Published in final edited form as:

Mol Cancer Ther. 2016 July ; 15(7): 1637–1647. doi:10.1158/1535-7163.MCT-15-1018.

PIM kinase inhibitors kill hypoxic tumor cells by reducing Nrf2 signaling and increasing reactive oxygen species

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Abstract

Intratumoral hypoxia is a significant obstacle to the successful treatment of solid tumors, and it is highly correlated with metastasis, therapeutic resistance, and disease recurrence in cancer patients. As a result, there is an urgent need to develop effective therapies that target hypoxic cells within the tumor microenvironment. The Proviral Integration site for Moloney murine leukemia virus (PIM) kinases is a pro-survival pathway that is upregulated in response to hypoxia, in a HIF-1-independent manner. We demonstrate that pharmacological or genetic inhibition of PIM kinases is significantly more toxic toward cancer cells in hypoxia as compared to normoxia. Xenograft studies confirm that PIM kinase inhibitors impede tumor growth and selectively kill hypoxic tumor cells *in vivo*. Experiments show that PIM kinases enhance the ability of tumor cells to adapt to hypoxia-induced oxidative stress by increasing the nuclear localization and activity of Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), which functions to increase the expression of antioxidant genes. Small molecule PIM kinase inhibitors prevent Nrf2 from accumulating in the nucleus, reducing the transcription of cytoprotective genes and leading to the build up of intracellular reactive oxygen species (ROS) to toxic levels in hypoxic tumor cells. This toxic effect of PIM inhibitors can be successfully blocked by ROS scavengers, including N-acetyl cystine and superoxide dismutase. Thus, inhibition of PIM kinases has the potential to oppose hypoxia-mediate therapeutic resistance and induce cell death in the hypoxic tumor microenvironment.

Keywords

Hypoxia; PIM kinases; Reactive oxygen species; Nrf2; oxidative stress

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The authors disclose no conflicts of interest.

Introduction

A significant obstacle to the successful treatment of advanced cancer is intratumoral hypoxia. Multiple clinical studies in prostate cancer have reported an association between hypoxia and poor clinical outcome in patients with various types of cancer following chemotherapy or surgery (1, 2). A study of 247 prostate cancer patients with localized intermediate-risk disease showed that tumor hypoxia is associated with early biochemical relapse and local recurrence in the prostate gland (3). Similarly, the expression of hypoxia-induced proteins (HIF-1 α and VEGF) predict treatment failure, independent of tumor stage, Gleason score, and serum PSA levels (4). Therefore, elucidating the molecular and cellular mechanisms controlling tumor cell survival in hypoxia is critical to the development of effective therapeutic strategies to kill hypoxic tumor cells.

Cellular hypoxia is a state of reductive stress that is characterized by the elevation of reducing equivalents (i.e., NADH and FADH₂) that build up in the mitochondria when insufficient O₂ is available for reduction by the electron transport chain (ETC) (5). Hypoxic cells rely largely on anaerobic glycolysis to generate ATP, while the remaining low level of oxygen supports the production of ATP through the tricarboxylic acid cycle and ETC. Electrons leaking from the mitochondrial ETC generate an excess of reactive oxygen species (ROS) (i.e., oxidative stress) (6, 7). Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) is a basic leucine zipper redox-sensitive transcriptional factor that regulates the expression of cellular antioxidant and cytoprotective genes (8). During normoxia, Nrf2 is held in the cytoplasm and by a cytoskeletal-associated inhibitory protein, Kelch-like ECH-associated protein 1 (KEAP1). KEAP1 promotes the degradation of Nrf2 via ubiquitination and rapid proteasomal degradation (9). In response to oxidative stress, thiol oxidation occurs in the hinge region of KEAP1, resulting in a conformational change and the loss of Nrf2 binding (10). Nrf2 then accumulates and translocates into nucleus where it binds to DNA containing antioxidant responsive elements (ARE/EpRE), activating the transcription of critical antioxidant genes, including heme oxygenase-1 (*HMOX1*), NAD(P)H dehydrogenase, Quinone 1 (*NQO1*), and Peroxiredoxin 1 (*PRDX1*) (11, 12). Thus, the Nrf2 signaling pathway represents the primary protective response against oxidative stress.

The Proviral Integration site for Moloney murine leukemia virus (PIM) kinases are implicated in cell survival and metabolism, and their expression is enhanced and correlated with poor prognosis in several types of cancer (13). The PIM kinase family is composed of three different isoforms (PIM1, PIM2 and PIM3) that are highly conserved throughout evolution (13). In contrast to many pro-survival kinases, PIM isoforms do not possess regulatory domains, so their expression levels are directly correlated with activity (14). The overexpression of PIM kinases has been observed in hematopoietic malignancies and prostate cancer, and mutations in PIM kinases have been identified in lymphoma (15, 16). Given the potential role of PIM in regulating malignant transformation, a number of newly developed pan-PIM kinase inhibitors are currently being tested in clinical trials (17), and these compounds have shown some success in multiple myeloma and hematopoietic cancers (18). It was previously reported that PIM1 expression is increased in response to chemically induced hypoxia, and it promotes resistance to chemotherapy (19, 20). However, the biological role of PIM kinases in regulating the protective cellular response to hypoxia and

the utility of small molecule PIM inhibitors as agents to target hypoxic tumor cells has not been examined.

