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RESEARCH ARTICLE

Phytohormone Signaling Induces Dormancy and Apoptosis in Prostate Cancer Disseminated Tumor Cells

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ABSTRACT

Prostate cancer (PCa) remains a major health concern, ranking as the second leading cause of cancer-related deaths in men in the United States. The dissemination of tumor cells (DTCs) from the prostate, their entry into circulation, and subsequent skeletal metastasis involve complex mechanisms that are not fully elucidated. Notably, DTCs demonstrate a remarkable ability to home to the hematopoietic stem cell niche within bone marrow, where they may remain dormant for years. Key signaling pathways implicated in DTC dormancy include TGF-β, BMP4/BMP7, GAS6/TAM receptors, and Wnt5a, each influencing cell cycle arrest, survival, and phenotype adaptation. Recent research has explored analogies between dormancy mechanisms in cancer and plant biology, particularly focusing on phytohormones such as abscisic acid (ABA) and gibberellins, which regulate plant stress responses and developmental dormancy. While plants utilize PYR1/PYL/RCAR receptors for ABA, mammals rely on LANCL2 and PPARγ. This study evaluated the effects of ABA, gibberellic acid, and the ABA agonist pyrabactin on human and murine PCa cell lines. Results demonstrated that gibberellic acid lacked proliferative effects and could not counteract ABA-induced growth arrest. In contrast, pyrabactin potently induced growth arrest and apoptosis, activating SMAC/Diablo cell death pathways independently of LANCL2 and PPARγ signaling. Further, the activity of ABA and pyrabactin depended on cellular uptake via SLC4A2 and SLC4A3 anion exchangers; downregulation of these transporters partially reversed their inhibitory effects. These findings suggest a mechanistic parallel between phytohormone-induced dormancy in plants and regulated dormancy and apoptosis in PCa, opening new avenues for therapeutic targeting of dormancy pathways in cancer metastasis.

Keywords: Prostate cancer, Disseminated Tumor Cells, Dormancy, Abscisic acid, LANCL2, PPARγ Pyrabactin, SLC4A2, SLC4A3, Bone marrow microenvironment.

Introduction

Prostate cancer (**PCa**) is a critical health challenge, ranking as the second leading cause of cancer-related death in men in the United States[1]. The mechanisms whereby disseminated tumor cells (**DTCs**) leave the prostate and enter circulation are not completely understood. Emerging evidence suggests that DTCs may leave the prostate early in the disease process, even before a primary tumor may become clinically detectable, with the risk of dissemination increasing as the tumor grows and invades nearby tissues[2]. The most frequent sites of PCa spread are the bones and lymph nodes, with other organs like the liver and lungs affected in more advanced cases. Unfortunately, PCa cells have a remarkable ability to survive in bone marrow, where DTCs may remain dormant for years. Despite advances, the precise mechanisms by which DTCs enter a dormant state and ultimately escape dormancy and form skeletal metastases remain elusive.

DTCs including those of PCa and breast cancer have been shown to specifically target and home to the hematopoietic stem cell (**HSC**) niche within the bone marrow[3,4]. Once in the niche, DTCs may alter their phenotype including the acquisition of cancer stem-like properties[5]. In addition to phenotypic changes, DTCs are influenced by the signaling mechanisms which regulate HSCs quiescence[6]. Multiple signaling pathways, including Transforming Growth Factor-beta (**TGF-β**), Bone Morphogenic Protein 4 and 7 (**BMP4, BMP7**), Growth Arrest-Specific 6 (**GAS6**), and Wnt5a are crucial in regulating DTC dormancy in the bone marrow. TGF-β signaling promotes tumor dormancy by inducing cell cycle arrest, senescence, or apoptosis in cancer cells. TGF-β may also regulate the tumor microenvironment, influencing the interactions between cancer cells and their surrounding stroma as well as impact immune regulation. Both BMP4 and BMP7 induce growth arrest in cancer cells, thereby contributing to the development of a dormant state. GAS6 is a protein that activates TAM receptors (Tyro3, Axl, Mer TK) and can influence cell survival and immune responses. The impact of GAS6 on PCa DTCs in the marrow is to induce cell cycle arrest or promote cell survival, leading to tumor dormancy[7-11]. Additionally, GAS6/TAM signaling has been implicated in promoting drug resistance and metastasis and tumor dormancy and recurrence. Wnt5a, a non-canonical member of the Wnt family, can induce a non-proliferative state in cancer cells, thought also to regulate dormancy[12-15]. It is important to note that the impact of each of these molecules is likely dependent on the context, such as the potential to promote tumor growth and angiogenesis.

Plant phytohormones, such as abscisic acid (**ABA**) regulate growth, stress responses, and dormancy, often by inducing cell cycle arrest and promoting cellular quiescence. Recent work in our group has drawn parallels between these mechanisms, suggesting that pathways governing phytohormone-induced plant dormancy share molecular similarities with those regulating tumor cell dormancy, such as stress signaling and metabolic adaptation. ABA and gibberellins are key phytohormones which regulate responses to environmental changes (e.g., drought, salinity, temperature changes) which antagonistically regulate several developmental processes[16]. Abscisic acid is primarily involved in regulating stress responses, stomatal closure and seed dormancy[17], while gibberellins promote stem elongation, seed germination, and flowering. Such that the ratio of ABA to gibberellins determines whether a seed will remain dormant or geminate. Together, these hormones help coordinate various physiological processes that enable plants to thrive in diverse environments.

In mammals, the major receptor for ABA appears to be LanC-like protein 2 (**LANCL2**) although LANCL1 a homolog, can also bind ABA albeit with lower affinity[18,19]. Some studies suggest ABA may modulate the activity of the nuclear hormone receptor protein peroxisome proliferator-activated receptors gamma (**PPARγ**) involved in glucose metabolism and adipogenesis[20-22]. In plants, the primary ABA receptors belong to the PYR1/PYL/RCAR family of proteins which were first identified with pyrabactin screening approach[23]. In contrast, gibberellin receptors have not been described in mammalian biology however gibberellic acid has been reported to be toxic to murine livers and kidneys[24-26].

This study aimed to expand our understanding of dormancy regulators by investigating the role of ABA, gibberellic acid and the ABA agonist pyrabactin in PCa cell lines. We found no significant proliferative activity of gibberellic acid on human PCa cell lines, nor was gibberellic acid able to

counteract the growth arrest induced by ABA. In stark contrast, the ABA agonist pyrabactin induced strong growth arrest on human and murine PCa cell lines as well as mesenchymal and hematopoietic cell lines. Furthermore, pyrabactin induced apoptosis of PC3 cells by initially activating SAMC/Diablo pathways and subsequently recruiting multiple cell death pathways. Interestingly, pyrabactin did not appear to exerts its effects on PCa cell lines using the ABA receptors Lancl2 or PPARγ. Moreover, given that Lancl2 and PPARγ receptors are located on the intracellular side of the plasma membrane, a transporter is necessary for the ABA to exert its effect[27]. We therefore targeted for deletion of the ABA transport exchanger SLC4A2 and SLC4A3 which encode members of the solute carrier (**SLC**) family of anion exchangers. The downregulation of SLC4A2 and SLC4A3 partially reversed ABA and pyrabactin's inhibitory activity on growth in PC3 cells. Together these observations suggest that ABA is transported across the cell membrane in some PCa cells using the transport exchangers, whereupon it activates Lancl2 and PPARγ to inhibit proliferation. Pyrabactin also uses the transport exchangers, but unlike ABA, activates cell death initially through SMAC/Diablo independent of Lancl2 and PPARγ signaling.

