoform may be sufficient to increase Pim-1 levels transcriptionally. This further
demonstrates that inhibition of AKT elevates Pim-1 through a transcriptional mechanism. To attempt to replicate these findings derived from treating prostate cancer cells with small molecules, PC3-LN4 cells are treated with siRNAs either to AKT 1, 2 or AKT 1, 2, and 3. Results demonstrate that the level of Pim-1 mRNA increases with a decrease in AKT, but suggests that knocking down 1, 2 and 3 was somewhat better than inhibiting the levels of 1,2 alone (Fig. 1). Interestingly Western blots demonstrate that AKT-3 levels vary but are present in 5 prostate cell lines, but that they are significantly less than the level of AKT 1 and 2. Taken together these results again validate the hypothesis that Pim-1 is regulated transcriptionally by AKT.

**Task 2, Year 1** – Task 2 was to determine whether FOXO transcription factors are capable of activating Pim-1 transcription. To evaluate the mechanism by which AKT inhibition activates the Pim-1 promoter, 3.0 kB of the Pim-1 upstream region was cloned into a firefly luciferase reporter plasmid and transfected into PC3-LN4 cells along with a renilla luciferase control plasmid. These cells were then treated with GSK690693 to block AKT and the ability of this agent to elevate Pim1 transcription evaluated. Results demonstrate that in a dose dependent fashion, AKT inhibition stimulates the activity of the Pim1 promoter (Fig 2). These results again demonstrate using a portion of the Pim1 promoter that inhibition of AKT stimulates the Pim1 transcription. These experiments set up a further analysis of which transcription factors might play a role in this stimulation.

Because AKT blocks the phosphorylation of the Foxo transcription factors, translocating these factors to the nucleus, the possibility that these proteins increased Pim1 transcription was next evaluated. qRTPCR demonstrates that transduction of a cDNA containing an activated mutants of FOXO transcription factors increases Pim1 transcription 4-6 fold. This result clearly points to a potential mechanism by which AKT inhibition activates Pim1 mRNA transcription. The experiment suggests that Foxo transcription factors play an important role here.

To validate the further genetic manipulation of FOXO transcription factors is essential for the control of Pim1, siRNAs that target FOXO 1 or 3 or the combination of these proteins were transfected into prostate cancer and then GSK690693, the AKT inhibitor, applied to these cells. Results demonstrate that the decrease in the FOXO transcription factors blocked the induction of the transcription of Pim-1 by this AKT inhibitor. Thus these results show by using two types of genetic manipulation that FOXO transcription factors are regulating Pim1 transcription. We suggest the following model. AKT inhibitors block the phosphorylation of FOXO transcription factors. This leads to their translocation to the nucleus and the binding to the Pim-1 promoter activating the transcription of the Pim-1 protein.

**Task 3, Year 1**- Task 3 was to examine whether Pim-1 induced increases in receptor tyrosine kinases (RTKs) is cell line specific. The addition GSK690693 an AKT inhibitor, induced up regulation of multiple RTK protein levels, including MET, EphA2, Her3, Her2, IGF1R, and the insulin receptor, in PC3-LN4, and as shown MET, HER2, HER3 and EPHA2 in DU145, 22RV1, and VCAP cells (Fig. 3). These results demonstrate that the regulation of Pim-1 by AKT inhibition is a general phenomenon that is not cell line specific. To document this further and demonstrate that Pim-1 plays an important regulatory role in the ability of AKT inhibitors to modulate RTKs, we determined the effects of Pim-directed siRNAs and small- molecule inhibitors on RTK levels. In all cell lines treated Pim-1 inhibitors blocked the ability of AKT inactivation to lead to increased RTK expression. Further, the use of siRNA directed at Pim-1 showed that a forced reduction in Pim-1 levels markedly reduced the ability of GSK690693 to elevate the protein levels of multiple RTKs, including MET and EPHA2, HER3, HER2, INSR, and IGF-IR, as well as the phosphorylation of ERK. Thus, Pim1 is shown to play a role in the regulation AKT inhibitor regulation of RTK levels. The data obtained comes from experiments with both small
molecules and genetic manipulation. This further establishes the role of Pim and the interaction with AKT.

**Task 4, Year 1** Task 4 was to study the activity of Pim and AKT inhibitors on the growth of PC3-LN4 cells placed orthotopically in mice.

To evaluate the possibility that combining an AKT and Pim inhibitor would have dual activity in killing cancer cells varying doses of GSK690693 plus SMI-4a were added to PC3-LN4 cells. Enhanced killing from this combination therapy was the result. With this data, a Chou-Talalay combination index (CI) (32) was calculated using the CalcuSyn from Biosoft and found to be < 0.5. A CI less than 1.0 denotes a high degree of synergism between agents. When PC3-LN4 was grown in soft agar with a combination of both agents, but neither agent alone, tumor cell growth was inhibited (Fig. 4). This combination therapy was then evaluated in an immunosuppressed mouse model by first injecting $10^{-6}$ PC3-LN4 cells subcutaneously. When tumors were palpable, animals were treated with GSK690693 30 mg/kg intraperitoneally for 7/7 days and SMI-4a 60 mg/kg orally twice a day for 5/7 days. The results demonstrate the ability of the combination, but not each agent alone, to inhibit tumor growth (Fig. 5).

