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TITLE: Pim Protein Kinase-Levels Correlate with Prostate Tumor Growth and Chemo Resistance-Potential Mechanisms

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### 14. ABSTRACT

Pim is a protein kinase that has been implicated to play a role in prostate cancer. Kaplan-Meier analysis demonstrated that those patients with high Pim-1 expression are at a significantly greater risk for developing metastatic cancer. In transgenic animal models, the levels of Pim-1 protein kinase are elevated in prostate tumors caused by over expression of the c-myc ongene, a gene overexpressed in human prostate cancer. In this proposal, we will examine whether Pim mimics Akt and TOR or modulates additional biochemical pathways and use knockout mice to dissect how myc and Pim collaborate to induce transformation and growth or prostate cancers. Pim inhibitors alone or in combination with TOR inhibitors will make a powerful chemotherapeutic strategy.

### 15. SUBJECT TERMS

Pim protein kinase, TOR, rapamycin, c-Myc, PTEN

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Introduction

The purpose of this research is to (1) determine using human prostate cancer samples whether Pim is over expressed in tumors with normal PTEN levels, (2) decipher the biochemical mechanism of action by which Pim regulates growth of prostate cancer cells, and (3) to use transgenic mice to understand whether Pim over expression is necessary for c-Myc induced growth of prostate cancer cells. The scope of this research involves studying normal prostate epithelial cells in tissue culture, malignant human prostate cancer cell lines, and nude mice with subcutaneous tumors, and transgeneic mice specifically expressing these genes in mice. The research sponsored by this proposal has led to the publication of an article in Molecular Cancer Research, and published in Oncogene both dealing with the mechanism of action of the Pim-1 protein kinase.

Body

Year 1

Figure 1

Task 1 – Complete multiplex PCR on human prostate samples and examine levels of Pim1, 2, c-Myc and Pten.

We have begun this analysis by first examining the levels of c-Myc in human prostate cancer cell lines that overexpress Pim. First, on the protein level, using western blots, we find that the level of c-Myc is elevated in cell lines that overexpress Pim (Figure 1).

This would appear to be secondary to decreased degradation. In contrast, when we examined the mRNA of c-Myc in these identical cell lines, the level of c-Myc was not markedly elevated (Figure 1). This experiment was done by QT-PCR. Slight elevations were seen in Pim-1 mRNA suggesting the possibility that Pim-1 and Pim-2 have different functions. These results are important because they tie c-Myc levels to the expression of Pim.

Task 2- Evaluate the ability of Pim to phosphorylate GSK3beta, Foxo transcription factors, and TSC both in cells and as GST fusion proteins.
The goal of this aim was to elucidate whether Pim would phosphorylate and modulate AKT substrates, including GSK3 beta, Foxo, and TSC-2. Although these are important experiments and still will be accomplished, we chose instead to look at the Akt substrate PRAS40. The reason we chose to look at this substrate is that it is involved in insulin signaling, but it is also part of the TORC1 complex. We have previously demonstrated that Pim-1 can stimulate the phosphorylation of 4E-BP-1 that is a TORC1 substrate. PRAS40 inhibits TORC1 activity but when phosphorylated by AKT it is released and that allows TORC1 to be activated. We hypothesized that Pim-1 would mimic Akt and thus activate TORC1 by phosphorylating PRAS40. Using an antibody that recognizes the phosphorylated site on PRAS40 modified by Akt, we find that Pim can phosphorylate this site \textit{in vitro} (Figure 2).

\textbf{Figure 2}

\textbf{Purified Protein} \hspace{1cm} \textbf{In vitro kinase assay}

\textbf{Figure 2} - Pim protein kinase phosphorylates PRAS40. PRAS40 was expressed in bacteria and purified to homogeneity on glutathione agarose. Two bands are shown with the lower being a degradation product. As controls wild type (WT) and mutant (4M) proteins are run. These GST fusions have a molecular weight of 40 kDa and demonstrate that the purified protein is not GST. The purified GST-PRAS 40 was then incubated with purified Pim-1 and ATP- gamma P32. The purified protein was run on SDS:PAGE and submitted to autoradiography. The phosphorylated PRAS-40 is shown in the right lane.