Materials and Methods

Cell Cultures.

Human PCa cell lines (PC3, C4-2B and DU145) and murine Myc-CaP and Raw264.7 cells were obtained from the American Type Culture Collection (Rockville, MD). Murine osteoblast cells (MC3T3-E1) were obtained from the American Type Culture Collection (Rockville, MD). All prostate cancer cell lines were routinely grown in RPMI 1640 (Life Technologies, Carlsbad, CA), and murine osteoblast (MC3T3-E1) cells were grown in α-MEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS, GEMINI Bio-Products, Sacramento, CA), 1% penicillin-streptomycin (P/S, Life Technologies) and maintained at 37°C, 5% CO_2, and 100% humidity. RAW264.7 cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin.

Cell Proliferation Assays.

To evaluate proliferation, 3,000 PCa cells/well in 100 µl of their respective media were added to 96-well plates in quintuplicate and allowed to adhere overnight. The next day, the cells were washed in PBS (Thermo Fisher Scientific, Inc.) and fresh medium was added. ABA was diluted in dimethyl sulfoxide (**DMSO**), and was added over the dose range of 0-200 µM for 24-72 h at 37˚C. Similarly, cultures were treated with Pyrabactin (B3438-10mg, Millipore-Sigma) or control for 24-72h. Gibberellic acid (Cat. G1025, Sigma-Aldrich) was added to the cultures at 0-100µM.

Proliferation was assessed using a Nikon Eclipse Ts2 inverted phase light microscope (Nikon Corporation) or using a colorimetric assay. For the colorimetric assays, 20 µl CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Reagent (cat. no. G5421; Promega Corporation) was added to the cultures for 2-4 h at 37˚C. The optical density (O.D.) of the cell culture plates was then evaluated using a plate reader (DU530 UV/Vis scanning spectrophotometer Biosciences at 450 nm or a Spectramax M2 microplate reader from Molecular Devices) at a wavelength of 490 nm.

Time course experiments were performed on DU145, C4-2B, PC3, MG63, MycCaP, MC3T3-E1, and Raw 264.7 cells. The cells were seeded in a $3.0x10^4$ cells/ml density, or 100µl in individual 96 well plates with 0-50µM of pyrabactin. Pyrabactin was added for the remaining 24, 48 or 72h of the culture. Twenty hours following the final dose of pyrabactin, 20 ul of XTT solution (VWR CellTiter 96® Aque 1000 assays Cat. # PAG5421) was added to each well.

To determine if pyrabactin permanently affected viability, cells were treated with pyrabactin for 72 h at 37˚C, washed, trypsinized and counted by Trypan blue exclusion. Equal number of cells ($1x10^5$ cells/well in 12-well plates, 1 ml of culture media) were then added back to culture for an additional 72 h at 37˚C and cell viability was evaluated colormetrically. Cell viability (%) was calculated as follows: (treatment group-background) / (control group-background) x100. The data are presented as the mean ± SEM.

Cell Cycle Assays

We engineered PC3 cells to stably express two fluorescent cell cycle reporters via lentiviral delivery ($PC3^{VC}$ cells), enabling real-time monitoring of cell cycle status and quiescence[28]. The first reporter, G0-Venus, is a p27 fusion protein that accumulates

in quiescence (G_0) and is degraded upon G_1/S transition[29]. The second, G1-Cherry, is a Cdt1 fusion protein, present during G_0 and G_1 and degraded in S phase[30,31]. High G_0-Venus with G_1-Cherry marks G_0, while G_1 is indicated by low G_0-Venus and high G_1-Cherry. These dynamics can be tracked by live imaging and flow cytometry. The generation of these cell lines were previously reported[28]. We performed cell cycle monitoring of $PC3^{VC}$ cultures treated with 50-100 µM abscisic acid (ABA).

shRNA and lentivirus preparation.

pLKO.1 lentiviral vector-based shRNAs targeting specific candidate genes and the non-specific (**NS**) control shRNA were obtained from Horizon Discovery (NS control and shRNA LANCL2) and MilliporeSigma (shRNA PPARγ). Lentivirus particles were prepared by transfecting 293T cells in 12-well plates with 0.5 µg either gene-specific shRNA plasmids (ShSLC4A2 Gene ID #6522; MilliporeSigma and ShSCL4A3 Gene ID #6508; MilliporeSigma) or NS shRNA plasmids along with lentiviral packaging plasmids [2nd generation psPAX2 packaging (plasmid #12260; Addgene, Inc.) and pMD2.G envelope (plasmid #12259; Addgene, Inc.) in a 1:1 ratio for 48 h at 37˚C. All lentiviral transfections were performed using Effectene Transfection Reagent. Stable cell lines were generated by infecting PCa cells (PC3 and C4-2B) with a multiplicity of infection of ~250 viral particles per cell in 200 µl (total collection volume, 1 ml) in 12-well plates for 24 h, followed by selection in puromycin (1 µg/ml) at 37˚C for 1 week. Thereafter reverse transcription-quantitative PCR (RT-qPCR) was used to assess the mRNA expression changes and western blotting was used to validate protein expression changes. After the initial assessment of the efficiency of shRNA knockdown (KD) of the target genes, SCL4A2 Clone ID TRCN0000043858; MilliporeSigma and SCL4A3 Clone ID TRCN0000045145; MilliporeSigma were selected for further studies. Thereafter, frozen stocks were established, and cells were used for experimentation after validation of the targeted KD within 2 weeks of transfection or thaw. The generation of LANCL2 and PPARγ shRNA knockdowns were previously reported[32].

RNA extraction, cDNA preparation and RT-qPCR.

Total RNA was extracted from target cells using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was purified using the RNeasy Mini Kit (Qiagen, Inc.). cDNA was synthesized using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Inc.) according to the manufacturer's instructions. qPCR was performed with gene-specific primers using the iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) on an Applied Biosystems 7500 thermocycler system (Applied Biosystems; ThermoFisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 15 min; 40 cycles at 95˚C for 15 sec, annealing at 60˚C for 30 sec and 72˚C for 30 sec; and a final extension step at 72˚C for 7 min. Results were normalized against β-actin levels using the formula ΔCq = Cq of target gene - Cq of β-actin. The mRNA expression levels of the control group were used to establish the baseline; therefore, ΔΔCq was calculated using the formula ΔΔCq = ΔCq of target gene - ΔCq of the baseline. The fold change of mRNA expression was calculated as fold = $2^{-\Delta\Delta Cq}$ [33]. The primer sequences for ShSLC4A2; 5'-TCCTCCCACCACATCCATCA-3' and 5'- CTCCTC AATGGTCGGGGTTTC-3' for ShSCL4A3; 5'-CCCC TCTAAGTCCAGACGTG-3' and 5'-CCGGTGAAAC TCAAAGTCCC-3' for β-actin; 5'-TCAGGACGGGA AGATCATTCA-3' and 5'-CAGAGCAGTCATGGG GATCAG-3'.