These novel biologic findings indicate that combination therapy aimed at inhibiting AKT and Pim kinases effectively blocks PCa growth, overcoming resistance mechanisms induced by AKTi therapy alone. This experiment also demonstrates that one can give combination therapy blocking both of these kinase pathways without harming the animals. As better inhibitors of both pathways are developed this combined therapy should become more powerful. Further experiments are carried out and described below which focus on a single receptor, MET, and how this receptor may be modulated biochemically by Pim.

The first paper written as a result of carrying out these tasks is “Cen B, Mahajan S, Wang W, and Kraft AS. Elevation of Receptor Tyrosine Kinases by Small Molecule AKT Inhibitors in Prostate Cancer Is Mediated by Pim-1. Cancer Research. 2013 Jun 1;73(11):3402-11”. In summary of the findings described above, we demonstrate that multiple AKT inhibitors in human clinical trials increase the levels of Pim protein kinase, including GSK690693, MK2206 and BEZ235 and that an increase in Pim protein kinase is sufficient to elevate multiple cell surface tyrosine kinases including c-MET, Her2, Her3, EGFR, and EPHA2. In parallel, it is shown that inhibition of AKT leads to increases in Bc1-2, c-Myc, VEGF, and HIF1α, all of which are known to be regulated by Pim and play an important role in stimulating tumor growth. Inhibiting Pim with small interfering (si) RNAs blocks this induced increase. To attempt to understand how Pim might be controlling the levels of this protein the laboratory hypothesized that the IRES found in a number of tyrosine kinase 5’ regions might be driven by Pim protein kinase. To investigate this possibility a number of IRES regions, c-Met, VEGF, HIF1α and c-Myc, were cloned into luciferase vectors and the ability of Pim-1 over expression or knockdown to regulate these IRES or 5’ upstream regions examined. Results demonstrate that overexpression enhanced or knockdown of Pim-1 blocked translation from these regions. The addition of low dose actinomycin D was used to induce ribosomal stress and lower the levels of RPS19 and RPS6. This inhibition decreased the AKT inhibitory induction of multiple RTKs, c-MET, Her3, and IGF-1R, suggesting that regulation of ribosomal proteins by AKT and Pim might be important for the induction of RTKs and their regulation by Pim-1 through the IRES.

The combination of an AKT and Pim inhibitor were used in animal studies as outlined in Task 4 Year 1 and the results published in this paper. This combination inhibited the subcutaneous growth of the human prostate...
tumor while causing a decrease in receptor tyrosine kinases. This experiment was done with an AKT inhibitor GSK 690693 and a Pim kinase inhibitor SMI-4A. Thus, this first publication validated the interaction between Pim and AKT and suggested that this could be targeted to inhibit tumor growth. The mechanism of Pim action was found to involve the 5’ upstream region and potentially the regulation of ribosomal subunit content.

Year 2-
In the second year of this proposal we focused on Tasks 3 and 4 of the Statement of Work. Tumors are complex with epithelial, stromal and vascular elements all contributing to the cancer growth. To be able to use Pim and AKT inhibitors together to treat cancer it is important to be able to define which cells within the tumor are being regulated by Pim and what is the outcome of treating animals with Pim inhibitors. To explore these tasks we first stained human tumor and normal samples with Pim1 antibodies. As can be seen (Fig. 6) Pim is overexpressed in tumor but not PIN or normal tissue.

Since Pim is increased in hypoxic cells, we then examined whether hypoxia sensitized cells to the Pim inhibitor AZD1208, which has been in human clinical trials. In Fig. 7, we demonstrated that hypoxia sensitizes to the growth inhibitory activity of Pim inhibitors. Viability of human prostate cancer cells was estimated in this experiment by using an MTS assay. Fig. 7 (left panel) shows that the induction in hypoxia in these cells is associated with a 100-fold increase in sensitivity of prostate cells to Pim inhibitor. In Fig. 7, the right panel using cell extracts from this experiment, we find that HIF1α is increased by hypoxia but decreased by the addition of the Pim inhibitor. Importantly the effect of Pim inhibitor on eIF4B phosphorylation is much more dramatic in the hypoxic cells when Pim levels are increased.

In an additional Western blot (Fig. 8 left panel), Pim inhibitor treatment of hypoxic versus normoxic prostate cancer cells, both HIF-1alpha and HIF-2alpha are shown to be decreased by Pim inhibitor AZD1208 treatment. No change was seen in the mRNA (Fig. 8 bottom left panel). The laboratory has derived mouse
embryo fibroblasts from mice that are knock-out all of the Pim enzymes. As demonstrated in Fig. 8 right panel, fibroblasts missing the Pim enzymes have low levels of HIF-1 and 2 alpha. Together these results suggest that the level of the HIF proteins is regulated by Pim. Along with the receptor tyrosine kinases, hypoxia Pim kinases importantly regulate the hypoxia inducible proteins that are key to the response of tumor cells to low oxygen tension.