Here, we investigate the role of PIM kinases in modulating the cellular and physiological response to intratumoral hypoxia. Both tissue culture and animal models demonstrate that hypoxia increases PIM1/2 expression, and in turn, PIM increases the nuclear localization of Nrf2, resulting in increased expression of cytoprotective proteins that combat the production of hypoxia-induced ROS. As a result, small molecule PIM kinase inhibitors dramatically enhance ROS levels in hypoxic tumor cells, accounting for their selective toxicity toward this population. Our findings describe a mechanistic role for PIM in controlling the amplitude of Nrf2 signaling and provide a foundation for the pharmacological inhibition of PIM kinases as a novel strategy for treating hypoxic tumors.

Materials and Methods

Plasmids and siRNA

HA-HIF-1 α (21) and HA-HIF-1 α (P402/564A) (22) constructs were purchased from Addgene. ARE-Luc was kind gift from Dr. Donna Zhang (University of Arizona) (23). The sequence of each of PIM1/2/3 siRNA are as follows: Pim1 sense 5'-GAUAUGGUGUGUGUGGAUA-3', Pim1 antisense 5'-UAUCUCCACACACCAUAUC-3', Pim2 sense 5'-ACCUUCUUCUCCGACCCUCA-3', Pim2 antisense 5'-UGAGGGUCGGGAAGAAGGU-3', Pim3 sense 5'-GCACGUGGUGAAGGAGCGG-3', Pim3 antisense 5'-CCGCUCUUCACACGUGC-3', and the scrambled sequence of nonsilencing control siRNA oligonucleotides are sense 5'-UUCUCCGAACGUGUCACGU-3' and antisense 5'-ACGUGACACGUUCGGAGAA-3'. HIF-1 α and HIF-2 α siRNA were purchased from Santa Cruz Biotechnology.

Reagents and antibodies

AZD1208 (17) and LGB321 (24) were provided by AstraZeneca and Novartis, respectively. Cycloheximide, propidium iodide and MG-132 were purchased from Sigma. CM-H₂DCFDA was purchased from Invitrogen. Antibodies to HIF-1 α and HIF-2 α were purchased from BD Transduction Laboratories and Novus, respectively. PIM1/2/3, IRS1 (S1101), cleaved Caspase-3, and Actin antibodies were purchased from Cell Signaling Technology. An anti-HA monoclonal antibody was purchased from Covance. Nrf2 antibodies for immunofluorescence and western blotting were purchased from Santa Cruz Biotechnology. The anti-pimonidazole mAb (Hypoxypoint-1) was purchased from Hypoxypoint, Inc. All other materials and chemicals were reagent-grade.

Cell transfection and immunoblotting

PC4-LN4 prostate cancer and HCT-116 colon cancer cell lines were maintained in RPMI medium (Cellgro) containing 10% FBS (Hyclone). MB-MDA-231 and MB-MDA-231-ARE-Luc breast cancer cells were maintained in DMEM containing 10% FBS. All cell lines were maintained at 37°C in 5% CO₂. All cell lines were authenticated by short tandem repeat DNA profiling performed by the University of Arizona Genetics Core facility-Arizona Research Laboratories Division of Biotechnology at the University of Arizona.

(<http://uagc.arl.arizona.edu/>). The cell lines were used for less than 50 passages, and they were routinely tested for mycoplasma contamination. When indicated, cells were maintained in a hypoxic environment (1% O₂) using a hypoxia chamber (In vivo2 400, Ruskinn, UK). Transient transfection of DNA and siRNA was carried out using Lipofectamine 3000 transfection reagent (Invitrogen), according to the manufacturer's protocol. Immunoblotting was performed as described previously (25). For cellular fractionation, nuclear and cytoplasmic extracts were isolated as previously described (26). Densitometric analysis was performed with the NIH ImageJ analysis software.

qRT-PCR analysis

Messenger RNA was isolated from cell lysates using the RNeasy mini kit (Qiagen), and cDNA was synthesized from each sample using the RT² first strand synthesis kit (Qiagen). Changes in gene expression in response to hypoxia and PIM inhibition was measured as follows: qRT-PCR reactions were performed with equal amounts of starting material (500 ng RNA) using Biorad SsoAdvanced Universal SYBR green supermix, according to the manufacturer's protocol. Validated primer sets (QuantiTech primer assays; Qiagen) for each of the following genes were purchased for qRT-PCR analysis of gene expression: PIM1, PIM2, PIM3, HIF-1 α , HIF-2 α , ANGPTL4, VEGF, HMOX1, NQO1, and *PRDX1*.

Luciferase, ROS and viability assays

For luciferase assays, approximately 3×10^5 MD-MBA-231-ARE-Luc were plated in triplicate into black 96 well plates and allowed to adhere prior to incubation in hypoxia and/or drug treatment. Firefly Luciferase was measured at the indicated times/conditions using the Luciferase assay system (Promega), according to the manufacturer's protocol. For ROS assays, PC3-LN4 cells were plated in 6 well plates and cultured in normoxia or hypoxia $\pm$ AZD1208 for the indicated times. Then, the cells were washed with PBS and incubated with CM-H2DCFDA (1 μ M in PBS), a general oxidative stress indicator. After 10 min, the CM-H2DCFDA was removed and the cells were washed once with PBS. Then, the cells were immediately imaged using a fluorescence microscope (EVOS, Invitrogen) or harvested by trypsinization, centrifuged, and resuspended in PBS for FACS analysis (FACSCanto II; Beckon Dickinson). To measure viability, cells were stained with crystal violet or sub-G1 DNA content was measured by FACS analysis after staining with propidium iodide, according to previously described methods (27, 28).