FACS

For evaluating of cell cycle phase, live cells were selected as DAPI-negative cells (cat. NBP2-31156, DAPI, NOVUS) were negatively gated for anti-mouse H-2kd (cat no. 116622, PE/Cy7, BioLegend, San Diego, CA), which were then positively gated for HLA-A,B,C (cat no. 311426, APC/Cy7, BioLegend). After these gates were applied, cells were plotted on the Venus-Cherry spectrum. Cell cycle was determined using FACS analyses using a FACS Aria three-laser flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and data were analyzed with DIVA software (Becton Dickinson).

Apoptosis Profile Arrays.

PC3 cells were seeded into T25 flasks (690160, VWR) at a density of $1.25x10^6$ cells/flask). Twenty-four hours after seeding, 50µM of pyrabactin (B3438-10 mg, Millipore-Sigma) or control were added the flasks. Cultures were further incubated for an additional 24 or 48 hours where upon the cells were recovered from culture using 0.25% Trypsin-0.1% EDTA (MT25053CI, Fisher) and then lysed, using the lysis buffer provide by the kit with

protease inhibitors, according to the instructions from the R&D Systems™ Proteome Profiler Human Apoptosis Array Kit (ARY009, Fisher). After extracting the proteins, the proteins were normalized and applied to the membranes per the manufacturers protocol. The membranes were imaged using an AnalyticJena UVP ChemStudio imager with exposures ranging from 7-10 minutes. Image analysis was performed using the ImageJ software.

Human Studies.

The studies were performed at the University of Michigan (IRB-Med, HUM00044196, 2014-2019), University of Alabama at Birmingham (IRB-300004457, 2019-2024) and Tufts University (IRB ID: Study0005280, 2024-present). The studies were evaluated by each of the respective Human Subjects Committees. Given that no subject interactions were planned, and the cell lines were to be purchased from commercial vendors, the studies were not deemed "Not Human Subjects Research" and considered exempt.

Statistical Analysis.

GraphPad Prism 5 was used for statistical analyses. All experiments were repeated, and the results are presented as the mean ± SEM. For each data point, a two-tailed, unpaired, Student's t-test was performed to determine the significance of the differences between two groups, and one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was performed to compare three or more groups. When analyzing multiple variables, a two-way ANOVA analysis followed by Bonferroni's post hoc test was performed to determine significance. $P<0.05$ was considered to indicate a statistically significant difference.

Results:

Impact of Gibberellic Acid on PCa Proliferation.

Plant phytohormones, such as abscisic acid (**ABA**) regulate growth, stress responses, and dormancy in plants, often by inducing cell cycle arrest and promoting cellular quiescence. Given that ABA is expressed in mammalian tissues[34], our lab has sought to draw parallels between ABA signaling and PCa dormancy in the bone marrow[32,35]. We believe that understanding these common signaling pathways will lead to the development of new therapeutic strategies to control cancer dormancy by mimicking or targeting these phytohormone-like pathways.

Gibberellic acid (**GA**) and ABA are two key plant hormones that interact through reciprocal regulation of various developmental processes, including seed dormancy and germination. ABA promotes seed dormancy and inhibits germination, whereas GA disrupts dormancy and promotes seed germination and growth by activating genes that encourage cell expansion and division. As a first step in understanding whether a reciprocal relationship exists in the cancer setting, we cultured PCa cells in 10% FBS for 72h. The cultures were then treated with XTT for 4h. The addition of GA to the human PCa cell lines PC3 (**Figure 1A**) and C4-2 (**Figure 1B**) had no significant impact on proliferation of either cell type.

Ki-67 is a protein that is used as a biomarker for cell proliferation. Ki-67 is present in the nucleus of cells that are actively dividing (i.e., those in the cell cycle phases G_1, S, G_2, and M) but is absent in resting (non-dividing) cells (G_0 phase). As a second approach to method to examine distinguish the impact of GA on PCa cells, PC3 cells were grown in 10% FBS (**Figure. 1C**). At 72h the cells were stained and Ki-67 staining evaluated by FACS. Reporting the data as % Ki67 negative staining as an indicator of cells in G_0, we observed that ABA increased the percentage of cells which did not stain for Ki67, GA had no impact, and the combination of equal doses of ABA and GA did not significantly differ from those cells treated with ABA alone.

Given that GA had no discernable impact on PCa cell proliferation *in vitro*, we next explored whether GA was able to reverse the growth inhibitory effect of ABA. To explore this possibility, we performed cell cycle monitoring of $PC3^{VC}$ cultures treated with increasing doses of ABA for 72h. Following culture, live cells were selected as DAPI-positive cells and cell cycle was determined using the fluorescent cell cycle reporters by FACS. As has been reported previously, ABA significantly increased the % of the cells in G_0 and decreased the % of cells in S/G_2/M phases of the cell cycle (**Figure 1D** (black bars)). In contrast, GA had little or no impact on the % of cells in G_0, G_1, and S/G_2/M (**Figure 1D** (open bars)) in keeping with the results on proliferation (**Figure**

1A, 1B, 1C). When ABA and GA were included in the culture, the impact of ABA was not significantly altered by the presence of GA (Figure 1C (hashed bars)). These results suggest that the receptors necessary for GA signaling are not present in PCa cells.