To examine whether decreases in the HIF proteins during hypoxia were biologically meaningful, we investigated the activity of a reporter with multiple HIF binding sites (HRE) in front of a luciferase reporter in cells that are treated with hypoxia with and without the Pim inhibitor. The addition of two different Pim inhibitors in a dose dependent fashion blocked the activity of the HRE to activate luciferase mRNA and protein production. This result demonstrates that the ability to inhibit HIF activity can be seen with multiple Pim inhibitors.

HIF regulates a number of important proteins that stimulate tumor growth in hypoxia. One of the most important for driving tumor growth by stimulating vessel growth into the tumor is VEGF. To examine whether Pim inhibitors block the induction of VEGF and other genes associated with the control of metabolism by HIF, prostate cells were placed in hypoxia and treated with various concentrations of AZD1208, the Pim inhibitor. The addition of Pim inhibitor blocked the induction of VEGF and inhibited the induction of specific enzymes that regulate metabolism. This data demonstrates that the inhibition of Pim by small molecule inhibitors blocks the ability of HIF to stimulate its target genes.

The mechanism by which Pim-1 regulates HIF activity was next investigated. Prostate cancer cells were first placed in hypoxia to induce HIF, then treated with cycloheximide to block translation. Either DMSO or AZD1208 was added to cells. The left panel in Fig. 9 demonstrates by Western blot that the half-life of both HIF-1 and 2 alpha was decreased by Pim inhibitor treatment. This decrease in protein half-life was quantitated in Fig. 9 (right panel) and shown to be significantly different. To further evaluate the mechanism for this change in half-life, prostate cells were either placed in normoxia or hypoxia and DMOG an inhibitor of the prolyl hydroxylase (PHD) that targets HIF proteins to degradation (Fig. 10 right panel). The Western blot demonstrates that the DMOG reverses the decreased amount of HIF (Fig. 10, left panel), suggesting that control of PHD activity may be the key to understanding Pim activity.

To examine this in animals, PC3-LN4 cells were injected subcutaneously in immunosuppressed mice (10 mice per group), and once the tumors grew to measurable size, the mice were treated with DMSO or AZD1208. This experiment demonstrates that Pim inhibitor treatment decreases the growth of these tumors. This decrease in tumor growth is significant. As predicted by the cell culture experiments, Western blots of extracts of these tumors demonstrate lower levels of both HIF1 and 2 alpha in treated tumors with increased Pim1 levels. The phosphorylation of two potential substrates of Pim, IRS1 and eIF4B are decreased in these tumor.

Figure 9. PC3-LN4 cells were placed in hypoxia and cycloheximide was added. Western blots were done at the times indicated (hrs) for the identified proteins. These blots were scanned and the expression plotted relative to actin (right panel).

Figure 10. PC3-LN4 cells were placed in normoxia or hypoxia and treated with Pim inhibitor AZD1208 with or without DMOG and propylhydroxylase inhibitor. Wester blots were carried out on these extracts. The HIF degradation pathway is shown in the right panel.
This data suggests a model wherein hypoxia in prostate tumors inhibits the activity or level of propyl hydroxylases. Pim inhibits this enzyme allowing the level of HIF and its substrates to increase. Pim inhibitors enhance PHD activity and lead to the degradation of HIF1 and 2 alpha and prevent the production of hormones, i.e. VEGF, a hormone that regulates the growth of vessels in prostate tumors.

This data has been published “Warfel NA, Sainz AG, Song JH, Kraft AS. PIM kinase inhibitors kill hypoxic tumor cells by reducing Nrf2 signaling and increasing reactive oxygen species. Mol Cancer Ther. 2016”

**Task 3 – Year 2.** Using IHC, examine whether in normal mice increases in Pim-1 lead to increases in receptor tyrosine kinases (RTKs).

Our preliminary results suggested that the Pim protein kinase could regulate multiple RTKs. However, it was not possible to analyze multiple proteins simultaneously. Because the MET tyrosine kinase drives the growth and metastasis of multiple human cancer types, we first examined whether the levels of Pim-1 correlates with the level of MET in prostate tumor cells.

Immunohistochemical analysis of a high-density tissue microarray representing tissues from 27 patients with newly diagnosed prostate cancer indicated that the levels of Pim-1 and MET are highly correlated (correlation coefficient (R) = 0.85 (Fig. 11).