Immunofluorescence

Twenty-four hours prior to the start of the assay, 2×10^6 PC3-LN4 cells were plated in 6 well plates containing microscope coverslips. Then, the cells were placed in normoxia or hypoxia in the presence or absence of 3 μ M AZD1208 or Sulforaphane for 24 h. Cells were fixed with 4% paraformaldehyde at room temperature for 10 min. Fixed cells were incubated for overnight with an Nrf2 antibody in PBS containing 5% normal goat serum. Coverslips were washed and incubated with AlexaFluor 488-conjugated anti-rabbit secondary antibody for 45 min, followed by DAPI to stain the nuclei. Coverslips were washed and mounted on glass slides, and images were obtained by fluorescence microscopy at 40x magnification. The images were captured and minimal alterations were performed on the digital images using NIS Elements software (Nikon).

In vivo studies

Male NOD/SCID mice (Charles River Labs) were housed and maintained in accordance with the Institutional Animal Care and Use Committee and state and federal guidelines for the humane treatment and care of laboratory animals. PC3-LN4 cells were injected subcutaneously into the rear flanks of mice, at a density of 2×10^6 cells per injection in PBS/Matrigel (v:v) in 200 μ L total volume. Once tumors reached a volume of approximately 250 mm³, the mice were separated into groups for treatment with vehicle (30% propylene glycol, 5% Tween-80, 65% of 5% dextrose in water, pH 4–5) or AZD1208 (30 mg/kg/day) by oral gavage once daily for 14 days. Tumor volumes were monitored over time by caliper measurement. At the end of the study, tumors were fixed, embedded in paraffin, and sectioned for staining with hematoxylin and eosin (H&E) or antibodies specific for Cleaved Caspase-3 and Hypoxyp probe-1. The percentage of CC3-positive cells was calculated using NIH ImageJ (4 fields were counted on 3 separate tumors from the Vehicle and AZD groups).

Statistical analysis

All western blots shown are representative of at least 3 independent experiments. Differences between independent groups were determined by the Student's t-test and linear regression analysis. Two-way analysis of variance (ANOVA) was used to analyze differences in survival between groups with 2 independent variables (i.e., normoxia vs. hypoxia). Three-way repeated measured ANOVA was used to analyze experiments with 3 independent variables (i.e., time, oxygen concentration, and drug treatment). The p-values were adjusted using Tukey adjustment. The data are presented as the mean $\pm$ S.E., and a p-value < 0.05 was considered to be statistically significant.

Results

Hypoxia sensitizes prostate cancer cells to small molecule PIM inhibitors

The PIM Ser/Thr protein kinases promote cell survival through pleiotropic mechanisms, and their role in hypoxic tumor cells has not been defined. It was previously reported that the expression of PIM1 is increased in hypoxia (14), but whether other PIM isoforms are also induced is unknown. The expression of all PIM isoforms was examined in prostate (PC3-LN4), colon (HCT116), and breast (MD-MBA-231) cancer cell lines. In response to hypoxia (6 h at 1.0% O₂), the protein levels of all PIM1 and PIM2, but not PIM3, were significantly increased across all cell lines examined (Fig. 1A, western blot). PIM mRNA levels remained relatively stable, suggesting that hypoxia regulates PIM at the protein level (Fig. 1A, bar graph). Next, the sensitivity of cancer cells to PIM kinase inhibitors was examined in hypoxia compared to normoxia. PC3-LN4 prostate cancer cells were treated for 72 h in normoxia or hypoxia with a dose range of AZD1208, a pan-PIM kinase inhibitor that has been used in preclinical (17, 29) and clinical trials (NCT01489722 and NCT01588548). Strikingly, the IC₅₀ of AZD1208 was nearly an order of magnitude lower in PC3-LN4 cells cultured in hypoxia compared to normoxia (IC₅₀ = 0.2 ± 0.1 vs. 16.8 ± 0.2 μ M, respectively; Fig. 1B). Similar results were obtained using HCT116 and MD-MBA-231 cells, demonstrating that the selective toxicity of PIM inhibitors toward hypoxic cancer cells is not specific to a particular cell line or type of cancer (Supplemental Fig. 1). Moreover, PARP cleavage, which is indicative of apoptosis, was present selectively in hypoxic cells after 48 h

of AZD1208 treatment (Fig. 1C). To demonstrate that this drug inhibited PIM activity in these cells, phosphorylation of IRS1 (S1101), a highly sensitive substrate PIM substrate was measured (30). Phosphorylation of this protein was inhibited in both normoxia and hypoxia following AZD1208 treatment (Fig 1C). Propidium iodide staining revealed that hypoxia alone did not significantly impact cell viability (approximately 11% apoptosis at 72 h). However, AZD1208 treatment caused a significant, time-dependent increase in the sub-G1 population, whereas this compound did not induce apoptotic cell death in normoxia. To confirm that the sensitivity of hypoxic cells to AZD1208 could be attributed to PIM inhibition and not off target effects of the drug, siRNA was used to knockdown each PIM isoform, and viability was measured after 48 h. In hypoxia, knockdown of PIM1 and PIM2 significantly reduced viability, whereas the loss of PIM isoforms was not toxic in normoxia (Fig. 1E, bar graph). In addition, we observed that knockdown of PIM1 and PIM2 reduced the protein levels of HIF-1 α and HIF-2 α (Fig. 1E, western blot). These data demonstrate that pharmacological or genetic inhibition of PIM1/2 selectively kills hypoxic cancer cells.

