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

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14. ABSTRACT Small molecule targeted at specific signal transduction pathways hold great promise for creating a new approach to prostate cancer treatment. However, resistance to these small molecule agents occurs through multiple mechanisms and restricts their therapeutic activity. Developing potential targets that can overcome this resistance by novel mechanisms is essential. In this proposal, the applicant research team demonstrates that resistance to small molecule AKT protein kinase inhibitors is potentially mediated by the Pim-1 protein kinase, and that unique Pim protein kinase inhibitors that can in turn synergize with AKT inhibitors to block prostate cancer growth overcome this resistance. The knowledge gained through the studies proposed in this application is essential for the development of this combined chemotherapeutic strategy.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	6
Publications	7
Conclusion	7
Supporting Data (Figures 1-12)	8

DOD Technical Report

Introduction

Over 90% of prostate cancer patients have abnormalities in the AKT signaling pathway. These abnormalities include mutations in the PTEN and AKT proteins as well as deletions and inversions. Protein kinases are targeted in an attempt to cure cancer, and small molecules that regulate this pathway have entered the clinic. Inhibition of AKT signal transduction pathway is not sufficient to inhibit prostate cancer growth. Inhibiting the AKT pathway leads to increases in receptor tyrosine kinases that have the capacity to block the action of this kinase. This grant proposal will explore the resistance to small molecule AKT protein kinase inhibitors mediated by the Pim-1 protein kinase, and examine in detail whether unique Pim protein kinase inhibitors that synergize with AKT inhibitors block prostate cancer growth overcome this resistance. The knowledge gained through the studies proposed in this application is essential for the development of this combined chemotherapeutic strategy.

To understand how Pim inhibitors collaborates with AKT inhibitors, it is essential to understand how Pim regulates translation and through what phosphorylation sites. This laboratory has in the past demonstrated that both Pim and AKT can regulate the phosphorylation of PRAS40, a protein that regulates the mTORC1 pathway. Both Pim and AKT modulate the phosphorylation of TSC-2 that also controls TORC1 activity. TORC1 plays a critical role in regulating 4E-BP1 phosphorylation, and thus modulates the activity of eIF4E and the translation of proteins that have a 5'Cap mRNA. Both of these results suggest that Pim can control protein translation and thus influence the growth of prostate cancer cells.

This work has is detailed in the publication from this second year of the grant. Experiments have been carried out to attack the tasks outlined for Year 1. A strategic decision was made to focus next on Task 3 from Year 2 of this grant followed by Task 1 of year 3. Although the order of analysis varies from the statement of work, these experiments have occurred in logical sequence and 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 thus controls 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.* The focus of the research carried out in the second year has been to publish these important findings which we have done in *Molecular and Cellular Biology* 2014, 34(13):2517. The second goal of these experiments was to extend these findings and develop a fuller understanding of Pim function with AKT inhibitors.

Body

In the second year of this proposal we have focused on Year 2, tasks 3 and 4 and Year 3 task 1. 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. 1) Pim is overexpressed in tumor but not PIN or normal tissue.

Tumor growth causes limitations on blood supply and hypoxic tumor region. *We questioned whether Pim levels and activity are associated with tumor normoxic or hypoxic tumor.* We have placed PC3-LN4 human prostate cells in hypoxia overnight and evaluated the level of Pim isoforms. In figure 2, we show that hypoxia increases the level of Pim1 in prostate cancer cells. Although Pim 1 and 2 are elevated by hypoxia, the level of mRNA is not greatly changed. The induction of hypoxia in these prostate cells is demonstrated by the increase in HIF-1alpha. In conclusion, hypoxia increases the level of Pim kinases. These are the prostate cells where Pim will be able to enhance receptor tyrosine kinase levels when AKT is inhibited.

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. 3, 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. 3 (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. 3, the right panel using cell extracts from this experiment, we find that HIF1alpha 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. 4 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. 4 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. 4 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. As can be seen in Fig. 5, 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. As seen in Fig. 6, 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. 7 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. 7 (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. 8 right panel). *The Western blot demonstrates that the DMOG reverses the decreased amount of HIF (Fig. 8, 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 is shown diagrammatically in Fig. 9. In Fig. 10, we demonstrate that Pim inhibitor treatment decreases the growth of these tumors. This decrease in tumor growth is significant (Fig. 10, right panel). 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 (Fig. 11). The phosphorylation of two potential substrates of Pim, IRS1 and eIF4B are decreased in these tumor samples (Fig. 11).