To determine whether Pim-1 expression has a direct effect on MET protein levels, we used several different approaches. RNA interference-mediated silencing of Pim-1 expression markedly reduced both the total and cell surface expression of MET in the prostate tumor cell line PC3-LN4. Moreover, although the overexpression of wild-type Pim-1 in DU145 prostate cancer cells resulted in increased levels of MET protein, the overexpression of kinase-dead Pim-1 did not. The phosphorylation of MET and AKT that occurs on culture of wild-type DU145 cells in the presence of HGF was enhanced in the Pim-1-overexpressing cells. This effect was specific as there was no difference in ERK phosphorylation between the over expressor and wild-type cell lines cultured in HGF. Conversely, in PC3-LN4 cells in which Pim levels were reduced using siRNA, the HGF-induced phosphorylation of MET and AKT was lower than those in control cells.

Potentially, Pim-1 could regulate MET protein levels without having a significant physiologically relevant effect on the response on the cells to HGF. HGF induces scattering of DU145 cells. Culture of the Pim-1-overexpressing DU145 cells with HGF resulted in a pattern of scattering of the cells similar to that observed in HGF-treated wild-type DU145 cells; however, scattering was observed at a lower concentration of HGF (0.25 ng/ml vs. 1 ng/ml). Pretreatment with a MET inhibitor (PHA665752) blocked the HGF-induced cell scattering in the Pim-1 overexpressing cells suggesting that this Pim-1 effect is mediated through the MET receptor. Taken together, these data suggest that the Pim-1-induced increase in the MET receptor enhances the ability of HGF to signal in these tumor cells.

Finally, to evaluate the ability of Pim-1 to modulate HGF-induced invasion of tumor cells, PC3-LN4 cells were placed in the upper chamber of a Boyden chamber in which the membrane had been coated with Matrigel, and HGF was added to the media in the lower chamber. In this assay, the Pim-1-overexpressing PC3-LN4 cells demonstrated increased invasion through the Matrigel-coated
membrane, and this invasion was blocked by treatment with the MET inhibitor PHA665752 (Fig. 12) or knockdown of Pim-1 expression by siRNA.

Collectively, these results demonstrate that Pim-1 levels are correlated to the MET RTK in prostate cancer, and Pim-1 can control signaling through the HGF/MET axis and that this affects HGF-induced cell motility and invasion tumor cells. The impact of these findings are such that they imply that if one regulates Pim-1 activity with small molecules drugs, it may be possible to modulate prostate tumor invasion and metastasis.

Year 3
Task 1. Year 3 - Examine whether Pim-1 kinases regulate the levels of RTKs by controlling transcription, protein translation, or the stability of these receptors.

Identification of the mechanisms by which Pim-1 controls the level of expression of MET requires a more detailed understanding of the mechanisms underlying translation of the MET protein. The 5’UTR of MET is relatively long (408 nt) and GC-rich, which is consistent with the possibility that this region functions as an internal ribosome entry site (IRES). Modeling of the secondary structure using the MFOLD program (5) indicated that the 5’UTR of MET is highly structured with the overall folding energy of the most stable predicted structure being -293.3 kcal/mol.

The translation initiation factor, eIF4B preferentially promotes the translation of mRNAs containing such complex secondary structures in their 5’UTRs. Notably, eIF4B has been identified as a potential substrate of Pim-1 kinase activity. Using in vitro kinase assays in which FLAG-tagged eIF4B was expressed in 293T cells and immunoprecipitated with anti-FLAG antibody, we confirmed that Pim-1 phosphorylated wild-type eIF4B. To identify the phosphorylation site(s), we transfected the cells with eIF4B constructs with S406A and S422A mutations, which prevent phosphorylation at these sites. Pim kinase was capable of phosphorylating an eIF4B construct with an S422A mutation, but not eIF4B with an S406A mutation (Fig. 13). Similar results were obtained using commercially available recombinant eIF4B with Pim-1 phosphorylating eIF4B at S406 and to a lesser extent at S422.

Phosphorylation at both these sites was inhibited by the small molecule pan-Pim kinase inhibitor GNE652 (produced by Genentech). In marked contrast, in agreement with a previous report (9), we found that AKT1 preferentially phosphorylated eIF4B at S422 and confirmed that this phosphorylation was inhibited by GSK690693, a small molecule AKT inhibitor.