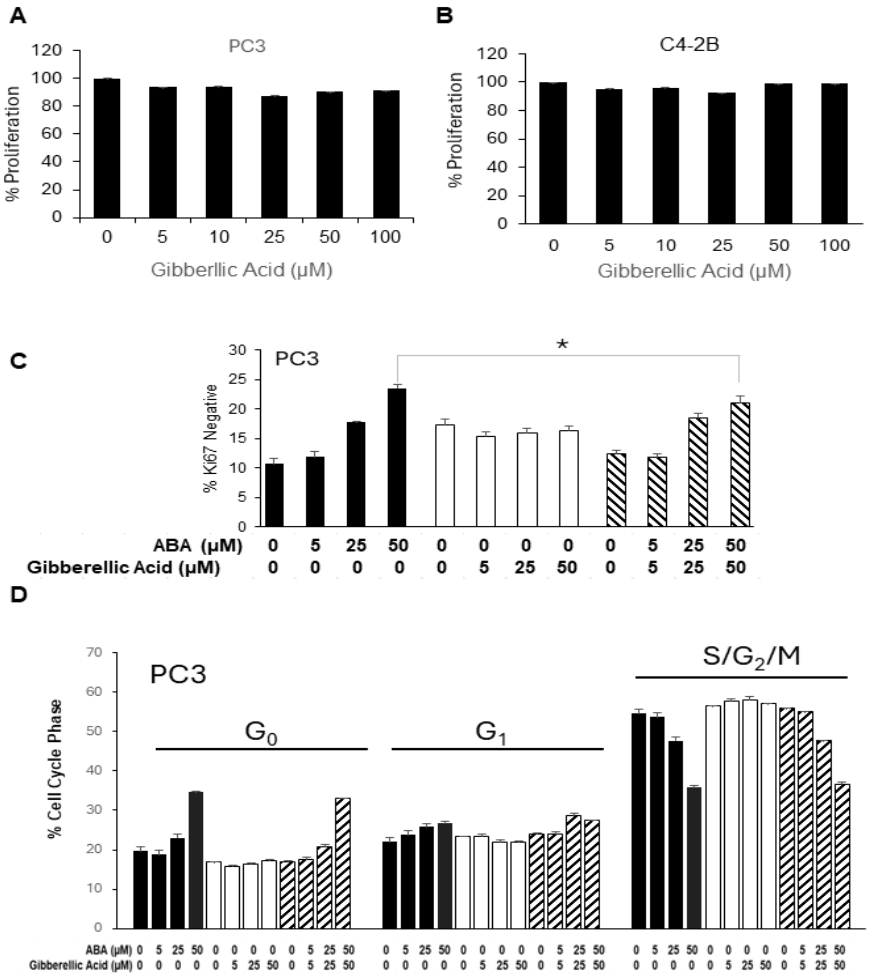

Figure 1: The Impact of Gibberellic Acid on Prostate Cancer Proliferation. (A) PC3 and **(B)** C4-2B cells (n=4) were cultured in 10% FBS with 0-100 mM of gibberellic Acid for 72h. The cultures were then treated with XTT for 4h and read at a wavelength of 450 nm. The data are reported as % mean ± standard deviation relative to 0 treatment control **(C)** PC3 cells were grown for 72h in the presence or absence of gibberellic acid (0-50μM) or ABA (0-50 μM), or a combination of both at equal doses and stained for Ki-67 and evaluated by FACS with at least 10,000 events recorded per condition. The data is reported as % Ki67 negative. *Indicates significant difference at $p<0.05$ (ANOVA) from ABA at 50 μM. **(D)** PC3 expressing Venus-cherry cell cycle reporters were cultured as in **(C)**. Once recovered from culture the cells were evaluated for those in G0, G1 or S/G2/M by FACS with at least 10,000 events recorded per condition. The data is reported as % Total cells/culture.

Impact of Pyrabactin on PCa Proliferation.

We next tested the impact of increasing doses of pyrabactin on a variety of human cell PCa lines including PC3, C4-2B, DU145 and the murine PCa cell line Myc-CaP using an XTT colormetric assays. In each case, significant growth inhibition was detected such that by 200μM all proliferative activities had been inhibited (Figure 2A, B, C, D). We next explored if the impact of pyrabactin on growth was specific for PCa epithelial cell lines by evaluating pyrabactin's activities on the human mesenchymal cells including the osteosarcoma cell line MG-63, the murine osteoblastic cell line MC3T3-E1 clone 4, and the murine macreophage-like cell line (Figure 2E, F, G). In each case, pyrabactin curtailed growth of the cell lines tested in keeping with its activities on PCa cell lines. Of the cells evaluated, RAW267 cells were the most resistant to pyrabactin's growth inhibition (Figure 2G).

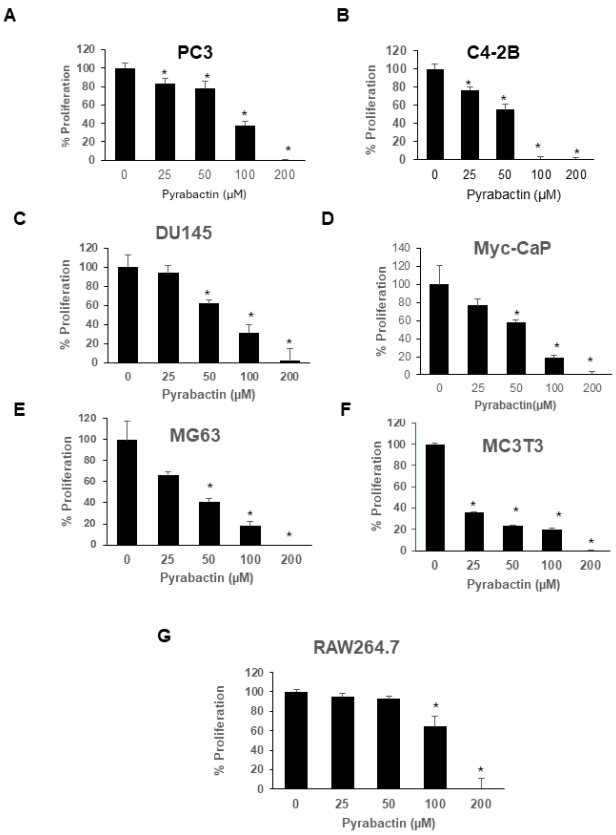

Figure 2: The Impact of Pyrabactin on Prostate Cancer and other Mesenchymal Cell Proliferation. Human **(A)** PC3, **(B)**, C4-2B, **(C)** DU145 and murine **(D)** Myc-CaP prostate cancer cell lines, and human **(E)** human MG-63 osteosarcoma cells **(F)** murine MC3T3-E1 osteoblast cells and **(G)** murine RAW264.7 monocyte were cultured 0-200 μM pyrabactin. The data are reported as % mean ± standard deviation relative to 0 treatment control. *Indicates significant difference treatment control at 0 μM pyrabactin at $p<0.05$ (ANOVA).

To evaluate the time course by which pyarabactin impacts PCa proliferation, a time course study using PC3, C4-2B and DU145 cells were conducted. In each case cells were cultured for 86 hours. In selected cases, the cells were plated overnight, washed and treated with 50μM pyrabactin for the final 24h-72h of culture prior to the addition of XTT to evaluate proliferation. For each cell type, the longer the exposure to pyrabactin, the more dramatic the growth inhibitory activity was observed (**Figure 3A, B, C**).