This data suggests a model wherein hypoxia in prostate tumors inhibits the activity or level of prolyl 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 (see Figure 12, model).

Key Research Accomplishments:

Published in year 2 of this grant – in Molecular and Cellular Biology (2014, 34(13):2517)

- 1- Inhibition of Pim by RNA interference or pharmacologic inhibition blocks AKT inhibitor-induced up regulation of RTKs in prostate cancer cells.
- 2- Pim-1 induced increases in receptor tyrosine kinases are not cell line specific.
- 3- Combination of Pim and AKT inhibitors resulted in synergistic inhibition of prostate cancer growth.
- 4- Pim protein levels correlate with MET levels in prostate cancer

- 5- Pim controls the phosphorylation of eIF4B
- 6- eIF4B phosphorylation regulates the association of this protein with the eIF3 complex
- 7- Pim-directed eIF4B phosphorylation modulates the translation and activity of MET

Novel Additional Findings in Year Two

- Hypoxia increases the level of Pim protein kinases.
- Pim inhibitors regulate the level of HIF1 and 2 alpha both in prostate cells in culture and in vivo tumor models.
- Pim inhibitors decrease the half-life of HIFs
- DMOG experiments suggest that Pim regulates the PHD protein levels or activity.
- Decreases in the level of HIF induced by Pim inhibitors blocks the production of VEGF

Publications:

Cen B, Xiong Y, Song JH, Mahajan S, DuPont R, McEachern K, DeAngelo DJ, Cortes JE, Minden MD, Ebens A, Mims A, LaRue AC, Kraft AS. The Pim-1 Protein Kinase Is an Important Regulator of MET Receptor Tyrosine Kinase Levels and Signaling. *Molec Cell Biol* 2014, 34(13):2517.

Conclusion:

The PI3K/AKT pathway is hyperactivated in prostate cancer but its effective therapeutic targeting has proven difficult. In particular, the antitumor activity of AKT inhibitors is attenuated by upregulation of receptor tyrosine kinases (RTK) through an uncharacterized feedback mechanism. Based on the data collected and supported by this proposal, we have published that RNA interference-mediated silencing or pharmacologic inhibition of Pim-1 activity curtails AKT inhibitor-induced upregulation of RTKs in prostate cancer cells.

Pim, through regulation of eIF4B phosphorylation, is able to control the synthesis of this protein and thus modulate the sensitivity to the HGF/MET axis in prostate cancer.

New data presented in this update demonstrates that Pim protein levels are regulated in prostate cancer through the induction of hypoxia. Increases in Pim could occur through the Foxo transcription factors or other mechanisms. Inhibition of Pim appears to regulate PHD proteins and thus control HIF activity. Inhibitors of Pim block HIF activity and thus function to inhibit tumor growth.

These new data suggest that the portion of the tumor that will be the most sensitive to the combination of AKT and Pim inhibitors will be the hypoxic portions. This is critical because the hypoxic areas of tumors are the most difficult to treat with standard chemotherapy and radiation.

PIM1 is overexpressed in human prostate tumor samples

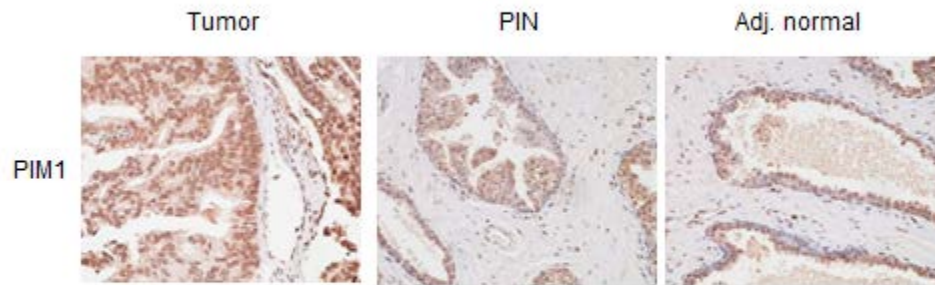


Fig. 1 – IHC staining shows that Pim levels are elevated in prostate cancer.