To determine whether the Pim kinase phosphorylation of eIF4B occurs in tumor cells, we analyzed Pim siRNA-transfected PC3-LN4 cells. The knockdown of Pim-1 expression was associated with reduced phosphorylation of eIF4B S406 but did not affect phosphorylation on S422. Insulin and serum- stimulation of serum-starved control tumor cells resulted in enhanced eIF4B phosphorylation at S406 and this effect was impaired in the Pim siRNA-transfected PC3-LN4 cells. Insulin and serum- stimulation of serum-starved control tumor cells also resulted in enhanced eIF4B phosphorylation at S422. In siRNA-transfected tumor cells a decrease in Pim levels impaired the insulin-treated S422 phosphorylation, but it did not affect the serum-stimulated levels. Interestingly, both insulin and serum increased the expression of Pim-1 protein, suggesting another level of regulation of eIF4B phosphorylation under these conditions. In these cells the phosphorylation of S406 and, to a lesser extent, S422 was blocked by the Pim inhibitor GNE652). Moreover, treatment of PC3- LN4 and DU145, with either of two pan-Pim inhibitors, GNE652 or AZD1208, reduced the phosphorylation of eIF4B S406.
Together these data suggest that S406 is the major Pim directed phosphorylation site on eIF4B, while S422 phosphorylation could occur as a result of interacting signaling pathways driven by specific growth factors in individual cell lines. As suggested in the introduction to this year 1 update it would appear that AKT and Pim are working together to regulate translation with Pim-1 phosphorylating the S406 site on eIF4B and AKT targeting S422. Importantly, these results point to eIF4B as a biomarker for both Pim-1 activity that can potentially be used to modulate the use of Pim-1 inhibitors.

It has been suggested that insulin-induced phosphorylation of eIF4B S406 is dependent on both MEK and mTOR activity (9). As described above, we found that treatment of PC3-LN4, and DU145, with GNE652 or AZD1208 (two Pim-1 inhibitors produced by Genentech and AstraZeneca) markedly reduced the phosphorylation of eIF4B S406. In contrast, treatment of these cell lines with the PI3K/AKT/mTOR pathway inhibitors BEZ235, PP242, or U0126 blocked the phosphorylation of eIF4B S422 (Fig. 14) and had no effect on S406. As the results could be affected by the experimental conditions, we analyzed eIF4B phosphorylation in PC3-LN4 that were either grown in serum-rich conditions or starved and insulin-stimulated. Again, we found that phosphorylation of eIF4B S406 was not inhibited by small molecules that block the PI3K/AKT/mTOR pathway (MK2206, rapamycin, PP242, and BEZ235) or the MEK pathway (U0126, and BI-D1870), when used alone or in combination. In marked contrast, the Pim inhibitors, GNE652 and AZD1208, clearly blocked phosphorylation of eIF4B S406. These results suggest that phosphorylation of eIF4B S422 is predominantly controlled by the PI3K/AKT/mTOR pathway, with MEK also playing a role in phosphorylation of S422 whereas phosphorylation of eIF4B S406 is predominantly controlled by the Pim protein kinases.

We have found previously that small molecule AKT inhibitors can induce Pim-1 expression. Consistent with this observation, GSK690693, an AKT inhibitor, increased the phosphorylation of eIF4B S406 while reducing the levels of eIF4B S422 phosphorylation. This AKT inhibitor-induced increase in phosphorylation of eIF4B S406 was blocked by GNE652, a Pim inhibitor, suggesting that under these circumstances the increased Pim-1 expression associated with inhibition of the AKT protein kinase plays a role in the phosphorylation of eIF4B S406.

These results clearly demonstrate that inhibitors of Pim-1 that are entering clinical trials can regulate the phosphorylation of the important translational control factor, eIF4B. Other inhibitors do not regulate the phosphorylation of this protein on S406 suggesting that Pim-1 inhibitors will play an important, if not essential, role in regulating translation in tumor cells.

The protein translation initiation complex consists of several proteins in the eEF family. In yeast and mammalian cells (9, 11), the binding of eIF4B to eIF3, a 13 member complex, enhances protein synthesis. It has been suggested that phosphorylation of eIF4B-S422 can regulate its interaction with the eIF3. To examine whether the association of eIF3 proteins with eIF4B is affected by eIF4B-S406 phosphorylation, we carried out co-immunoprecipitation experiments using cells transfected with the phosphorylation site S406A and S422A mutant constructs as well as S406D and S406E mutant constructs, which carry negative charges and thus mimic phosphorylation of eIF4B at S406. 293T cells were first transfected with wild-type FLAG-eIF4B or the phosphorylation site mutant constructs, starved of serum overnight, and then stimulated with insulin to promote the formation of the eIF3 complex. Consistent with the reported effects, insulin treatment stimulated
an enhanced interaction of wild-type eIF4B with eIF3A and eIF3B and this interaction with eIF3A was absent when the cells were transfected with the S422A or S406A/S422A mutants (Fig. 15). This interaction also was abrogated when the cells were transfected with the S406A construct; moreover, the S406D and S406E mutant constructs, mimicking the Pim phosphorylation site, formed a complex with eIF3A or eIF3B as efficiently as wild-type eIF4B.

Pretreatment of 293T cells with the pan-Pim inhibitor GNE652 or the PI3K/mTOR blocker BEZ235, or both agents prior to insulin addition blocked the ability of eIF4B to bind to eIF3B. Thus, eIF4B S406 phosphorylation plays an essential role in the formation of the translation initiation complex.