Secondly, we find that cells that express Pim when starved of growth factors continue to contain highly phosphorylated PRAS40. These data suggest that one mechanism by which Pim may be regulating 4E-BP1 phosphorylation is by phosphorylating PRAS40 releasing it from the TOR complex and thus allowing TOR to remain active even when AKT activity is decreased.

Task 3- Carry out animal experiments to evaluate the ability of chemotherapy to kill prostate tumors expressing Pim.
   a) Establish DU-145 cells that express high levels of Pim1, Pim2, and Pim2 K/A
b) Examine the ability of doxorubicin to kill tumors established from wild type PC-3, LNCaP, and DU-145 tumors or those that express Pim.

We attempted to accomplish this aim with DU-145 cells, but were more successful in creating PC-3 cell lines that expressed Pim1, Pim2 and Pim 2 K/A. These cell lines were placed subcutaneously and grew tumors (see Figure 3).

As can be seen in the Figure, prostate cancer cells that overexpress Pim grew much faster as tumors. Interestingly, those PC-3 cells that expressed kinase-dead Pim had a lower take rate and grew poorly in the animals and formed tumors at a much lower rate. This data strongly suggested that Pim could regulate tumor growth in animals.

As prescribed by this task, we next tested the ability of these tumors to be killed by doxorubicin. Although this chemotherapeutic agent was able to decrease the growth of wild type PC-3 tumors in animals (p<0.009), PC-3 Pim-1 or 2 tumors continued to grow rapidly even when treated with chemotherapy (figure 4).
Figure 4 - Lack of inhibition of tumor growth by doxorubicin in Pim containing tumors. PC-3/neo, PC3/Pim1, PC3/Pim2 and PC3/Pim2 K/A cells (2 x 10^6 per xenograft) were injected in the flanks of BALB/c nu/nu mice with mice mice in each group with four tumors injected in each mouse. The tumor volume was calculated from caliper measurements in two dimensions at the indicated time points. Points are the mean of 20 measurements, bars are SD. Mice treated with Doxorubicin (Dx) received drug 40mg/Kg weight once per week for a total of six doses.

Although the tumor growth was inhibited by doxorubicin this chemotherapeutic compound did not inhibit the growth of PC-3- Pim tumors slightly it was not able to kill the tumors. These data suggest that Pim cannot only stimulate tumors to grow more rapidly but also makes tumors more resistant to chemotherapy induced cell death.

Task 4- Develop transgenic mice that overexpress Pim2 or Pim 2 K/A in the prostate.

We spent significant effort attempting to create transgenic mice that overexpress Pim or mutant Pim in the prostate. However, we were not successful. To accomplish this task we created a vector that contained 2 copies of the probasin promoter, which has mutiple androgen responsive elements. These
elements will stimulate the production of Pim in male mice that have reached puberty. The probasin promoter is only active in the prostate. However, although the transgenic mice were constructed we did not see staining for Pim protein in the prostate. RT-PCR did demonstrate small amounts of mRNA present, but Pim protein could not be found by histology.

In contrast to our efforts, one of our collaborators, Dr. Michael Lilly, a coauthor on both of the DOD publications, was able to construct transgenic Pim-1 mice with expression in the prostate. He has found that while these mice do not develop specific tumors, they do form prostatic intraepithelial neoplasia. These mice will be available to our laboratory through collaboration.

**Year 2**

Task 1- Using tissue microarrays examine the levels of Pim1, Pim2, myc, and Pten protein. Compare these results to those obtained by multiplex PCR.

We attempted to carry out this aim using antibodies to Pim1 and Pim2 that we had published previously. However, because the levels of Pim were too low to measure on human microarrays with this reagent we placed this aim on hold.