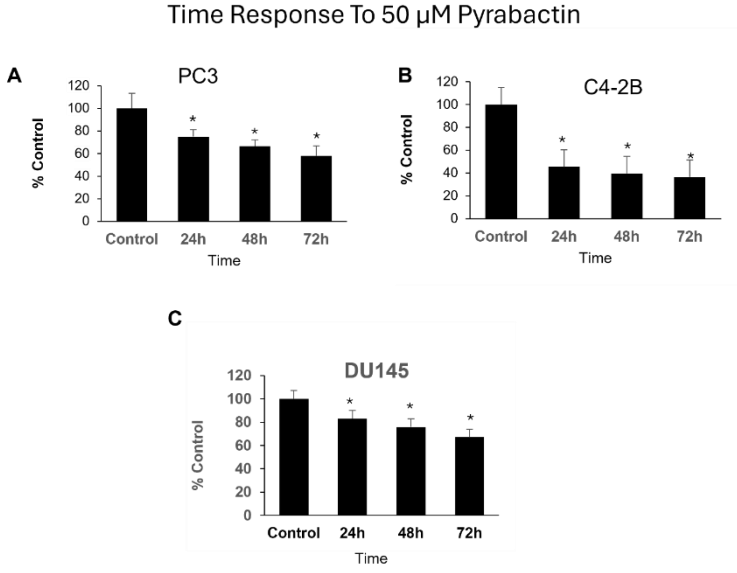

Figure 3: Time Course of Pyrabactin Inhibition of Prostate Cancer Cell Line Proliferation. A time course study using **(A)** PC3, **(B)** C4-2B and **(C)** DU145 cells was conducted over the course of 72h. The cells were plated overnight, washed and treated with 50μM pyrabactin for the final 24h-72h of culture prior to the addition of XTT to evaluate proliferation. *Indicates significant difference treatment control at 0 μM pyrabactin (Control) at $p<0.05$ (ANOVA).

To further explore the impact of pyrabactin on PCa cell proliferation, we performed cell cycle monitoring of $PC3^{VC}$ cultures treated with increasing doses of pyrabactin for 72h. Following culture, FACS was used to select DAPI-positive cells to distinguish live vs dead cells. As shown in **Figure 4A,** pyrabactin had a significant negative impact on the number of DAPI positive cells at 50μM. Pyrabactin significantly increased the % of the cells in G_0 and decreased the % of cells in $S/G_2/M$ phases of the cell cycle at 50 and 100 μM (**Figure 4B** black bars and hashed bars).

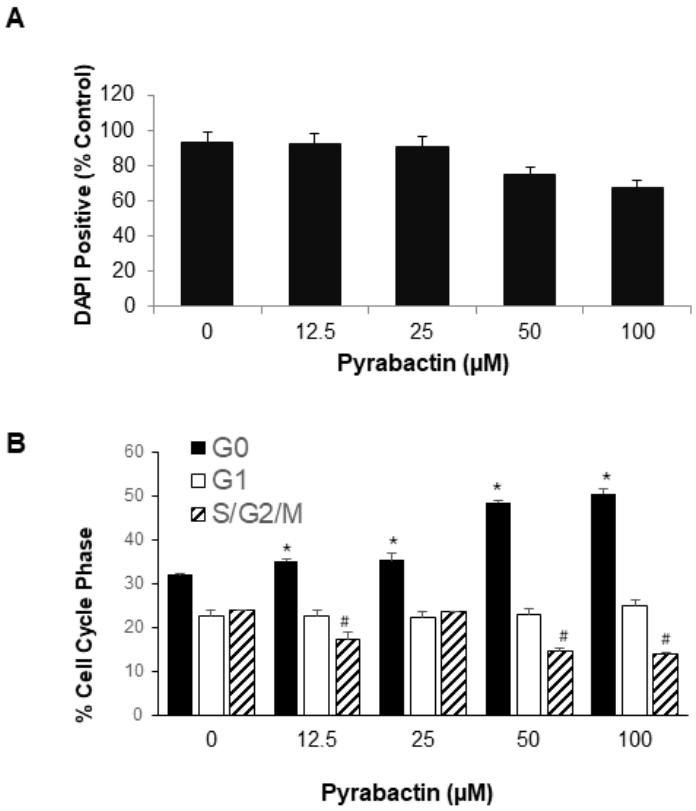

Figure 4: Pyrabactin on PC3 Cell Cyle and Proliferation. (A) PC3 expressing Venus-cherry cell cycle reporters were cultured 0-100 μM of pyrabactin for 72h and then stained with the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) and evaluated by FACS. The data are reported as % mean ± standard deviation relative to 0 μM pyrabactin treatment control. **(B)** PC3 Venus-cherry cells were evaluated for cell cycle phase (G_0, G_1 or $S/G_2/M$) by FACS. The data is reported as % Total cells/culture. *Indicates significant difference at $p<0.05$ (ANOVA) from 0 μM pyrabactin for cells in G_0, and # indicates significant difference at $p<0.05$ for cells in $S/G_2/M$.

In previous work we determined that the growth inhibition of ABA was reversable following exposure to the hormone[32]. Here to determine if the inhibition of proliferation by pyrabactin was reversible, a pulse-chase type of investigation was performed. The metastatic PCa cells were treated with vehicle or 50 μM of pyrabactin for 72h. After the initial primary culture, the cells were washed, trypsinized and secondary cultures were established without additional pyrabactin treatments. To account for the differences in viability that pyrabactin may have induced, the secondary cultures were established with equal numbers of viable cells. As expected, the pyrabactin-treated PC3 and C4-2B primary cultures had fewer cells relative to the vehicle-treated cultures (**Figure 5A and 5C**). In the secondary cultures, the cells grew regardless of their prior exposure in primary culture to pyrabactin. In fact, C4-2B cells grew significantly better than the untreated cells from the primary culture. These data demonstrate that the pyrabactin-induced inhibition of proliferation was potentially reversable at 50 μM during a 72h exposure (**Figure 5B and 5D**).

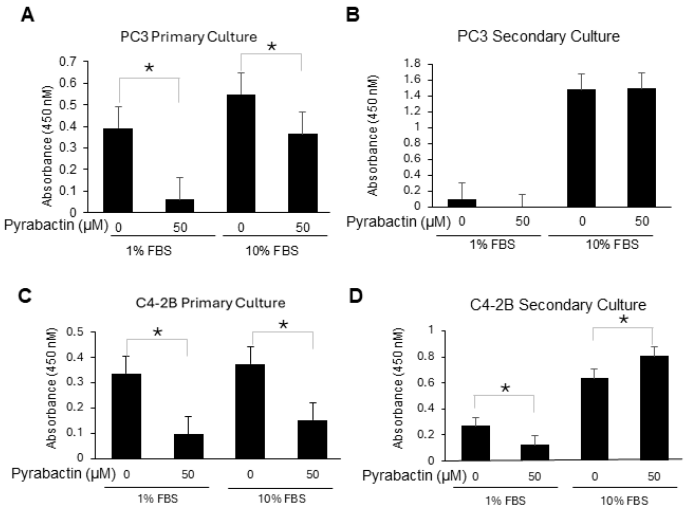

Figure 5: Recovery of PCa Cell Proliferation Post-Exposure to Pyrabactin. Human **(A)** PC3, and **(C)** C-4B prostate cancer cell lines were treated with 50μM pyrabactin for 72h. Selected initial cultures were evaluated proliferation by XTT assay. A second cohort of cells were recovered from primary cultures, normalized and replated for secondary culture without additional pyrabactin for an additional 72h. The secondary **(C)** PC3, and **(D)** C4-2B cultures were evaluated for proliferation. *Indicates significant difference treatment control at 0 μM pyrabactin at $p<0.001$ (ANOVA).