PIM inhibitors kill hypoxic cancer cells in a HIF-1-independent manner

Because HIF-1 is widely considered the master regulator of the cellular response to hypoxia and knockdown of PIM1/2 reduced the protein levels of HIF- α isoforms, we asked whether the HIF-1 signaling pathway is essential for PIM inhibitor-mediated toxicity. First, we examined the effect of PIM inhibition on the expression of HIF- α isoforms in hypoxia. A panel of cancer cell lines was incubated in hypoxia $\pm$ AZD1208 for 6 h, and western blotting used to monitor HIF- α protein. In all the cell lines tested, AZD1208 significantly reduced HIF-1 α and HIF-2 α protein levels (Fig. 2A). To determine whether PIM inhibitors kill hypoxic cells in a HIF-dependent manner, PC3-LN4 cells were transfected with siRNA to knockdown HIF-1 α , HIF-2 α , or the combination of both, effectively eliminating HIF-1 signaling. These cells then were incubated in hypoxia for 72 h in the presence or absence of AZD1208. Knockdown of HIF-1 α , HIF-2 α , or both did not significantly reduce PC3-LN4 cell viability after 72 h in hypoxia, providing evidence that HIF-1 signaling is not essential for this cell line to survive in hypoxia (Fig. 2B, compare DMSO treated cells). Furthermore, crystal violet staining revealed that AZD1208 reduced cell viability to a similar extent in cell lacking HIF- α isoforms, indicating that PIM inhibitors are toxic toward hypoxic cells even in the absence of HIF-1 signaling (Fig. 2B, compared DMSO vs. AZD). Similar results were observed using HCT116 and MD-MBA-231 cells (Supplemental Fig. 2). In addition, the protein levels of PIM1 and PIM2 were unchanged in response to knockdown of HIF-1 α isoforms, demonstrating that PIM levels are not regulated by the HIF-1 pathway in hypoxia (Fig. 2B, western blot).

To further establish that the ability of PIM inhibitors to kill cells was independent of their effect on the HIF-1 pathway, we utilized a HIF-1 α mutant in which Pro402 and Pro564 were mutated to alanine (P/A). This construct is unable to be hydroxylated by prolyl hydroxylase domain-containing proteins (PHD1, 2, 3), making it highly stable. To determine whether PIM inhibitors could reduce the expression of this mutant, PC3-LN4 cells were transfected with HA-tagged WT HIF-1 α or the P/A mutant, placed in hypoxia $\pm$ AZD1208 for 6 h, and exogenous HIF-1 α expression monitored using an anti-HA antibody. WT HIF-1 α was reduced by AZD1208, but the P/A mutant was unchanged, indicating that PIM inhibitors

likely reduce HIF-1 α levels by interfering with its canonical degradation pathway (Fig. 2C, western blot). Importantly, AZD1208 significantly reduced the transcriptional activity of WT HIF-1 but not the P/A mutant, as determined by the expression of HIF-1 target genes, Angiopoietin-Like 4 (ANGPTL4) and vascular endothelial growth factor (VEGF) (Fig. 2C). To determine if a HIF-1 construct that is refractory to AZD1208-mediated downregulation could block the toxic effect of this drug in hypoxia, PC3-LN4 cells were transfected with WT HIF-1 α or the P/A mutant, which is not degraded in response to AZD1208 treatment. The next day, the cells were treated with DMSO or AZD1208 and incubated in hypoxia for 48 h. AZD1208 reduced the viability of prostate cancer cells overexpressing the P/A mutant to the same extent as cells expressing the vector and WT HIF-1 (Fig. 2D). Taken together, these data demonstrate that PIM inhibitors kill hypoxic cancer cells through a mechanism that is unrelated to their ability to reduce HIF-1 activity.

PIM inhibitors are toxic toward hypoxic cells in a ROS-dependent manner

Similar to the metabolic phenotype associated with hypoxia, the loss of PIM kinases was recently shown to alter the cellular redox state by reducing the levels of metabolic intermediates in the glycolytic and pentose phosphate pathways, increasing the production of reactive oxygen species (ROS) (31). Treatment with PIM inhibitors could therefore increase the ROS levels resulting from hypoxic stress, leading to tumor cell death. To assess ROS production, PC3-LN4 cells were placed in normoxia or hypoxia $\pm$ AZD1208 for 24 h. After incubation for the indicated time, cells were loaded with a chloromethyl derivative of H₂DCFDA (DCF), an intracellular dye that indicates ROS levels, and harvested for FACS analysis or imaged under a fluorescence microscope. At 24 h, ROS levels were significantly increased in hypoxia compared to normoxia (Fig. 3A, compare DMSO). Strikingly, two structurally unrelated pan-PIM kinase inhibitors, LGB321 and AZD1208 (Supplemental Fig. 3), caused a further increase in ROS levels, especially in hypoxia (7-fold increase compared to hypoxia alone). AZD1208 increased ROS levels by 2-fold in normoxia, whereas LGB321 had no effect, indicating that PIM activity is particularly important for controlling ROS levels in the context of hypoxia (Fig. 3A, bar graph). If AZD1208-induced ROS production accounts for the decrease in cell viability in hypoxic prostate cancer cells, ROS scavengers, N-acetyl cysteine (NAC) and polyethylene glycol-superoxide dismutase (PEG-SOD), should reduce intracellular ROS and rescue viability in AZD1208-treated cells. To evaluate this possibility, PC3-LN4 cells were treated with AZD1208, NAC, PEG-SOD or the indicated combinations for 48 h in hypoxia, and viability was measured by crystal violet staining. DCF staining confirmed that both NAC and PEG-SOD significantly reduced ROS levels in AZD1208 treated cells in hypoxia (Fig. 3B). As expected, AZD1208 significantly reduced cell viability (Fig. 3C; compare bars 1 and 2). However, NAC and PEG-SOD largely rescued AZD-induced cell death, increasing viability to approximately 85% (Fig. 3C; compare bar 2 to bars 5 and 6). These data show that PIM inhibition selectively kills cancer cells in a ROS-dependent manner. Moreover, NAC and PEG-SOD did not rescue the AZD1208-mediated downregulation of HIF- α expression, providing further evidence that PIM inhibitors kill hypoxic cells through a mechanism that is distinct from any effect on HIF-1 (Fig. 3C, western blot).