These data suggested that both the eIF4B S406 and S422 phosphorylation sites contribute to the binding of eIF4B to eIF3B and that inhibition of phosphorylation of both sites resulted in greater inhibition of binding than inhibition of either site alone. We therefore extended the studies to determine whether the phosphorylation state of eIF4B affects the formation of translation initiation complex at the 5’ cap structure. The m7-GTP-sepharose binding assay was used to identify the proteins that bound to the 5’ cap structure. Insulin treatment of starved PC3-LN4 cells enhanced the binding of both eIF4B and eIF3B to the m7-GTP beads. Treatment with the Pim inhibitor GNE652 reduced binding of both eIF4B and eIF3B to the m7-GTP beads as did treatment with the PI3K/mTOR pathway inhibitor. BEZ235. Concomitant treatment with both GNE652 and BEZ235 resulted in the same or better reduction in binding. The inhibitors did differ, however, in terms of their effects on the binding of other components of the translation complex; BEZ235 enhanced the interaction of 4EBP1 with the complex, but GNE652 did not. Consistent with this observation, BEZ235 treatment also resulted in a decrease in the binding of eIF4G, whereas GNE652 did not.

These findings suggest eIF4B S406 phosphorylation is an important regulatory element in controlling the protein binding of eIF4B to the eIF3 complex and that eIF4B S406 and S422 phosphorylation may play different roles in the formation of the translation initiation complex.

The conclusions from this data demonstrate the biologic importance of eIF4B phosphorylation by Pim-1. They suggest that this phosphorylation of this protein is key in regulating translation through binding to the eIF3 complex.

The experiments described above established that Pim-1 kinase activity may affect translational activity directly. Furthermore, we have previously demonstrated that Pim-1 does not affect the transcription of MET (3). To specifically determine the role of eIF4B S406 phosphorylation in the regulation of the translation of MET, we utilized the eIF4B mutant constructs. Transfection with the wild-type eIF4B construct increased the expression of MET as compared to the vector control (Fig. 16) whereas transfection with the S406A and S406A/S422A mutants failed to do so. The levels of MET protein were increased to the levels seen on transfection with wild-type eIF4B after transfection with eIF4B-S422A (Fig 16), S406D, or S406E mutants. Furthermore, treatment with the small molecule Pim inhibitor, AZD1208, caused down regulation of MET

**Figure 15.** Phosphorylation of eIF4B regulates its association with the eIF3 translational complex. FLAG-tagged wild-type (WT) eIF4B and its mutants were expressed in cells. Cells were stimulated with insulin (1 µg/ml) for 15 minutes. Anti-FLAG antibodies were used to immunoprecipitate the translation complex. Immunoprecipitates were Western blotted with antibodies as shown. Cell lysates used in these immunoprecipitations were immunoblotted as shown.

**Figure 16.** eIF4B Ser406 phosphorylation positively regulates the MET expression. Cells were transfected with pcDNA3 (Vector), or plasmids expressing wild-type eIF4B (WT) and its mutants for 48 hours. Cell lysates were analyzed by immunoblot assays using indicated antibodies.
expression that was reversed by transfection with wild-type eIF4B, eIF4B S406D or S406E, but not by eIF4B S406A. Thus, the eIF4B S406 phosphorylation site is critical for the regulation of MET translation in this cell line.

To determine the effects of eIF4B S406 phosphorylation on translation of MET protein, we monitored new protein synthesis rates by labeling PC3-LN4 cells with [35S]-methionine. Treatment of both PC3- LN-4 with the pan-Pim inhibitor AZD1208, but not the PI3K/mTOR inhibitor BEZ235, reduced MET expression, but had no effect on ERK protein synthesis. While in comparison, cycloheximide treatment decreased the levels of both MET and ERK (data not shown). In the same cell system, overexpression of wild-type eIF4B, S406D and S406E mutants, but not the S406A protein, increased the rate of MET protein synthesis.

These experiments demonstrate that eIF4B phosphorylation by Pim-1 plays a significant role in regulating eIF4B phosphorylation. The phosphorylation of this translation factor then controls the production of MET protein while having no effect on the levels of the ERK serine threonine protein kinase. In comparison, cycloheximide that is a general translation regulator controls the expression of both of these proteins.

Importantly we have discovered a new substrate for the Pim-1 protein kinase eIF4B that is essential for controlling the translation of the MET protein. Inhibiting this phosphorylation in prostate cancer cells decreases MET levels and consequently diminishes the activity of the MET/HGF axis with diminished invasion and motility of these tumor cells. AKT is shown to share yet another substrate with Pim and thus enhance our hypothesis that inactivating both of these pathways will be needed to block prostate cancer growth.

Task 2,3 Year 3
The combination of an AKT and Pim inhibitor were used in animal studies as outlined in Task 4 Year 1 and the results published in this paper. This combination inhibited the subcutaneous growth of the human prostate tumor (Fig. 17) while causing a decrease in receptor tyrosine kinases. This experiment was done with an AKT inhibitor GSK 690693 and a Pim kinase inhibitor SMI-4A. To attempt to use compounds that are entering the clinic, the experiment was repeated with a Novartis compound BKM120, a broadly active PI-3Kinase inhibitor and AZD1208, a Pan-Pim kinase inhibitor (Fig. 18). Thus, this first publication validated the interaction between Pim and AKT and suggested that this could be targeted to inhibit tumor growth. The mechanism of Pim action was found to involve the 5’ upstream region and potentially the regulation of ribosomal subunit content.