Instead, we followed up on an observation that led us to an understanding of how Pim levels are regulated by a specific enzyme that is essential for the Myc pathway. The goal of this task was to determine how Pim-1 levels are regulated in prostate cancer tissues. In the first year of this proposal we have seen that Pim appears to interact with PP2A. This is an important interaction because it suggests that an enzyme, PP2A, that both controls the c-Myc and TOR pathways may also be regulating Pim protein kinase.

**Figure 5**

Pim-1 protein levels are negatively regulated by PP2Ac.

(a) Increased cellular PP2A activity decreases Pim-1 and Pim-2 protein levels. HEK-293T cells were co-transfected with 0.5 µg pcDNA3/ Pim-1 or Pim-2, Runx1, and increasing amounts of pD30-PP2A-HA-C, 0.5 µg, 1 µg to 2 µg, as indicated. Control empty pcDNA3 vector was added to ensure that the total amount of plasmid DNA per transfection was identical. Whole-cell lysates were collected at 36 h post-transfection. Western blotting was carried out using anti-HA (for exogenous PP2Ac), anti-PP2A (for total PP2A), anti-Pim-1, anti-Pim2, and anti-His for the detection of the Runx1 control, and anti-GAPDH another control.

To evaluate the effect of PP2A on the levels of Pim we transfected 293T cells and prostate cells (data not shown) with Pim proteins and also with HA-PP2Ac. This result can be seen in Figure 1 where increases in PP2A led to decreases in Pim-1 protein levels. This decrease in Pim-1 protein levels is associated with a decrease in the ability of Pim-1 to phosphorylate its substrates (Figure 2). Thus, increases in PP2A activity enhance both the degradation of Pim-1 and the c-Myc protein.
Figure 6 PP2A dephosphorylates Pim-1 *in vitro*, and decreases Pim-1 kinase activity. *In vitro* dephosphorylation assay. HEK 293T cells were transfected with 2 µg pcDNA3/Flag-Pim-1. After 36 h transfection, the Flag-Pim-1 proteins were labeled by incubating the cells in media containing $[^{32}P]$ orthophosphate for 4 h followed by the immunoprecipitation of Flag-Pim-1 proteins. The $[^{32}P]$ orthophosphate labeled-Flag-tagged immunoprecipitates were treated with or without recombinant PP2A (A/C dimer) in an *in vitro* phosphatase reactions. 40% of the reaction products were analyzed by autoradiography (upper row), 10% were used in a Western blotting for measuring the amount of Pim-1 in each group by using anti-Pim-1 antibody (bottom row), and 50% of immunoprecipitate was washed and then subjected to a kinase assay with histone H1 as substrate (middle row).

The PP2A protein is composed of an A, B, and C subunit. The A subunit works as a scaffold, while the B subunit targets this phosphatase to specific substrates, and the C subunit is the catalytic moiety. To discover how Pim is regulated by PP2A we have incubated Pim with varied PP2A beta subunits. We find that Pim-1 binds tightly to the B56beta subunit as shown in this coimmunoprecipitation experiment illustrated below in Figure 7.

Figure 7

Figure 7 Pim-1 specifically associates with B56 *in vivo*. (a) Pim-1 coimmunoprecipitates with the B56β subunit. Pim-1 was cotransfected with 0.5 µg HA tagged B56α, β, γ, δ, ε subunits into 293T cells. Whole-cell lysates were collected at 48 h posttransfection and immunoprecipitated with anti-HA beads. 5% of the lysates were saved for input detection and 50% of the immunoprecipitation samples were analyzed by Western blotting with anti-HA to recognize the B56 subunits, and anti-Flag to measure Pim-1.

To examine what portion of the Pim-1 molecule bind to PP2A we generated cut backs in Pim-1 protein cDNA and expressed these in cells along with PP2A. We find by this technique that the sequence between 140 and 177 is essential for the interaction of these two proteins. This result is illustrated in Figure 4. These studies demonstrate the close physical interaction between PP2A and Pim-1.