PIM kinase activity regulates the cellular antioxidant response by controlling NRF2 signaling

A major mechanism in the cellular defense against oxidative stress is activation of the Nrf2-antioxidant response element signaling pathway, which controls the expression of genes that eliminate ROS and enhance the cellular antioxidant capacity. To determine whether PIM kinases alter Nrf2 signaling, the effect of AZD1208 on Nrf2 DNA binding capacity and its transcriptional activity was measured. First, PC3-LN4 cells were incubated for 24 h in normoxia or hypoxia $\pm$ AZD1208. Nuclear extracts were isolated and the relative amount of Nrf2 binding to a specific dsDNA sequence containing the Nrf2 response element was measured by a colorimetric assay. No change was observed following AZD1208 treatment in normoxia, but there was approximately a 40% decrease in Nrf2 DNA binding following AZD1208 treatment of hypoxic cells (Fig. 4A). This result indicates that less Nrf2 is present in the nucleus to bind the promoters of target genes in the absence of PIM activity. Next, Nrf2 transcriptional activity was measured using a breast cancer cell line (MD-MBA-231) stably expressing a luciferase reporter construct fused to the ARE of hNQO1 (ARE-Luc). Luciferase expression in these cells is directly correlated with Nrf2 transcriptional activity (23). MD-MBA-231-ARE-Luc cells were incubated in normoxia or hypoxia for 24 h in the presence or absence of AZD1208, LGB321, or a known inducer of Nrf2 signaling, sulforaphane (SULF). In normoxia, neither PIM inhibitor significantly altered luciferase expression, whereas there was a 3-fold increase in response to SULF. In contrast, both PIM inhibitors significantly reduced luciferase expression in hypoxia, demonstrating that PIM inhibition reduced the transcriptional activity of Nrf2 in hypoxia (Fig 4B). In agreement with previous reports, we observed that hypoxia increased the expression of several Nrf2 target genes (*NQO1*, *HMOX2*, and *PRDX1*) (7). Treatment with AZD1208 reduced the hypoxia-mediated increase in Nrf2 target genes back to basal levels. This result suggests that PIM expression is necessary for the full activation of Nrf2 in hypoxia (Fig. 4C). Moreover, we demonstrate that PIM1 overexpression was sufficient to increase the expression of Nrf2 target genes in normoxia (Fig. 4D).

PIM regulates Nrf2 activity by controlling its cellular localization

Nrf2 activation is associated with its nuclear translocation. In response to oxidative stress, Nrf2 escapes its interaction with KEAP1 in the cytoplasm and enters the nucleus to enhance the transcription of antioxidant genes (12). Therefore, PIM inhibitors might blunt Nrf2 activity by reducing its nuclear accumulation. To test this hypothesis, we performed immunofluorescence staining to monitor the cellular localization of endogenous Nrf2 in response to hypoxia and PIM inhibition. Similar to previous reports, endogenous Nrf2 was evenly distributed throughout the cell at basal conditions (Fig. 5A) (32, 33). After 24 h in hypoxia, Nrf2 accumulated in the nucleus of the cell, and treatment with a chemical activator of Nrf2, sulforaphane, further increased its nuclear localization (Fig. 5A, compare normoxia to DMSO and SULF in hypoxia). In contrast, treatment with AZD1208 blocked the hypoxia-mediated accumulation of Nrf2 in the nucleus, as demonstrated by a decrease in nuclear localization compared to the DMSO or SULF treated cells in hypoxia. Interestingly, bright punctate staining of Nrf2 was observed throughout the cytoplasm and perinuclear regions of the cell following AZD1208 treatment, suggesting that a majority of the protein is

sequestered outside the nucleus and it may be forming aggregates in the cytoplasm (Fig 5A, compare AZD to DMSO).