New unpublished data generated by Dr. Song (Figure 3) in the laboratory demonstrates that the overexpression of Pim blocks the activity of inhibitors of AKT/PI3K when applied to prostate cancer cells. As in the above research, Pim is shown to regulate Nrf-2 and to increase proteins that block the generation of ROS, e.g. HMOX1. If this effect is reversed, then it is possible for AKT/PI3K agents to enhance the killing of these prostate cancer cells.

Opportunities for training and professional development provided
This project has led to the professional development for Dr. Jin Song. Based on the work he accomplished on this project he has been promoted to a Research Assistant Professor and submitted his own DOD grant. This project has given him the chance to explore the control of metabolism by Pim and AKT and learn techniques including measuring oxygen and lactate production and assays for GSH, GSGS, ATP and NADPH. He has explored this Pim effect first in genetically engineered models isolated from Pim KO mice and then in prostate cancer cells with activated AKT. This novel research results should allow him to generate sufficient data that can be used for further individual applications for grant funding. This research has clearly generated a member of the next generation of scientists.
In summary, we have made the following observations/accomplishments in this grant period:

1- Inhibiting Pim blocks the AKT inhibitor induced increase in tyrosine kinases.
2- Pim kinase is capable of stimulating increases in tyrosine kinases and blocking the action of tyrosine kinase inhibitor, e.g. c-MET, that target RTKs that activate AKT.
3- Tyrosine kinases that are translated by Pim over expression may be stimulated by modulation of the IRES.
4- Pim phosphorylates eIF4B and this phosphorylation controls eIF4A activity and likely 5’ IRES translation.
5- Pim protein kinase controls metabolism and regulates ROS levels through the modulation of Nrf-2 location.
6- The induction of inhibitors of ROS by Pim blocks the ability of AKT/PI3K inhibitors to kill prostate cancer.

How were the results disseminated to communities of interest?
The results of this research were disseminated widely in the peer reviewed literature. In addition, abstracts of this work were presented at National meetings, including the American Associate of Cancer Research.

4. Impact
What was the impact on the development of the principal discipline(s) of the project?
These results have focused attention in prostate cancer on the continued development of the combination of Pim and AKT/PI3K inhibitors in the clinic. Recent results in breast cancer have shown that the patients that are resistant to PI3K inhibitors that they have increased levels of Pim protein kinase. Inhibition of Pim sensitized cells to these inhibitors. We have detailed a path forward for the use of Pim inhibitors. We have demonstrated the following:

1- Inhibiting Pim blocks the AKT inhibitor induced increase in tyrosine kinases.
2- Pim kinase is capable of stimulating increases in tyrosine kinases and blocking the action of tyrosine kinase inhibitor, e.g. c-MET, that target RTKs that activate AKT.
3- Tyrosine kinases that are translated by Pim over expression may be stimulated by modulation of the IRES.
4- Pim phosphorylates eIF4B and this phosphorylation controls eIF4A activity and likely 5’ IRES translation.
5- Pim protein kinase controls metabolism and regulates ROS levels through the modulation of Nrf-2 location.
6- The induction of inhibitors of ROS by Pim blocks the ability of AKT/PI3K inhibitors to kill prostate cancer.

These results will have direct impact on the ability to design new therapies for patients. First, it is clear that resistance to AKT inhibitors may be overcome by the addition of the addition of Pim inhibitors. In addition, the activity of specific inhibitors of receptor tyrosine kinases that activate AKT may also be enhanced by the addition of Pim inhibitors. Mechanistically this may be due to the ability of Pim to regulate eIF-4B phosphorylation. This result could mean that targeting translation down-stream of the 5’Cap could be a useful strategy to block Pim kinase action. Finally, we show that Pim kinases block AKT/PI3K inhibitor action by regulating Nrf-2 activity, potentially modifying its translocation to the nucleus. This finding suggests that other agents that target this pathway could overcome Pim inhibitory effects.

What was the impact on other disciplines?
The discovery of two new and novel substrates will make it considerably easier to begin to develop more highly active Pim inhibitors. The previous in vivo substrate for Pim was the BAD protein also discovered by our laboratory. However, this protein is rare in most cell types and to use it as a biomarker over expression is really needed. In contrast both IRS-1 and eIF-4B are found in reasonable levels in many cell types and will be better biomarkers for cell screens and in vivo in patients.

What was the impact on technology transfer?
None

What was the impact on society beyond science and technology?
An extension of our research will be clinical trials in humans. These trials are poised to make large differences for patients. This data will enhance the potential to combine Pim and AKT inhibitors and make a difference for patients in
the future. The ability of Pim to regulate HIF-2α will further research to examine the role of this enzyme in vessel growth and thus potentially in regulating other non-cancer related fields.