Pyrabactin Does Not Signal Through LANCL2 and PPARγ.

In previous work we reported that ABA through lanthionine synthetase C-like protein 2 (**LANCL2**) and peroxisome proliferator activated receptor γ (**PPARγ**) receptors. LANCL2 and PPARγ induces activation of p38MAPK resulting in dormancy of PCa metastatic cells. To determine whether pyrabactin signals through the same mechanisms, PC3 and C4-2B cells in which the receptors were downregulated using short hairpin RNAs were evaluated for their sensitivity to pyrabactin. As shown in **Figure 6A** for PC3 and in **Figure 6B** for C4-2B, reduced expression of LANCL2 or PPARγ had minimal impact on the overall growth inhibition induced by pyrabactin. These data suggest that pyrabactin signaling is independent of the ABA receptors LANCL2 and PPARγ.

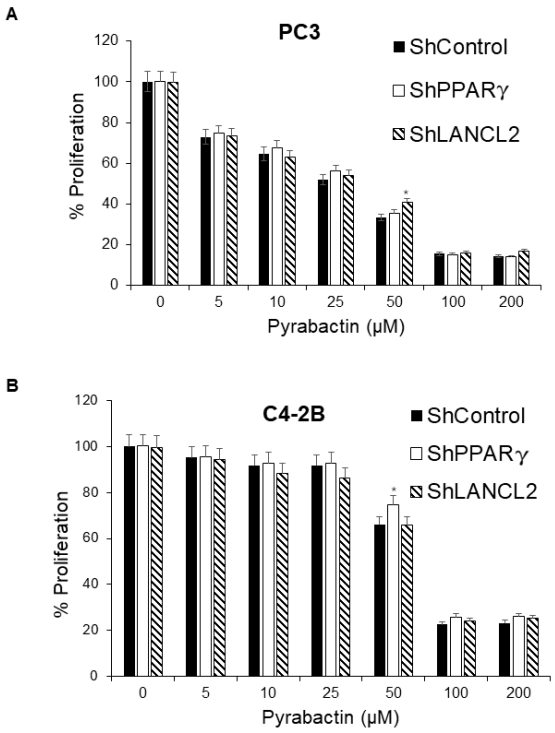

Figure 6: Pyrabactin does not signal through LANCL2 and PPARγ. (**A**) PC3 and **(B)** C4-2B expressing short hairpin RNAs targeting LANCL2, PPARγ or control were evaluated for their sensitivity to pyrabactin following exposure of 0-200μM pyrabactin for 72h and evaluated by XTT assay at a wavelength of 450 nm. The data are reported as % mean ± standard deviation relative to 0 treatment control. *Indicates significant difference treatment control at 0 μM pyrabactin at $p<0.01$ (ANOVA).

Pyrabactin's Impact on Membrane Transport.

As the mammalian ABA receptors LANCL2 and PPARγ are located inside the plasma membrane, a membrane transporter is needed for ABA to function. Recently two anion exchangers were identified as ABA transporters in human cells including members of the AE family, specifically the proteins SLC4A2 and SLC4A3[27], have been implicated which are primarily involved in bicarbonate transport and pH regulation[27].

To explore the role that membrane transport plays in ABA signaling and the response of PCa cells to pyrabactin, SLC4A2 and SLC4A3 shRNAs and negative-control shRNAs (shControls) were obtained and used to transfect PC3 and C4-2B cells and establish stable clones. As shown in **Figure 7A**, for SLC4A2 TRCN0000043858 (labeled 58 in the figure) and for SLC4A3 Clone TRCN0000045145 (labeled 45 in the figure) were selected for further study given the most robust reduction in the mRNA expression. Ultimately, we were able to establish stable and control lines for both transporters for PC3, but only SLC4A2 in C4-2B, the reason for which remains unclear (**Figure 7B**). Nevertheless, we used these cells in proliferation assays in the presence of 100 μM ABA and 25 or 50μM pyrabactin. As expected, inclusion of ABA in the cultures reduced PC3 proliferation by 25%, whereas the impact of ABA on C4-2B was approximately 15%. For ABA, loss of SLC4A2 and SLC4A3 resulted in significant reversal of the growth arrest activity of ABA for PC3 cells. Loss of SLC4A2 and SLC4A3 partially reversed the growth arrest induced by pyrabactin on PC3 cells, yet the response to C4-2B to ABA or pyrabactin was not altered with the loss of SLC4A2 expression (**Figure 7C, D**).

Previously we had noted that ABA inhibited migration of PC3 and C4-2B cells[32]. We examined the impact of pyrabactin and the membrane transporters (SLC4A2, SLC4A3) ability to alter cell migration of PC3 cells across a porous micromembrane in response to serum after 24h of culture (**Figure 7E, F**). Significant impacts on cell migration were noted between the shControl PC3 cells and PC3 cells with SLC4A2 and SLC4A3 down regulated. Migration of the cells at 100 μM, pyrabactin inhibited PCa migration to serum, and loss of the membrane transporters further accentuated the inhibitory effect (**Figure 7E, F**).

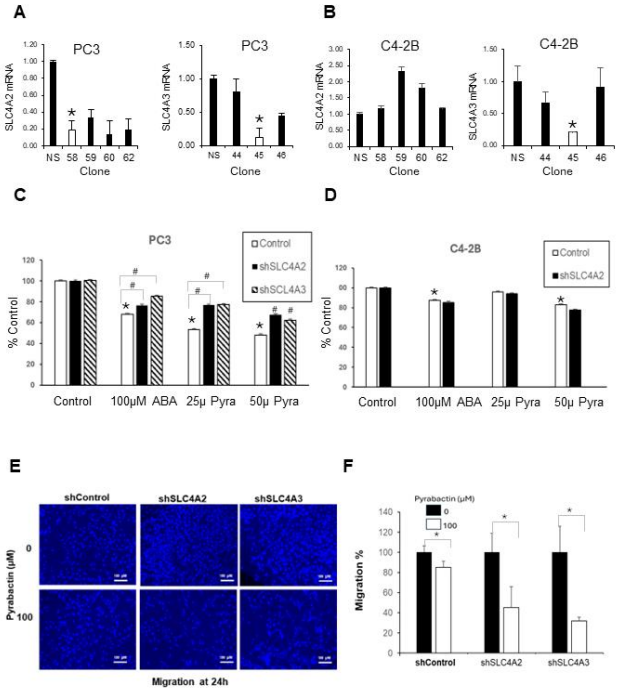

Figure 7: ABA and Pyrabactin Signaling in Response to Reduced Expression of Membrane Transporters SLC4A2 and SLC4A3. (A) To explore the role that membrane transport plays in ABA signaling SLC4A2 and SLC4A3 siRNAs and negative-control shRNAs (shContols) were used to establish stable clones. Clone TRCN000004385858 for SLC4A2 (labeled 58 in the figure in red and asterix*) and Clone TRCN000004514545 zSLC4A3 (labeled 45 in the figure) were selected for further study given the most robust reduction in the mRNA expression. **(B)** Unfortunately, we were not able to generate stable knockdowns of SLC4A3 in C4-2B. **(C)** PC3 and **(D)** C4-2B were exposed to 100μM ABA or pyrabactin (**Pyra**) (25 or 50μM) for 72h (n=5). *Indicates significant difference treatment control at 0 μM pyrabactin at $p<0.05$ (ANOVA), and #indicates significant difference ShControl at $p<0.05$ (ANOVA). **(E)** Migration assay of PC3 cells expressing the transporter shRNAs or controls cells in response to serum at 24h in Transwell plates. Cells were stained with DAPI and imaged at 100x. Bar=100μm. Representitive images are presented, and all presented images were lighted by 20% in Photoshop. **(F)** Quantification of migration presented in (E).