To confirm that PIM kinase activity alters the cellular localization of Nrf2 and to quantitatively compare the amount of Nrf2 in the cytoplasm and nucleus, biochemical fractionation was performed. PC3-LN4 cells were treated with DMSO, AZD or SULF for 24 h in normoxia or hypoxia, and the cytosolic and nuclear fractions were isolated as described in the materials and methods section. Equal amounts of nuclear and cytoplasmic protein were loaded on SDS-PAGE gels, and western blotting was used to determine the amount of Nrf2 in each cellular compartment. The nuclear to cytoplasmic ratio was determined using densitometry. To verify that we effectively isolated the indicated cellular fractionations, we probed for Lamin A, a protein specifically found in the nucleus, as a control. Nrf2 was relatively evenly distributed between the cytosol and nucleus in normoxic conditions, with a slightly higher amount of protein in the cytoplasm (Fig. 5B, normoxia ratio = 0.6). As expected, Nrf2 exposure to hypoxia as well as treatment with sulforaphane caused a majority of Nrf2 to translocate to the nucleus (Fig. 5B, hypoxia DMSO ratio = 2.0 and SULF ratio = 4.4). In contrast, Nrf2 levels increased in the cytosol following AZD1208 treatment, demonstrating that PIM inhibition retains this protein in the cytoplasm (Fig 5B, AZD ratio = 0.3). This data is consistent with the results of our immunofluorescence staining. Consistent with a role of PIM kinases in affecting the nuclear translocation of Nrf2, PIM1 expression caused the opposite effect; Nrf2 was accumulated in the nuclear fraction in the PIM1 overexpressing cell line compared to the vector control (Fig. 5C, VEC vs. PIM ratio = 0.6 and 2.1, respectively). Together, these data demonstrate that PIM kinases control Nrf2 activity by regulating its cellular localization.

AZD1208 reduces tumor growth and selectively kills hypoxic tumor cells in vivo

To investigate the effect of PIM inhibitors on hypoxic tumor cells *in vivo*, PC3-LN4 cells were injected subcutaneously into each flank of 4-6 week old, male NOD-SCID mice. The tumors were allowed to grow to an average size of 250 mm³, when regions of intratumoral hypoxia have formed. Then, the mice were randomly segregated into two groups (n = 5 mice / 10 tumors) that received vehicle or AZD1208 (30 mg/kg/day). Tumor volume was measured every other day by caliper measurement for 14 days. Forty-five minutes prior to sacrifice, the mice were injected with pimonidazole (i.p.), a marker of hypoxia. Mice were sacrificed by cervical dislocation to avoid any ischemic artifact resulting from CO₂-induced asphyxiation, and the tumors and mouse tissue were harvested for biochemical and histological examination. Treatment with AZD1208 significantly reduced tumor volume, especially at later time points, and the average tumor weight at the end of the study was markedly lower in the AZD-treated cohort (Fig. 6A and B, respectively). To account for intrinsic differences in tumor size (e.g., larger tumors are more hypoxic), tumors with approximately the same volume and weight were selected for biochemical analysis at the time of harvest. These tumors were immediately frozen in liquid nitrogen and stored at -80°C until lysis and subsequent western blot analysis. Confirming that AZD1208 effectively inhibited PIM kinases *in vivo*, IRS1 (S1101) phosphorylation was abolished in AZD1208-treated mice (Fig. 6C). Moreover, the protein levels of both HIF-1 and HIF-2 were significantly reduced in the tumors of AZD1208 treated mice (Fig. 6C). To further

investigate the effect of PIM inhibition on survival in normoxic vs. hypoxic regions of the tumor, serial sections of vehicle and AZD1208-treated tumors were stained with hypoxyprobe-1 (HP1; anti-pimonidazole), a marker of hypoxia and cleaved caspase 3 (CC3), a marker of apoptosis. CC3 staining was more widespread in PIM inhibitor-treated tumors, and the number of apoptotic cells was significantly increased in regions of hypoxia compared to normoxia, demonstrating that hypoxic cells are preferentially killed by AZD1208 *in vivo* (Fig. 6D and E). In combination with our cell culture studies, these findings demonstrate the potential value of small molecule PIM kinase inhibitors as hypoxia-targeting agents for use in solid tumors.

Discussion

A majority of the research on tumor hypoxia has focused on HIF-1, a master regulator of the transcriptional response to low oxygen. In contrast, the identification of HIF-1-independent signaling pathways that regulate survival in hypoxia has been elusive. Here, we establish and characterize one such pathway and show that the PIM kinases are critical for survival in the hypoxic tumor microenvironment (Fig. 7). Our experiments demonstrate that PIM1 and PIM2 are induced in response to hypoxia, independent of HIF-1-mediated transcription (Fig. 1A). We find that hypoxia markedly increases the sensitivity of cancer cells to genetic or pharmacological inhibition of PIM1/2, indicating that these enzymes play an important role in cellular adaptation and survival in hypoxia (Fig. 1B-D). Importantly, in a prostate cancer xenograft model treated with AZD1208, we observed a dramatic increase in apoptosis specifically in hypoxic regions (Fig. 6), validating the utility of small molecule PIM kinase inhibitors as hypoxia-targeting agents *in vivo*. Interestingly, AZD1208 reduced the protein levels of HIF-1 α and HIF-2 α (Fig. 2A and 6C), suggesting that PIM kinase activity also influences HIF-1 signaling in hypoxia. However, the ability of AZD1208 to kill hypoxic cells was not altered by siRNA knockdown of HIF- α isoforms, and the overexpression of a constitutively active HIF-1 α that could not be downregulated in response to AZD1208 did not rescue the toxic effect of PIM inhibition (Fig 2B and C). These data demonstrate that the toxicity of PIM inhibitors toward hypoxic cells is independent of the HIF-1 signaling pathway.