5. Changes/Problems
Changes in approach and reasons for change
None

Actual or anticipated problems or delays and actions or plans to resolve them
During this project period the PI moved from the Medical University of South Carolina to the University of Arizona Health Sciences Center. The transfer of funds to the new University took significant time and delayed the research slightly as the new laboratory was put together.

Changes that have a significant impact on expenditures
None

Significant changes in use or care of human subjects and vertebrate animals
None

6. Products
Publications, conference papers, and presentations

Journal Publications

Abstracts
3. Song JH, Kraft AS. Insulin Receptor Substrate (IRS) 1 is a Key Substrate for Pim Protein Kinases. AACR Annual Meeting, New Orleans, LA, April 16-20, 2016.
7. Participants and Other Collaborating Organizations
Individuals who have worked at least one person month per year during reporting period

Andrew Kraft, MD  Principal Investigator  No Change
Jin Song, PhD  Co-Investigator  No Change
Jin Zhou, PhD  Biostatistician  No Change
Libia Llувano  Research Specialist  No Change

Has there been a change in other active support?
There is no overlap in any of these projects.

IRG-16-124-37-IRG (Kraft, PI)  01/01/16 – 12/31/18
American Cancer Society
American Cancer Society Institutional Research Grant
The purpose of this award is to provide funds to institutions as block grant to provide seed money for newly
independent investigators to initiate research projects

5R01 CA173200 (Kraft, PI)  09/17/13 – 07/31/18
NIH/NCI
Targeting the Pim 1 Protein Kinase to Overcome Resistance to AKT Inhibitors
The goal of this project is to focus attention feedback resistance mechanisms between different kinase inhibitors.

W81XWH-15-1-0512 (Kraft, PI)  09/15/15 – 09/14/18
DOD
Regulating Cancer-Associated Fibroblast Biology in Prostate Cancer
These studies will accelerate and significantly advance the rational development of targeted agents that inhibit the
activity of cancer associated fibroblast cell function for the treatment of prostate cancer, and by measuring the scope of
proteins secreted by these cells using novel technology, develop potential urine biomarkers of treatment success.

1R21 CA178324-01A1 (Kraft, PI)  06/01/15 – 05/30/17
NIH/NCI
PIM 1 Protein Kinase in Regulating Stromal Cell Biology in Prostate Cancer
These studies will markedly increase the understanding of the role of Pim1 in prostate cancer fibroblast activity and
develop the rationale for using Pim inhibitors as a potential method of interrupting stromal epithelial interaction inhibiting
prostate tumor growth.

What other organizations have been involved?
Because of the move of the PI, this work was done at both the Medical University of South Carolina and the University of
Arizona.

8. Special Reporting Requirements
None

9. Appendices
23585456, PMCID: PMC3680595
Mims A, LaRue A, Kraft AS. The Pim-1 Protein Kinase Is an Important Regulator of MET Receptor Tyrosine
PMCID: PMC4054323
Elevates the Cellular Levels of Reactive Oxygen Species and Sensitizes to K-Ras-Induced Cell Killing. Oncogene
2015 July; 34(28):3728-36, PMID 25241892
doi: 10.1016/j.phaarnthera.2015.03.001. Epub 2015 Mar 5. Review. PMID: 25749412


Please see this response.

Thanks.

Andrew

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On 8/29/17, 4:38 AM, "Clevenger, Wendy L CIV USARMY MEDCOM USAMRMC (US)" <wendy.l.clevenger.civ@mail.mil> wrote:

Classification: UNCLASSIFIED
Caveats: NONE

Review of the report has been completed. The report is UNACCEPTABLE as written. We have included the reviewer's comments in the body of this email and ask that you revise the report to incorporate the reviewer's recommendations.

Reviewer's Comments:
This report was DISAPPROVED as of 07/13/2017

Not Scientifically acceptable in accordance to the contract Statement of Work. Return report to Principal Investigator for rewrite with an UNLIMITED distribution (Approved for Public Release; Distribution Unlimited)

Comments to PI - Thank you for submitting the final report. The final report is not accepted because the final report is not cumulative. This final report is not comprehensive of all tasks and the entire POP as is the requirement for final reports. In addition the report is not clearly and rigorously aligned with the work as outlined in the approved statement of work. Please submit a revised final report that covers progress over the entire award period toward the approved SOW and not just progress that was made since the second annual report.
Sincerely,

Ramachandran Arudchandran
Science Officer

Use this URL: https://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting to obtain a copy of the format requirements and to view a sample cover, Standard Form 298 and table of contents, on the U.S. Army Medical Research and Material Command web site.

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***The revised report is due in this office not later than: 09/27/2017 ***

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The true soldier fights not because he hates what is in front of him, but because he loves what is behind him. G. K. Chesterton

Classification: UNCLASSIFIED
Caveats: NONE