Figure 8

Figure 8 Identification of Pim-1 domain responsible for banding to B56β. (a) Structural domains of Pim-1 and Pim-1 deletion mutants use in these experiments are represented as black bars. (b) B56β binds the hinge region of Pim-1. HEK-293T cells were cotransfected with the HA-B56β (full length) along with the empty cDNA (mock) or the indicated Flag-tagged Pim-1 deletion mutants. The Flag-Pim-1 proteins were immunoprecipitated with anti-Flag beads and immunoblotted with anti-HA antibody to detect B56β or anti-Flag to measure Pim-1.
First, we demonstrated that Pim-1 is ubiquinated so that the degradation of Pim-1 is clearly mediated by the proteasome. The levels of ubiquination of Pim-1 is mediated by the presence of absence of PP2A B56 beta protein. Knockdown of this subunit using RNAi clearly demonstrates that the ubiquination of Pim-1 decreases (Figure 9).

Figure 9

Secondly, we find that Pim-1 binds to the prolyl isomerase Pin-1. The binding of Pin-1 to Pim-1 appears to decrease the level of Pim-1 in the cells. The results of experiments demonstrating these points are seen in Figure 10.

Figure 10

Based on these results we have derived a model of how the level of Pim-1 protein may be controlled in prostate tumor cells. Lesions in any of these control mechanisms could elevate the levels of Pim-1 protein. This mechanism suggests that Pin1 binds to Pim-1 isomerizing the protein. This leads to...
PP2A binding, dephosphorylation of Pim-1, and the ubiquination of the Pim-1 protein. Degradation of Pim-1 then follows (see Figure 11 below). Clearly, a decrease in the activity of PP2A will lead to increases in Pim-1. These increases would occur in parallel with those in c-Myc.

Figure 11

Hypothetical model of Pim-1 degradation by the proteasome mediated by Pin1 and PP2A.

Task 2- Examine using cell culture models of prostate cancer the exact sites of phosphorylation induced by Pim on the 4E-BP1 mutants.

We have spent considerable effort mapping the Pim regulated 4E-BP1 phosphorylation site. By combining purified Pim-1 enzyme and 4E-BP1 protein we have demonstrated that Pim will not phosphorylate either 37, 46, 65 or 70 phosphorylation sites on this protein. These are major sites of phosphorylation by TOR protein kinase, although other protein kinases including Akt and Erk have also been implicated.

To attempt to identify the phosphorylation site induced by Pim we have cut the 4E-BP1 band and sent it for mass spectrometric analysis. This was done twice and yielded S44 and S45 on sequential analysis. To follow up this task further we made mutants in the S44 site and attempted to determine whether this would prevent phosphorylation or Pim dependent phosphorylation. We transfected 4E-BP1 with an S44 mutation into 293T cells and PC-3 cells. This mutant protein still appears to be phosphorylated in vitro, suggesting that S45 may be the site. Further experiments will be necessary to decipher whether this is indeed the Pim phosphorylation site.

Task 3 – Carry out further animal studies of the effect of chemotherapy on Pim containing tumors examining additional agents that are active in prostate cancer including estramustine and docetaxol.

To attempt to further develop an approach to the therapy of Pim containing tumors, we decided to use the effort involved in this task to develop novel and specific Pim inhibitors. We felt that in human tumors that overexpress Pim that the use of docetaxol or estramustine would not be curative and so felt
a new approach to inhibiting Pim was necessary. We therefore decided to develop our own unique inhibitor of the Pim protein kinase.

Using the S6 peptide as a substrate in an in vitro reaction containing Pim1 protein, we screened a 26,000 compounds. This procedure led to the designation of two D5 and 801 as compounds that inhibited Pim-1 in vitro in low nanomolar amounts. Unfortunately, these compounds do not inhibit Pim-2. We find that incubation of PC-3 human prostate cancer cells with each of these compounds inhibits their growth 50% as demonstrated by an MTS assay. Flow sorting suggests that there are increased numbers of cells in G1 but no obvious apoptosis. This may mean that Pim plays an essential role in regulating the cell cycle.