One mechanism by which hypoxia damages normal cells and tissue is through increased ROS production. As a result, tumor cells must buffer this excess ROS to maintain non-toxic levels in the context of acute and/or chronic hypoxia. Our data suggest that a critical function of PIM kinases in hypoxia is to protect tumor cells against hypoxia-induced ROS production. A recent study reported that TKO MEFs (lacking all three PIM isoforms) have decreased expression of several key ROS scavenger proteins, including superoxide dismutase 2 (SOD2) and Peroxiredoxin-3 (Prdx3), resulting in increased basal ROS levels (31). We observed that hypoxia increased ROS levels in prostate cancer cells (Fig. 3A). Strikingly, treatment with chemically distinct PIM inhibitors increased ROS to a much greater extent in hypoxia compared to normoxia, leading to cell death (Fig. 3A). Thus, in the presence of a PIM inhibitor, hypoxic cancer cells are no longer able to maintain ROS at physiological levels, resulting in apoptosis due to excessive free radicals. These data support a model where hypoxia increases mitochondrial ROS generation, and to combat the toxic effects of excess ROS, hypoxic cancer cells increase PIM protein levels. This enzyme then

functions to increase the expression of detoxifying proteins to reduce the accumulation of free radicals and support survival. This mechanism may explain the selective toxicity of PIM inhibitors toward hypoxic tumor cells.

This study is the first to demonstrate that PIM kinases influence the cellular antioxidant defense system via enhancing the activity (Fig. 4) of and cellular localization (Fig. 5) of Nrf2. Combined with the fact that PIM kinases are upregulated in hypoxia, this finding poises PIM kinases as a key player regulating the cellular cytoprotective response to low oxygen, which is prevalent in solid tumors. Nrf2 expression levels are known to influence both tumor progression and therapeutic efficacy. For example, Nrf2 has been shown to be elevated in lung, breast, and prostate cancers, and its expression is correlated with poor prognosis (34, 35). Moreover, Nrf2 overexpression is associated with chemoresistance in many cancers, including colon, breast and prostate cancers (36). Several mechanisms have been proposed to explain the activation of Nrf2 in cancer, such as mutations that disrupt KEAP1 binding and upregulation via amplification of oncogenic proteins, including *Myc* and *Kras* (37). We demonstrate that overexpression of PIM kinases, which is frequently observed in cancer, is sufficient to activate Nrf2. This data provides a new mechanism by which PIM kinases promote therapeutic resistance, independent of their role in regulating cell cycle progression and survival. Mechanistically, PIM kinases promote the nuclear translocation of Nrf2 (Fig. 5). Thus, by enhancing PIM expression in response to hypoxia, cancer cells increase Nrf2 activity, allowing for more rapid and robust detoxification of the cell, and increased survival in response to the harsh tumor microenvironment and/or therapeutic interventions. Previous reports have described that Nrf2 can be directly phosphorylated by PKC (38) and MAPKs (39), leading to dissociation from KEAP1 and accumulation in the nucleus resistance to therapy; although, the importance of these phosphorylation events for Nrf2 activity remains unclear. Further studies are warranted to determine whether PIM kinases directly phosphorylate Nrf2 or act through an indirect mechanism to promote its accumulation in the nucleus.

In summary, we have identified a novel mechanism by which PIM kinases regulate NRF2 activity and tumor cell survival in hypoxia (Fig 7). Inhibiting PIM in solid tumors has the unique advantage of opposing the oncogenic properties of hypoxia by selectively killing hypoxic tumor cells. These findings provide new insights into pathways regulating the cellular response to oxidative stress as well as a HIF-1-independent mechanisms that are essential for survival in hypoxia. The biochemical and *in vivo* results presented here support the development of PIM kinase inhibitors as a novel therapeutic strategy to increase ROS-mediated killing specifically in hypoxic tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Dr. Donna Zhang for providing ARE-Luc and MD-MBA-231 (ARE-Luc) cells. We thank Dr. Ningfei An at the University of Chicago for her technical assistance with *in vivo* experiments. We appreciate the support of AstraZeneca and Novartis for making the PIM inhibitors available for these studies.

This work was supported by University of Arizona Cancer Center support grant P30CA023074, NIH award R01CA173200, and DOD award W81XWH-12-1-0560 to A.S. Kraft.

Abbreviations

PIM	proviral Integration site for Moloney murine leukemia virus
Nrf2	nuclear factor-erythroid 2 p45-related factor 2
ROS	reactive oxygen species
HIF-1	hypoxia inducible factor-1
ETC	electron transport chain
KEAP1	kelch-like ECH-associated protein 1
HMOX1	heme oxygenase-1
NQO1	NAD(P)H dehydrogenase, Quinone 1
PRDX1	peroxiredoxin 1
VEGF	vascular endothelial growth factor
ANGPTL4	angiopoietin-like protein 4
HP-1	hypoxypromote-1
CC3	cleaved Caspase 3
SCID	Severe combined immunodeficiency
DCF	H ₂ DCFDA
NAC	N-acetyl cysteine
PEG-SOD	polyethylene glycol-superoxide dismutase
SULF	sulforaphane

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Figure 1. Hypoxia increases PIM protein levels and sensitizes prostate cancer cells to AZD1208

A) PC3-LN4, HCT116, and MD-MBA-231 cells were cultured in normoxia or hypoxia, and protein and mRNA were harvested to assess the expression of PIM isoforms. B) PC3-LN4 cells were treated with a dose range of AZD1208 in normoxia or hypoxia for 72 h, and cell viability was measured by crystal violet staining (* $p < 0.05$; normoxia vs. hypoxia). C) In parallel, cells were cultured in normoxia or hypoxia $\pm$ AZD1208 (3 μ M) for 48 h, and protein levels were monitored by western blotting. D) PC3-LN4 cells were treated with DMSO or AZD1208 (3 μ M) for the indicated times, and samples were stained with propidium iodide for FACS analysis (* $p < 0.05$; compared to normoxia at each time point). E) siRNAs targeting PIM1, PIM2, and PIM3 were transfected into PC3-LN4 cells. The cells were incubated for 48 h $\pm$ AZD1208 (3 μ M), and viability was measured at 48 h (* $p < 0.05$; n.s. = not significant). N = normoxia; H = hypoxia.