Hypoxia increases PIM isoform expression

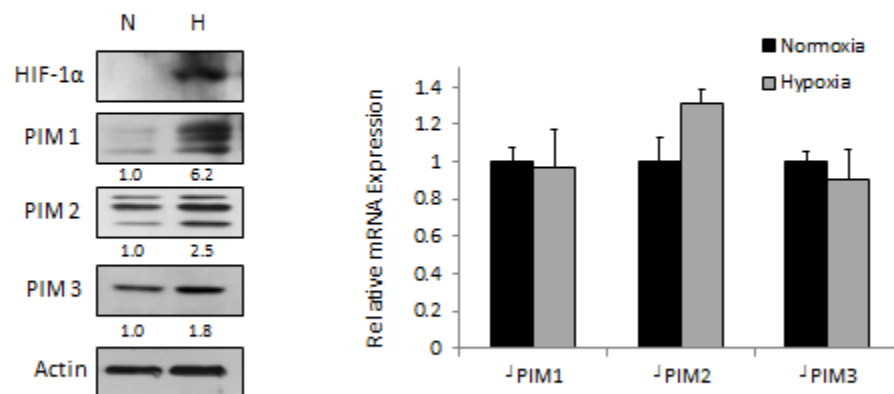


Fig. 2- Human prostate cancer cells PC3-LN4 were placed in hypoxia for 16h and extracts were subjected to Western blots. mRNA was extracted and qRT-PCR performed for the three Pim isoforms.

Hypoxia sensitizes prostate cancer cells to PIM inhibitors

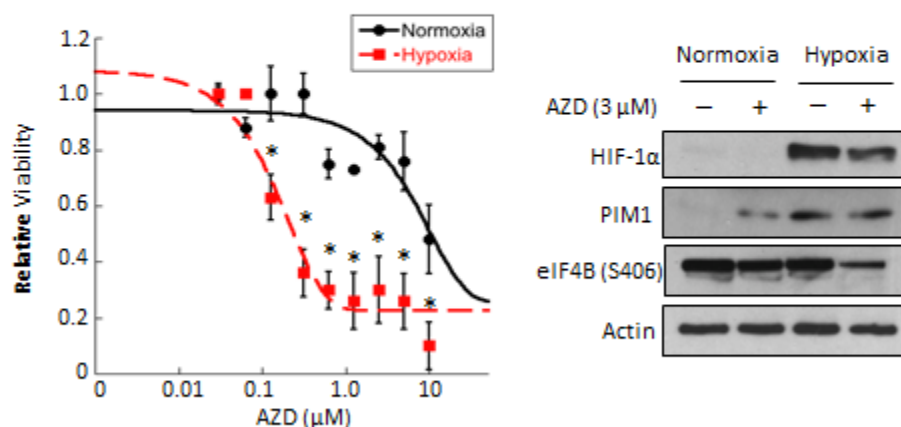


Fig. 3- PC3-LN4 cells were placed in normoxia or hypoxia and simultaneously treated with AZD1208 for 16h. Cell viability was estimated by the MTS assay. Extracts of cells treated with AZD for 8h were examined by Western blot.

PIM increases the protein levels of HIF-1α and HIF-2α

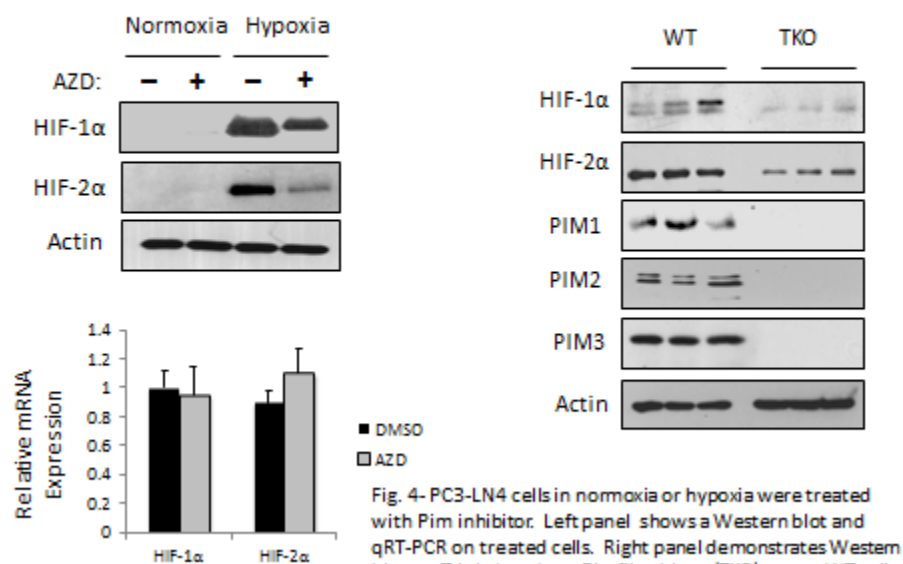


Fig. 4- PC3-LN4 cells in normoxia or hypoxia were treated with Pim inhibitor. Left panel shows a Western blot and qRT-PCR on treated cells. Right panel demonstrates Western blot on Triple knockout Pim fibroblasts (TKO) versus WT cells.