Although not as yet completed in prostate cancer cells, in leukemic cells MV4;11 cells in serum free media, both 801 and D5 compounds will inhibit the phosphorylation of 4E-BP1 on S37, T46. In that these are TOR sites and clearly not phosphorylated by Pim, this result suggests that these compounds somehow are inhibiting the TOR pathway. Additional experiments demonstrate that these compounds will inhibit the phosphorylation of the BH3 protein BAD, a well known substrate of Pim. Addition of these agents to FDCP-1 cells expressing Pim also markedly decreases the phosphorylation of PRAS40, a known Akt substrate that contains a consensus sequence that would allow it to be a Pim substrate.

The compounds 801 and D5 have the potential to teach us a great deal about how Pim functions and whether the development of a clinically useful compound might make a significant impact on prostate cancer treatment.

Task 4- Begin mating experiments with transgenic Pim and Myc mice and determine the incidence of prostate cancer.

Our collaborator Dr. Michael Lilly has mated the mice expressing Pim in the prostate with mice that express Myc or Akt in the prostate. Normally the myc containing mice will develop tumors in a year, while the Akt containing mice just develop prostate intraepithelial neoplasia. He finds that in both matings lead to the rapid development of prostate tumors. Further matings will be necessary to determine the mechanism by which Pim enhances the growth of these tumors.

Year 3

Task 1- Carry out animal experiments examining the ability of CCI-779 alone or in combination with chemotherapy to inhibit tumor growth in PC-3, LNCaP, and Du145 cells.

To complete this task, we focused our efforts on whether CCI-779 (rapamycin) alone or in combination with D5 would inhibit the growth of tumor cells. Using the leukemic cell line MV4;11 we demonstrate that the combination of rapamycin and D5 gave a synergistic inhibition of cell growth. Rapamycin alone at the dose of 50 nM gave only a 50% inhibition of cell growth. Thus, inhibition of Pim and TOR together give a synergistic and almost complete inhibition of cell growth.
Figure 12

![Graph showing cell survival](image)

**Figure 12.** Effect of Pim inhibitors (D5, 628, 801, 802) on MV4;11 cells (human leukemic cell line containing the FLT3/ITD mutation). Cells were treated with the Pim inhibitors (5 µM) alone (black bars) or in combination (grey bars) with the mTOR inhibitor rapamycin (5 nM) and cell survival measured 72 hours later (shown as a percentage normalized to survival of cells treated with 0.2% DMSO).

Task 2 - Analyze Pim/Myc crossed mice for tumor spread and incidence of metastasis.

We do not have any data on whether the tumor will spread and metastasize. The tumors that are created from these matings are very large and the possibility of spread is present but no data has been obtained.

Task 3 - Mate Myc transgenic mice with Pim 1/Pim 2 KO mice.

We have carried out mating of Pim 1/Pim2 KO mice supplied by Dr. Craig Thompson, University of Pennsylvania, with mice that are over express Myc in the prostate supplied by Dr. Charles Sawyers, now of Memorial Sloan Kettering. Because the Pim2 gene is on the X chromosome we were able to obtain Myc transgenic mice that were Pim2 KO from mating female KO mice to Myc over expressor males. The male offspring of these matings were Pim2 KO and 50% of these were found to be Myc overexpressors. The Pim-1 KO in the Myc overexpressor backgroung was harder to obtain but with successive matings these were obtained.

Task 4 - Examine Myc/Pim KO/Pim 2 KO mice for the rate of development and the incidence of tumor formation.

We allowed Myc overexpressor Pim1/Pim2 KO mice to age. At 3, 6, 9, and 12 months the prostate was dissected and subjected to pathologic examination. Slides from blocks made of these prostates were sent to Baylor Medical Center and analyzed in a blinded fashion. The analyzed results demonstrate that the Myc gene induces PIN and tumorigenesis even in the background of Pim1 and Pim2 KO mice.