Figure 2. PIM inhibitors selectively kill hypoxic cancer cells in a HIF-1-independent manner

A) PC3-LN4, HCT116, and MDA-MB-231 cells were placed in hypoxia $\pm$ AZD1208 for 6 h, and protein was collected to assess the expression of HIF- α isoforms. B) PC3-LN4 cells were transfected with the indicated siRNA and incubated in hypoxia $\pm$ AZD1208 (3 μ M) and viability was measured by crystal violet; lysates were collected in parallel for western blotting. C) PC3-LN4 cells were transfected with WT HIF-1 or the HIF-1 (P/A) mutant and incubated in hypoxia for 6 h $\pm$ AZD1208 and protein and mRNA were collected for analysis. D) In parallel, transfected cells were incubated in hypoxia $\pm$ AZD1208 (3 μ M) for 48 h. * p < 0.05; n.s. = not significant.

Figure 3. PIM inhibitors kill hypoxic tumor cells by increasing ROS

A) PC3-LN4 cells were cultured in normoxia or hypoxia for 24 h $\pm$ LGB-321 (1 μ M) or AZD1208 (3 μ M). DCF staining was imaged using a fluorescence microscope and quantified by flow cytometry (histograms). The geometric mean fluorescence intensity (MFI) was calculated from 3 independent experiments. B) PC3-LN4 cells were placed in normoxia or hypoxia and treated with AZD1208 alone or in combination with NAC (5 mM), or PEG-SOD (150 U/mL) for 24 h prior to DCF staining. C) PC3-LN4 cells were treated with AZD1208, NAC, PEG-SOD or the indicated combinations for 72 h in hypoxia, and viability was assessed by crystal violet staining; in parallel, lysates were collected at 24 h to assess HIF- α protein levels. * p < 0.05.

Figure 4. PIM regulates Nrf2 activity

A) PC3-LN4 cells were incubated in normoxia or hypoxia $\pm$ AZD1208 (3 μ M) for 24 h. Nuclear lysates were isolated and Nrf2 DNA binding was measured. B) MD-MBA-231-ARE-Luc cells were incubated in normoxia or hypoxia $\pm$ AZD1208 (3 μ M), LGB321 (1 μ M), or sulforaphane (15 μ M) for 24 h, and luciferase expression was measured. C) PC3-LN4 cells were placed in normoxia or hypoxia $\pm$ AZD1208 for 24 h, and mRNA was collected and gene expression compared to normoxia was measured by qRT-PCR (* $p < 0.05$ compared to normoxia; + $p < 0.05$ compared to hypoxia). D) mRNA and protein were collected from PC3 cells stably expressing Vector or PIM1, and gene expression was measured by qRT-PCR. * $p < 0.05$.

Figure 5. PIM kinase activity regulates the cellular localization of Nrf2

A) PC3-LN4 cells were incubated in normoxia or hypoxia $\pm$ AZD1208 (3 μ M) or Sulforaphane (15 μ M) for 24 h. Cells were fixed, and Nrf2 localization was monitored by immunofluorescence. B) PC3-LN4 cells were incubated in normoxia or hypoxia $\pm$ AZD1208 (3 μ M) or Sulforaphane (15 μ M) for 24 h. Nrf2 levels were monitored in nuclear and cytoplasmic extracts by immunoblotting. C) PC3 cells stably expressing Vector or PIM1 were harvested, and Nrf2 levels were monitored by immunoblotting. * $p < 0.05$.

Figure 6. AZD1208 selectively kills hypoxic cells *in vivo*

A) Tumor volumes were monitored by caliper measurements (line graph). B) At the end of the study, tumors from each group were harvested and weighed (bar graph). C) Size-matched tumors from each cohort were homogenized in lysis buffer and subjected to western blotting with the indicated antibodies. D) Tumors from each cohort were immunostained with hypoxypoint-1 (HP1) and cleaved caspase-3 (CC3). Dotted lines delineate regions of hypoxia in serial sections. E) Percent CC3+ area was determined as described in the materials and methods section. * $p < 0.05$.

Figure 7. PIM kinase inhibitors selectively target hypoxic cancer cells by reducing Nrf2 activity and increasing ROS to toxic levels

Model depicting the role of PIM kinases in controlling cell Nrf2 signaling and survival in hypoxia. A) Hypoxia upregulates PIM and ROS levels. In turn, PIM promotes the nuclear translocation of Nrf2, leading to the transcription/translation of cytoprotective genes that recued ROS levels and allow for cell survival. B) Pharmacological inhibition of PIM blocks the nuclear translocation of Nrf2, leading to a significant increase in ROS levels that ultimately kills hypoxic cancer cells. Thus, PIM inhibition represents an attractive strategy to oppose hypoxia-mediated therapeutic resistance.