Impact of Pyrabactin on Apoptosis.

While studying the impact of pyrabactin on PCa cells, we observed that under a light microscope PCa cells frequently were rounded up and frequently were detached from the culture surface (**Figure 8A**). Given the cellular appearance (**Figure 8A**), the decrease in cell numbers (**Figure 2**), we speculated that pyrabactin may be inducing apoptosis of the PCa cells. To formally explore the impact of pyrabactin on PCa cell apoptosis, PC3 cells were incubated in 50 µM pyrabactin for 24 and 48h. Thereafter the cells were lysed and normalized protein evaluated using a membrane-based sandwich immunoassay proteome array kit (R&D Systems) and visualized using chemiluminescence (**Figure 8B**). We observed that at 24h, pyrabactin treatment resulted in a significant decrease in the expression of SMAC/Diablo (**Figure 8C**). By 48h, significant changes in many apoptosis pathways were observed including Bad, pro- and cleaved caspas3, cLAP-1 and -2, claspin, Trail R1 and R2, CD95, FAD, Hif-1α, H0-2, HSP27, HSP60, HSP70, PON2, SMAC/Diablo, surviving, and Xiap (**Figure 8D**). These results, in conjunction with those of our pulse chase experiments (**Figure 5**) suggest that for those cells which are susceptible to pyrabactin, multiple pathways are activated to induce apoptosis after an initial decrease in expression of SMAC/Diablo.

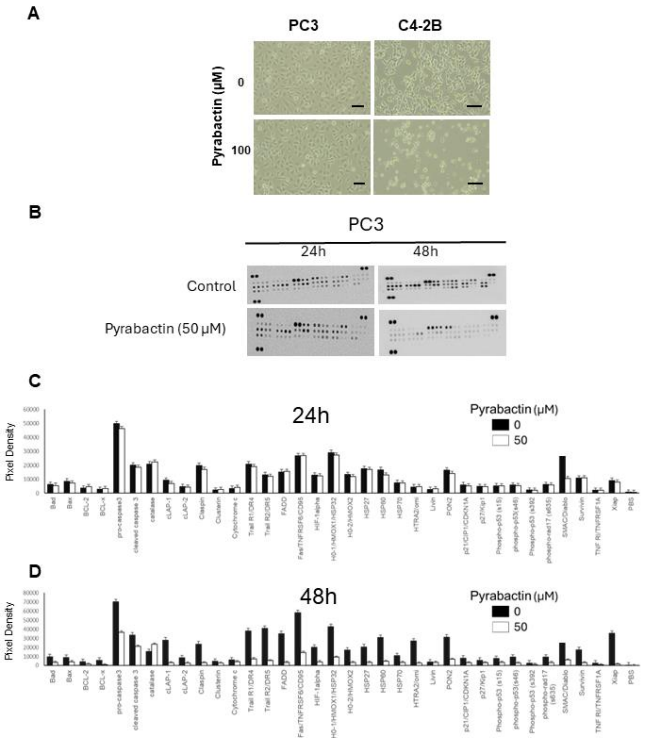

Figure 8: Pyrabactin Induces Apotosis of PCa Cells. (A) PC3, and C4-2B cells were treated with 100 µM pyrabactin for 72h of culture and imaged by light microscopy. Bar=100µm. **(B)** To explore the impact of pyrabactin on PCa cell apoptosis, PC3 cells were incubated in 50 µM pyrabactin for 24h and 48h. R&D Systems™ Proteome Profiler Human Apoptosis Array Kits were used to evaluate down stream signaling in PC3 cells following pyrabactin treatment at **(C)** 24h and **(D)** 48h. Image analysis was performed using the ImageJ software.

Discussion:

Standard therapy for PCa varies based upon stage, grade, patient's age and the health, and patient preferences. Typically standard treatments such as surgery, chemotherapy, and radiation are designed to target actively dividing cancer cells[34], these therapies are largely ineffective against DTCs that enter a dormant state, sometimes for years, before they reactivate and cause metastatic disease[35-37]. Given that ABA is expressed in mammalian tissues[38,39] and predominantly down regulates proliferation in plants[40], our lab has sought to draw parallels between ABA signaling and PCa dormancy in the bone marrow. We believe that understanding these common signaling pathways will lead to the development of new therapeutic strategies to control cancer dormancy by mimicking or targeting these phytohormone-like pathways.

Abscisic and gibberellins are key phytohormones which regulate plant responses to environmental changes (e.g., drought, salinity, temperature changes) which antagonistically regulate several developmental processes[16] Abscisic acid is primarily involved in regulating stress responses, stomatal closure and seed dormancy[17], while gibberellins promote stem elongation, seed germination, and flowering. Although ABA was first characterized in plant tissues, it has subsequently been identified to play a role in regulating several biologic processes in mammals[39,41,42]. ABA is produced by mammalian cells although ABA is also consumed by humans during ingestion of fruits and vegetables[43]. In preclinical studies, ABA has been demonstrated to modulate macrophage and granulocyte functions, immune cell migration and the production of cytokines, contributes to wound healing and inflammatory responses[44-46]. ABA supplementation has been shown to enhance glucose uptake in adipose and muscle cells and stimulate islet cells to secrete insulin, suggesting a potential role in glycemic control and diabetes[21,43,47-49]. In contrast, gibberellins have not been reported to be produced by mammalian cells[24,40]. However, humans are exposed to gibberellins either as part of agricultural processes or as residues plant-based foods. ABA and gibberellins are of great agricultural interest as they can be used to improve crop yield and quality, especially under challenging climate conditions such as drought, salinity or to synchronize ripening[25]. In fact, synthetic molecules which mimic gibberellins or ABA including the synthetic ABA agonist pyrabactin, can be sprayed on crops to enhance agricultural performance.