Unfortunately, this negative result was not possible to publish but does suggest the very interesting conundrum that Pim is not necessary for tumorigenesis induced by overexpression of Myc in the prostate. However, Pim definitely enhance the transformation of the prostate gland in the presence of overexpression of Myc and Akt.

The effects of Pim occur even when the prostate will not become tumors, as in the case of Akt.
**Research Accomplishments**

- Construction of prostate cancer cell lines that express Pim proteins
- Demonstration that the expression of Pim makes tumors growth much faster while dominant negative Pim inhibits tumor take.
- The growth of Pim containing cells is faster in culture with no decrease in apoptosis.
- Pim stimulates increases in the phosphorylation of 4E-BP-1 and S6 kinase
- Pim increases the level of c-Myc p62S phosphorylation without increasing p58 phosphorylation
- We did not see any major increase in c-Myc on QT-PCR, suggesting that the increase in levels of c-Myc arise from stabilization of proteins.
- Increased PP2A activity negatively regulates Pim-1 protein levels
- PP2A dephosphorylates Pim-1 and decreases Pim-1 kinase activity
- The PP2A B56 beta subunit associates with Pim 1 in cells
- Knockdown of B56 beta increases Pim-1 protein expression
- B56beta affects the half-life and ubiquitinylation of Pim-1
- The Pin1 propyl isomerase associates with Pim-1 in vivo and facilitates Pim-1 degradation.
- Knockdown of B56 beta increases cell viability in Pim containing but not negative cell lines
- A new model can be constructed to understand control of Pim levels.
- Screen a 26,000 compound library for inhibitors of Pim and discover novel Pim protein kinase inhibitors.
- Demonstrate that these inhibitors block the growth of PC-3 human prostate cancer by 50% over 72 hours in serum containing medium.
**Reportable Outcomes**


Conclusions

The results from year 1 of this proposal clearly demonstrated that Pim-1 makes prostate cancer grow faster and makes the tumors resistant to chemotherapy. The biochemical mechanism for this effect is suggested by the observation that the Pim protein kinase appeared to regulate the TOR pathway and inhibit the activity of PP2A. Inhibition of PP2A was felt to increase the activity of c-Myc.

In year 2 to understand in more depth how Pim-1 is functioning, we have focused on the regulation of Pim-1 by the PP2A phosphatase. We find that PP2A activity decreases both the level and activity of the Pim protein kinases. Okadaic acid and the SV40 small T antigen that are known modulators of PP2A inhibit this effect. Our results demonstrate that Pim-1 binds to the beta subunit of PP2A. Based on the observation that the Pin-1 prolyl-isomerase binds Pim-1 and decreases the level of this protein we have arrived at the following hypothesis. We believe that Pim-1 is phosphorylated, allowing the binding of Pin-1. Isomerization of the molecule then allows the interaction with PP2A B subunit. Pim-1 is then dephosphorylated and this then allows the molecule to be ubiquinated and degraded. Since PP2A plays an essential role in regulating the TOR pathway, these observations connect growth factor signal transduction and Pim levels in prostate cancer.

In year 3 we have screened a 26,000 compound library to attempt to develop small molecule inhibitors of Pim that could be used clinically. We have developed two compounds, D5 and 801, that inhibit Pim-1 protein kinase in vitro at nM levels. In cell culture in the presence of serum these small molecules inhibit the phosphorylation of 4E-BP-1. This result correlates with our earlier findings in year 1 that Pim-1 increases the phosphorylation of this protein. We also find that these two small molecules will inhibit the phosphorylation of PRAS40, an Akt substrate. Additionally these molecules block the phosphorylation of the BH3 protein BAD. Inhibiting BAD phosphorylation by
Pim allows this protein to induce apoptosis. Finally, in culture both compounds inhibit the growth of prostate cancer cells by 50%. These small molecule Pim inhibitors have potential as chemotherapeutic agents either alone or in combination.