PIM inhibitors decreases HIF-1 activity

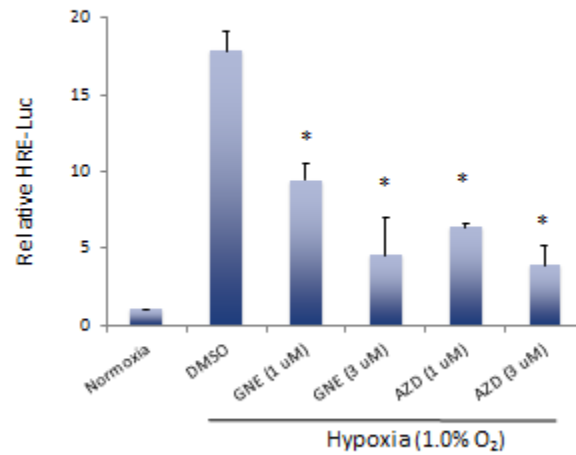


Fig. 5- PC3-LN4 cells were transfected with a vector having a HIF responsive promoter (HRE) with a luciferase reporter (LUC). These cells placed in hypoxia and were treated with DMSO or the Genentech (GNE) Pim inhibitor or AZD inhibitor in varying doses.

PIM inhibition blunts the expression of HIF-1 target genes

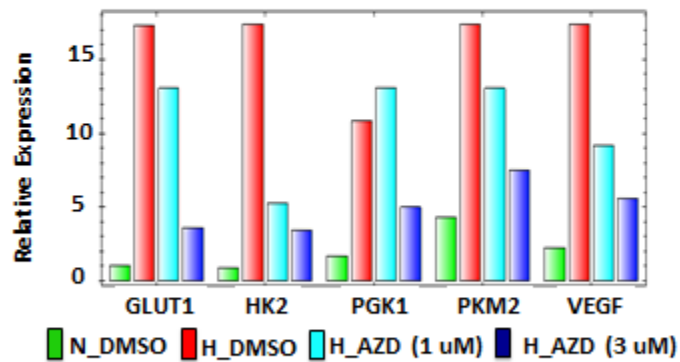


Fig. 6- PC3-LN4 cells were placed in normoxia or hypoxia overnight and treated with or without Pim inhibitor. QRT-PCR was performed for Glut1, hexokinase (HK2), phosphoglycerokinase (PGK1), PKM2, and vasculature endothelial growth factor (VEGF).

PIM inhibitors reduce HIF-1α and HIF-2α stability

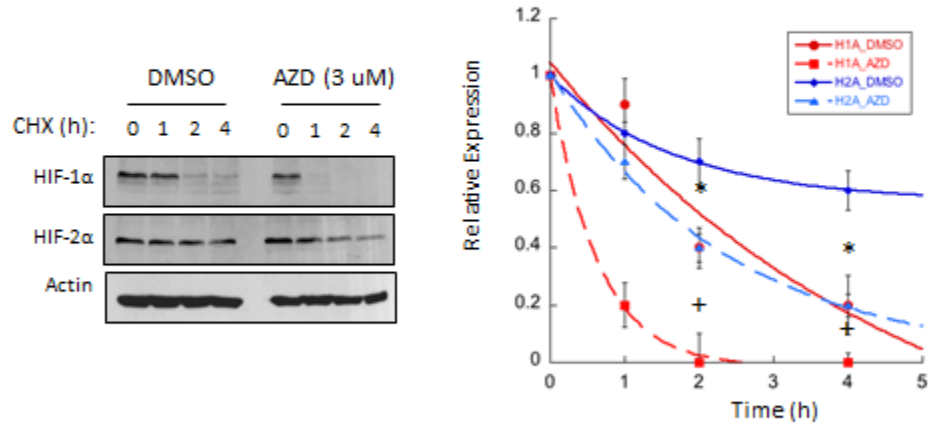


Fig. 7- PC3-LN4 cells were placed in hypoxia and cycloheximide was added. Western blots were done at the times indicated (hrs) for the identified proteins. These blots were scanned and the expression plotted relative to actin (right panel).