We observed that gibberellic acid had little to no impact on PCa cell line proliferation or entry/exit from different cell cycle states and did not appear to counteract the growth inhibition of ABA. Our observations are in keeping with reports of an absence of specific receptors for gibberellic acid in mammalian biology. Yet gibberellic acid has been reported to be toxic to murine livers and kidneys[24-26]. In other reports gibberellic acid fed to control and streptozotocin-induced diabetic rats resulted in enhanced lipid peroxidation in erythrocytes and reduced antioxidant defenses compared to control animals[50]. In microglial cells gibberellic acid inhibits the release of TNF-α, IL-6, and IL-1β in stimulated with LPS in part by activating the NF-κB signaling pathway. As a result GA enhances the survival of septic mice and mitigates post-sepsis cognitive impairment[51]. In the context of cancer, GA is reported to improve metabolic functions in patients with lung cancer in combination with several chemotherapy modalities in patients with lung cancer[52] and derivatives of GA have shown some efficacy in treating oral cancer *in vitro* [53]. In an in-silico study of glioblastoma in which three mRNAs (STX1A, PTX3, MMP9) were used to identify potential therapeutic drugs, gibberellic acid was among predicted therapeutic agents which may have therapeutic benefit[54]. Why PC3 and C4-2B cells failed to respond to gibberellic acid by changing proliferation or cell-cycle state remains unclear.

We found that pyrabactin significantly down regulated PCa cell proliferation and migration in response to serum. Pyrabactin is a synthetic chemical initially identified as an agonist of ABA receptors in plants[55]. Pyrabactin was developed to study the ABA signaling pathway in plants and to manipulate plant stress responses through binding to PYR/PYL/RCAR receptors, mimicking ABA and influencing growth, seed dormancy, and drought resistance. We therefore evaluated pyrabactin as a possible treatment for PCa given its reported ABA agonist activity. Unlike ABA, pyrabactin did not signal through Lancl2 or PPARγ and inhibition of SLC4A2 and SLC4A3 membrane transport proteins which facilitate ABA's entry into PCa cells, inhibition of pyrabactin membrane transport was cell type dependent. Where down regulation of SLC4A2 in PC3 cells partially ameliorated the growth inhibition in PC3 cells but not C4-2B. Unfortunately, we were unable to establish stable clones for reducing SCL4A3 in C4-2B cells, but in PC3 cells down regulation of the transporter reduced growth inhibition by both ABA and pyrabactin.

In our assays we observed that pyrabactin treatment of the PCa cells led to cell rounding and detachment from the culture surface. Based on these morphological changes and the observed reduction in cell numbers, we hypothesized that pyrabactin may induce apoptosis in PCa cells. To directly investigate the effect of pyrabactin on PCa cell apoptosis, PC3 cells were treated with pyrabactin for 24 hours and representative apoptosis pathways analyzed using a membrane-

based sandwich immunoassay proteome array kit. We observed an initial reduction in the expression of SMAC/Diablo. By 48 hours, however, nearly all apoptotic pathways represented in the assay were significantly altered. These include death receptor signaling modulation (TRAIL-Receptors, CD95, FADD), mitochondrial pro-apoptotic signaling (Bad, SMAC/Diablo), blocking proteins (IAPs and HSPs) and stress response players (HIF-1α, HO-2, PON2), and active (cleaved) caspase-3 are all altered.

We did not have the opportunity to investigate the mechanisms whereby SMAC/Diablo activates mitochondrial or death receptor-mediated apoptosis. It is known that SMAC/Diablo leads to activation of caspase-9 through down regulation of inhibitors of apoptosis including XIAP, cIAP-1 and cIAP. Further, SMAC/Diablo activates the effector caspases (Caspase-3, Caspase-7), and Trail-R and Fas signaling activates Caspase-8 which further activates downstream effector caspases[56-59]. These data, together with data from our pulse-chase experiments, indicate that in cells susceptible to pyrabactin, multiple apoptotic pathways are activated, proceeding with an initial decrease in SMAC/Diablo expression. The pulse chase experiments also demonstrate that to some extent sub populations of PCa cells are resistant to pyrabactin at the levels used in the studies.

PCa is a heterogeneous disease which can manifest a wide spectrum of clinical outcomes ranging from curable early-stage disease to advanced, terminal metastases. Bone is the predominant site of PCa metastasis, and notably, most men who die from metastatic PCa have significant bone involvement. A key feature of bone metastasis is the ability of DTCs to remain dormant for years or decades before reactivation. Dormancy in PCa is intricately regulated by the interplay between tumor-intrinsic factors and the bone marrow microenvironment, which is designed primarily to promote the quiescence and survival of hematopoietic and mesenchymal stem cells. For instance, osteoblasts and other cells are involved in the formation of hematopoietic stem cell (HSC) niches secrete GAS6, a factor that not only regulates HSC function but also restricts the proliferation of prostate cancer (PCa) cells both *in vitro* and *in vivo*. Additionally, we have demonstrated that GAS6 and its receptors AXL and Mer are essential for the suppression of PCa cell proliferation and reactivation[7,8,10,60]. Furthermore, GAS6 function is partially mediated by TGFβ2, with AXL playing a role in upregulating the expression of both TGFβ and its receptor, TGFβ receptor 2[61]. We believe that tumor cells exploit these regulatory bone marrow niches, using signals such as GAS6 produced by osteoblasts and other niche cells in hematopoietic stem cell niches to suppress their own proliferation and promote dormancy[62].

Our recent studies reveal that ABA, a molecule traditionally studied in plants, can also impact PCa cell proliferation and dormancy[32,63]. ABA exerts its effects through signaling via the LANCL2 and PPARγ receptors, activating downstream effectors including p38MAPK and cell cycle inhibitors like p27, p16, and p21[32]. Experimental knockdown of these receptors abrogates the dormancy-inducing response to ABA, supporting their central regulatory roles[32,63]. The cellular heterogeneity among PCa cells suggests possible involvement of additional ABA receptors such as LANCL1 and CD38, though their functions require further elucidation[32]. Ultimately, ABA signaling might either keep DTCs dormant or, when interfered with or combined with chemotherapy, selectively kill these cells. These insights emphasize the urgent need for therapeutic approaches targeting PCa dormancy and underscore how environmental and cell-intrinsic signals converge to regulate metastatic cell fate in the bone marrow niche.

In summary we found that pyrabactin, a synthetic ABA agonist, significantly inhibited PCa cell proliferation, migration, and induced apoptosis, with effects mediated by various apoptotic pathways and membrane transport proteins, though some cell populations were resistant. The proliferation of other cell types including osteoblasts, and cells of hematopoietic origin were similarly impacted by pyrabactin. Unlike ABA and pyrabactin, gibberellic acid showed little effect on PCa cell cycles, although it has shown toxicity in other mammalian systems and possible therapeutic benefits in different cancer contexts. Together these studies expand our understanding of phytohormone signaling in PCa cells.

Conflict of Interest Statement:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

The authors have significantly contributed to the article's development and writing. A Rodriguez: data curation, data analysis, writing. Y Jung: Conceptualization, data curation and analysis, writing. KR Parajuli: data curation and analysis, writing. RS Taichman: Conceptualization, analysis, writing.

Declaration of AI use

The authors did not use AI-assisted technologies to create this work.

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