PDH activity is required for PIM inhibitors to reduce HIF-1α

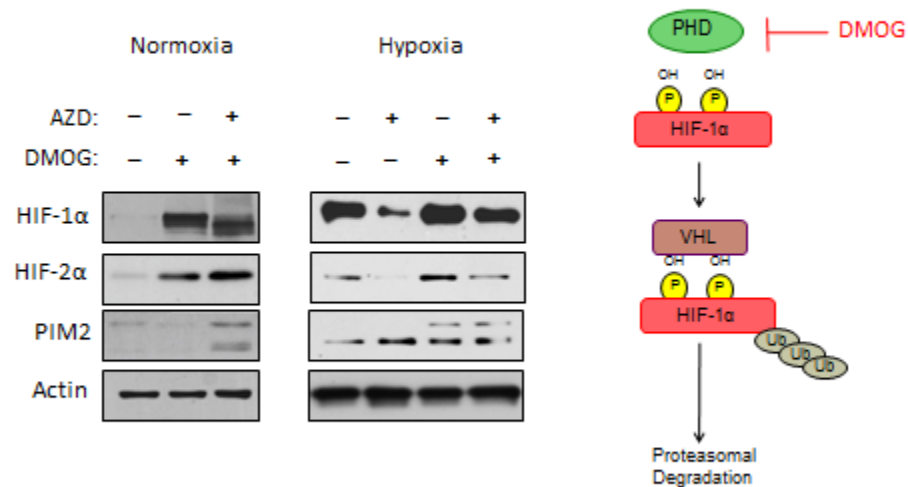


Fig. 8- PC3-LN4 cells were placed in normoxia or hypoxia and treated with Pim inhibitor AZD1208 with or without DMOG and propyl hydroxylase inhibitor. Western blots were carried out on these extracts. The HIF degradation pathway is shown on the right panel.

Does AZD1208 inhibit tumor growth and HIF signaling *In vivo*

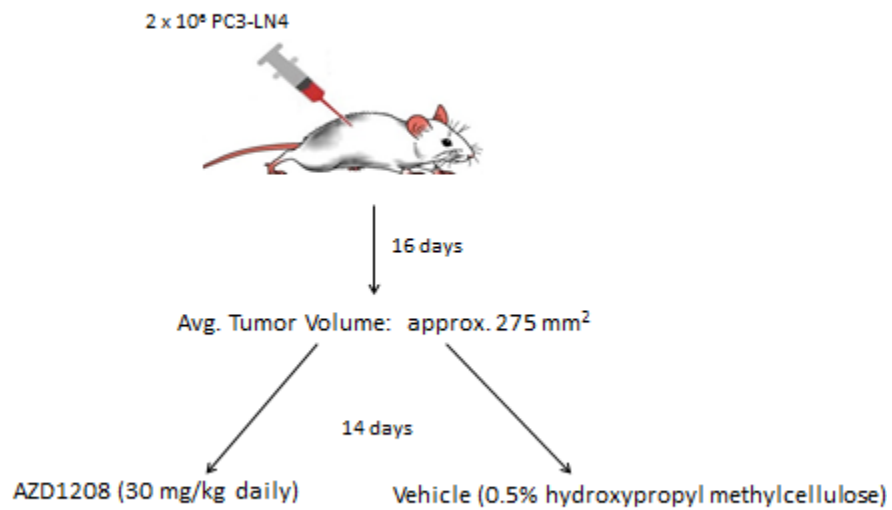


Fig. 9- Schema for animal experiment evaluating the activity of Pim inhibitors in hypoxia.

AZD1208 inhibits PC3-LN4 tumor growth *in vivo*

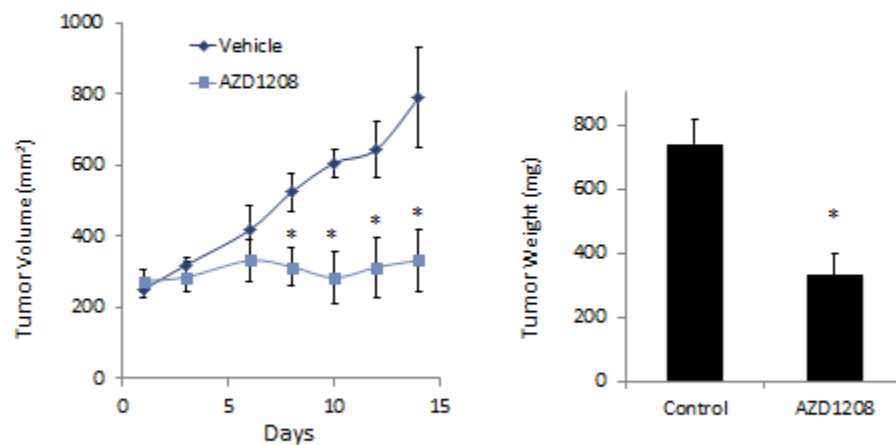


Fig. 10- Growth curve of PC3-LN4 cells treated with AZD 1208 (left panel). The weight of tumors at the time of necropsy on day 15 is shown. The standard deviation of measurements of 10 mice per group is shown. The star denotes significance in a student t-test.

AZD1208 inhibits PC3-LN4 tumor growth *in vivo*

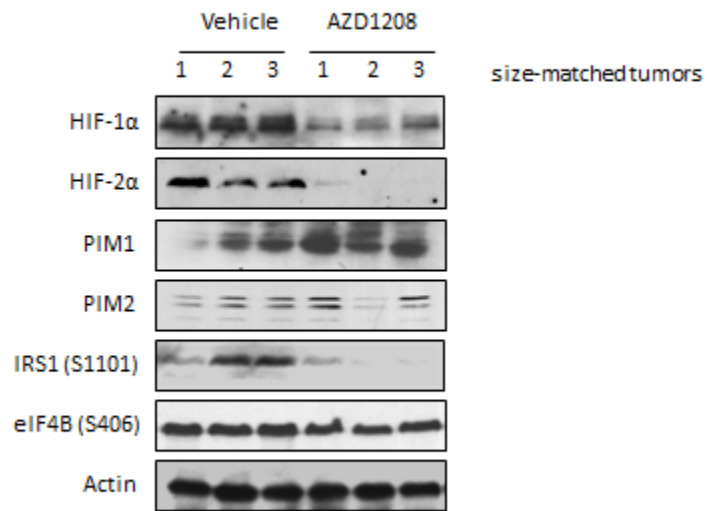


Fig. 11- Western blot of tumors from mice treated with vehicle or Pim inhibitor.

PIM stabilizes HIF-1α via inhibition of prolyl hydroxylases

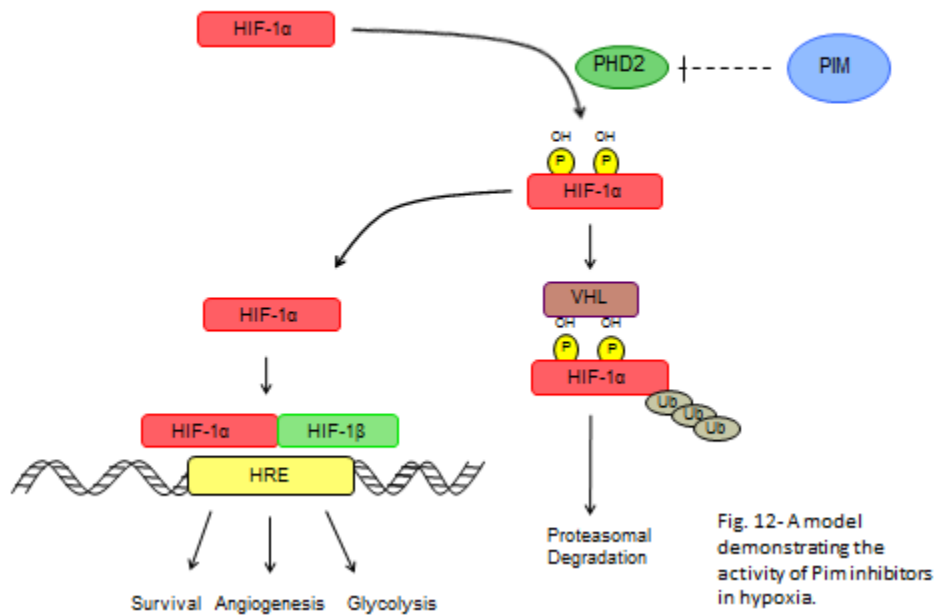


Fig. 12- A model demonstrating the activity of Pim inhibitors in hypoxia.